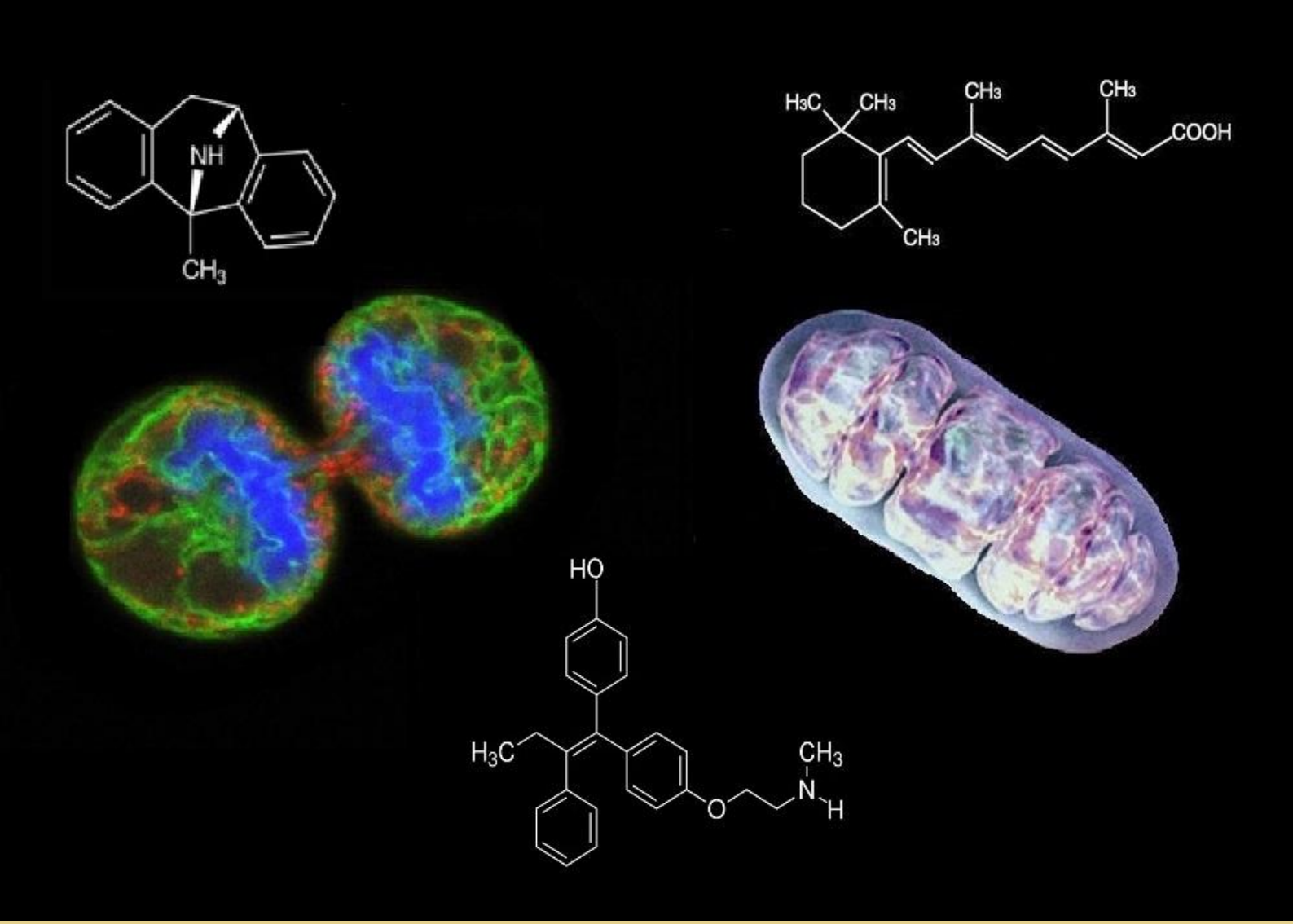

Mariana Ponte Cardoso Ribeiro

TREATMENT STRATEGIES FOR MELANOMA: PHARMACOLOGICAL AND TOXICOLOGICAL ACTION MECHANISMS OF COMBINED THERAPIES

Tese de Doutoramento em Ciências Farmacêuticas orientada pelo Professor Doutor José Barata Antunes Custódio e pela Professora Doutora Armanda Emanuela Castro e Santos e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro de 2013

· U C ·

Universidade de Coimbra

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Treatment strategies for melanoma: pharmacological and toxicological action mechanisms of combined therapies

Tese de Doutoramento em Ciências Farmacêuticas, na especialidade de Bioquímica, apresentada à Faculdade de Farmácia da Universidade de Coimbra para obtenção do grau de Doutor

Orientadores: Professor Doutor José Barata Antunes Custódio e Professora Doutora Armanda Emanuela Castro e Santos

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Figuras na capa:

Human melanoma cell dividing
Cortesia de Paul J. Smith and Rachel Errington. Wellcome images
images.wellcome.ac.uk

Mitochondria and free radicals - Healthy mitochondrion
Cortesia de National Institute on Aging/National Institutes of Health
nia.nih.gov/alzheimers/scientific-images

Aos meus pais

À Mafalda

Ao Raul

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CHAPTER I

Abbreviations

ADP	adenosine 5’-diphosphate
AF-1	activator function 1
AF-2	activator function 2
AIF	apoptosis-inducing factor
Akt	protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	one-way analysis of variance
ANT	adenine nucleotide transporter
APL	acute promyelocytic leukemia
APV	D-(-)-2-amino-5-phosphonopentanoic acid
AP-1	activator protein 1
ATP	adenosine 5’-triphosphate
BAEC	bovine aortic endothelial cells
BCA	bicinchoninic acid
B-Raf	serine/threonine-protein kinase B-Raf
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CATR	carboxyatractyloside
cdk	cyclin-dependent kinase
CK	creatine kinase
CNS	central nervous system
Co-A	coactivator
Co-R	corepressor
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CRE	cyclic adenosine monophosphate response element
CREB	cyclic adenosine monophosphate-responsive element-binding protein

CTGF	connective tissue growth factor
CyA	cyclosporine A
CYP	cytochrome P450
CypD	cyclophilin-D
Cys	cysteine
DBD	DNA binding domain
DIABLO	direct inhibitor of apoptosis-binding protein with low pI
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECF	enhanced chemifluorescence
EDTA	ethylenediaminetetraacetic acid
EDX	endoxifen
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ER+	estrogen receptor-positive
ER−	estrogen receptor-negative
ERE	estrogen response elements
ERK	extracellular signal-regulated kinase
ETS	E26 transformation specific
E2	17β-estradiol
$FADH_2$	flavin adenine dinucleotide
FBS	fetal bovine serum
FDA	Food and Drug Administration
Glu/Mal	glutamate/malate
GluR	glutamate receptor
GPCR	G protein-coupled receptor
GPER1	G protein coupled estrogen receptor 1

GPR30	G protein-coupled receptor 30
GPR30+	G protein-coupled receptor 30-positive
GSH	glutathione
HB-EGF	heparin-bound epidermal growth factor
HDAC	histone deacetylase
HEPES	4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid
HKII	hexokinase II
IGF	insulin-like growth factor
IGF-1R	insulin-like growth factor-1 receptor
iGluR	ionotropic glutamate receptor
IMM	inner mitochondrial membrane
IMS	intermembrane space
KA	kainate
KAR	kainate receptor
Ki	inhibitor constant
LBD	ligand binding domain
LDH	lactate dehydrogenase
LHON	Leber's hereditary optic neuropathy
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy associated with ragged-red fibres
MiTF	microphthalmia-associated transcription factor
Mitoc	mitochondria
mGluR	metabotropic glutamate receptor
MMP	matrix metalloproteinase
MOMP	mitochondrial outer membrane permeabilization
MOPS	3-[N-Morpholino]propanesulfonic acid
MPT	mitochondrial permeability transition
mRNA	messenger RNA
mtDNA	mitochondrial DNA

mTOR	mammalian target of rapamycin
NAC	*N*-acetyl-L-cysteine
NADH	nicotinamide adenine dinucleotide
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline
NF-κB	nuclear factor-κB
NMDA	*N*-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptor
N-Ras	GTPase N-Ras
OHTAM	4-hydroxytamoxifen
Olig	oligomycin
OMM	outer mitochondrial membrane
PAGE	polyacrylamide gel electrophoresis
PBR	peripheral benzodiazepine receptor
PBS	phosphate-buffered saline
PIP2	phosphatidylinositol-4,5-biphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphate
PI3K	phosphoinositide-3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLZF	promyelocytic leukemia zinc finger
PML	promyelocytic leukemia
PMSF	phenylmethanesulphonyl fluoride
PPAR	peroxisome proliferator-activated receptor
pRb	retinoblastoma protein
PTEN	phosphatase and tensin homolog
PTP	permeability transition pore
PVDF	polyvinyldiene fluoride
RA	all-*trans*-retinoic acid
Raf	RAF proto-oncogene serine/threonine-protein kinase
RAR	retinoic acid receptor

RARE	retinoic acid response element
Ras	Ras GTPase
RCR	respiratory control ratio
RNA	ribonucleic acid
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
RXR	retinoid X receptors
SDS	sodium dodecyl sulphate
S.E.M.	standard error of the mean
SERD	selective estrogen receptors down-regulators
SERM	selective estrogen receptors modulators
Smac	second mitochondria-derived activator of caspase
SRB	sulforhodamine B
SRE	serum response element
SRF	serum response factor
Succ	succinate
TAM	tamoxifen
TBARS	thiobarbituric acid reactive substances
TBS	tris-buffered saline
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor-β
TMB	3,3',5,5'-tetramethylbenzidine
TPP^+	tetraphenylphosphonium cation
VDAC	voltage-dependent anion channel
VDR	vitamin D receptor
WB	western-blot
ΔpH	pH gradient
$\Delta\mu H^+$	electrochemical proton gradient
ΔΨ	mitochondrial membrane potential

Resumo

A terapêutica disponível para o melanoma apresenta elevada toxicidade e uma baixa eficácia. O desenvolvimento e a progressão do melanoma envolvem diferentes mecanismos e a combinação de fármacos permitirá obter tratamentos mais efetivos e com efeitos adversos menos acentuados.

O antiestrogénio tamoxifeno (TAM) é amplamente prescrito para pacientes com carcinoma da mama, mas induz vários efeitos adversos e a sua eficácia é limitada pelo metabolismo interindividual ou pela coadministração de medicamentos que possam diminuir os níveis dos seus metabolitos ativos, o endoxifeno (EDX) e o 4-hidroxitamoxifeno (OHTAM). O TAM tem sido também usado no tratamento do melanoma, todavia a sua utilização e os mecanismos de acção nesta patologia permanecem controversos. Assim, dado que alguns dos efeitos adversos do TAM resultam de indução de disfunção mitocondrial, investigámos os efeitos do EDX em mitocôndrias de fígado, individualmente ou em combinação com o ácido *trans*-retinóico (RA) para clarificar se o EDX é uma alternativa menos tóxica; posteriormente, estudámos também os efeitos do EDX numa linha celular de melanoma, individualmente ou em combinação com o RA ou com o antagonista dos recetores do *N*-metil-D-aspartato (NMDA) MK-801, para avaliar a eficácia do EDX no melanoma relativamente ao TAM. Estudámos também a expressão e a função do recetor associado à proteína G 30 (GPR30) nas células de melanoma para clarificar o mecanismo de ação dos antiestrogénios.

Os resultados obtidos demonstram que o EDX apenas afecta a função bioenergética mitocondrial em concentrações superiores às atingidas nos tecidos, induzindo uma ligeira alteração na integridade da membrana mitocondrial, contrariamente aos efeitos acentuados do TAM. O EDX também inibiu a permeabilidade transitória mitocondrial (MPT) induzida por cálcio e fosfato.

O RA é um eficiente citostático cuja utilização clínica é limitada pelos seus efeitos adversos, incluindo hepatotoxicidade, que está possivelmente associada à indução da MPT. Uma vez que as combinações de RA com o TAM apresentam ação sinérgica em células do cancro da mama e que os nossos resultados demonstram que o EDX é menos tóxico para a mitocôndria, avaliámos os efeitos do RA em combinação com o EDX na bioenergética mitocondrial e na MPT. O EDX, assim como o TAM e o OHTAM, preveniu a MPT induzida

pelo RA, podendo contribuir para reduzir a sua hepatotoxicidade. Os efeitos do RA na bioenergética mitocondrial não foram potenciados pelo EDX. Deste modo, a combinação de EDX com o RA poderá apresentar vantagens na terapêutica do melanoma. Assim, e considerando que o RA e o TAM individualmente diminuem a proliferação e a migração das células de melanoma, estudámos os efeitos do EDX combinado com o RA nestas células. A combinação de RA com EDX potenciou significativamente a ação antiproliferativa de ambos os compostos, induzindo bloqueio do ciclo celular em G1, indicando que os seus mecanismos de ação convergem na regulação do ciclo celular. Contudo, verificámos que o TAM não potenciou os efeitos antiproliferativos do RA. A combinação do RA com EDX diminuiu ainda a migração das células de melanoma em concentrações que não afetaram a proliferação de células não-neoplásicas.

Recentemente, foi demonstrado que o MK-801 diminui a proliferação de células do melanoma. Após testarmos os efeitos dos antagonistas dos recetores ionotrópicos do glutamato em células do melanoma, selecionámos o MK-801 para estudar em combinação com o TAM, OHTAM e EDX. Os antiestrogénios atuaram sinergicamente com o MK-801 na diminuição da proliferação das células do melanoma, sendo as combinações com EDX e OHTAM as mais eficazes, bloqueando a progressão do ciclo celular em G1.

O papel emergente do GPR30 no cancro levou-nos a investigar a sua expressão e função no melanoma, uma vez que este recetor é ativado pelo TAM e pelo OHTAM. Verificámos que a linha celular de melanoma expressa o GPR30 e que o seu agonista seletivo G-1 reduziu drasticamente a proliferação celular, sugerindo que o GPR30 poderá ser um novo alvo terapêutico no melanoma e, possivelmente, estar envolvido na ação antiproliferativa dos antiestrogénios nestas células. Estudámos ainda os efeitos dos antiestrogénios na via das cinases reguladas por sinal extracelular (ERK) e verificámos que nas condições experimentais utilizadas o EDX é mais potente do que o TAM na redução da fosforilação da ERK 1/2, podendo justificar a maior eficácia do EDX em relação ao TAM no melanoma, individualmente ou em combinação com o RA ou com o MK-801, que também modulam esta via de sinalização.

Assim, os resultados obtidos indicam que o EDX poderá ser uma alternativa ao TAM no tratamento do cancro e mais eficaz no melanoma maligno, particularmente em associação com outros fármacos.

Palavras-chave: endoxifeno; ácido *trans*-retinóico; antagonistas dos recetores do glutamato; mitocôndria; melanoma.

Abstract

The available treatments for melanoma rely on aggressive strategies that fail to alter the history of the disease. The onset and progression of melanoma involves a complex molecular machinery and, therefore, the use of combinations of drugs is a rational approach to build more effective treatments and to minimize side effects.

The antiestrogen tamoxifen (TAM) is widely prescribed for patients with breast carcinoma, but induces several adverse effects. Moreover, the efficacy of TAM is limited by the individual metabolism or by coadministered medication that may decrease the effective levels of TAM active metabolites, endoxifen (EDX) and 4-hydroxytamoxifen (OHTAM), which are responsible for the anticancer action of TAM. TAM is also used in the treatment of melanoma, but its inclusion in therapeutic regimens and its action mechanisms remain controversial.

In the present work we had the following goals: as some of TAM adverse effects result from mitochondrial damage, we investigated the effects of EDX on liver mitochondrial function, either individually or in combination with all-*trans*-retinoic acid (RA), to clarify whether EDX is a less toxic drug; afterwards, we studied the effects of EDX on a melanoma cell line individually or in combination with RA or with the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801, to assess the efficacy of EDX in melanoma in comparison with TAM. Finally, we evaluated the expression and role of the G protein-coupled estrogen receptor 30 (GPR30) in melanoma cells to characterize the action mechanisms of antiestrogens.

Our results show that EDX compromised mitochondrial bioenergetics at concentrations higher than those reached in tissues and mildly affects mitochondrial membrane integrity in comparison with TAM, suggesting that the adverse effects related with mitochondrial damage can be minimized by using EDX instead of TAM. Furthermore, like TAM or OHTAM, EDX prevented the mitochondrial permeability transition (MPT) induced by Ca^{2+} and phosphate.

RA is a powerful anticancer agent, but its clinical utilization is limited by its adverse effects, including the hepatotoxicity, which is probably related with MPT induction. Since the combinations of RA with TAM demonstrated a synergism of action in breast cancer cells and our results show that EDX is less toxic to mitochondria, we monitored the effects of RA

in combination with EDX on mitochondria. EDX, similarly to TAM or OHTAM, prevented RA-induced MPT. Moreover, RA compromised mitochondrial bioenergetics in a concentration-dependent manner, but its effects were not enhanced by EDX. Thus, the combination with EDX may contribute to reduce the hepatotoxicity of RA and low concentrations of both drugs might be given together with acceptable toxicity.

Considering these promising results and that RA and TAM decrease the proliferation and migration of melanoma cells individually, we investigated the effects of EDX in combination with RA on melanoma cells. RA combined with EDX significantly enhanced the antiproliferative activity of the drugs alone by inducing cell cycle arrest in G1, indicating that their mechanisms of action converge in the regulation of the cell cycle. In contrast, TAM was unable to potentiate the actions of RA. The combination of RA with EDX also decreased melanoma cell migration at concentrations that do not affect the proliferation of non-neoplasic cells, suggesting that the combined treatment of RA and EDX may be devoid of significant toxicity.

Recently, it was reported that MK-801 decreases melanoma cell proliferation. Thus, after investigating the effects of ionotropic glutamate receptor antagonists on melanoma cells, we chose MK-801 to combine with TAM, OHTAM and EDX. The antiestrogens acted synergistically with MK-801 to decrease melanoma cell proliferation and the combinations with EDX and OHTAM were more effective than the combinations with TAM, inducing cell cycle arrest in G1.

The emerging role of GPR30 in cancer, which is activated by TAM and OHTAM, led us to investigate its expression and function in melanoma. The melanoma cell line used expressed GPR30 and its activation by the selective agonist G-1 markedly decreased cell proliferation, pointing out that the GPR30 is a new target in melanoma and it is possibly involved in the antiproliferative effect of antiestrogens on melanoma cells. We also addressed the effects of antiestrogens on the activation of the extracellular signal-regulated kinase (ERK) pathway and we found that, in our experimental setting, EDX was more effective than TAM in the reduction of ERK 1/2 phosphorylation, providing the first clues on the superior efficacy of EDX over TAM in melanoma, either individually or in combination with RA or MK-801, which also target this pathway.

Altogether, the data obtained support that EDX may be an alternative to TAM in cancer therapy and a more effective drug for the management of malignant melanoma, particularly in therapeutic association.

Keywords: endoxifen; all-*trans*-retinoic acid; glutamate receptor antagonists; mitochondria; melanoma.

CHAPTER I

GENERAL INTRODUCTION

1.1 - Introduction

Malignant melanoma is one of the most aggressive and treatment-resistant of human cancers. Melanoma incidence has increased by approximately 2.8 % annually since 1981 (Fig. 1.1) and it is estimated that almost 2 % of Americans will receive a melanoma diagnosis during their lifetime (Jemal et al., 2010; Little and Eide, 2012). The incidence and the mortality of melanoma are generally greater in men than women (29.1 per 100 000 in males compared with 19 per 100 000 in women, in 2008) (Fig. 1.1) (Little and Eide, 2012). In fact, male gender was associated with a greater incidence of unfavorable primary tumor characteristics (Scoggins et al., 2006) and increased rate of metastasis, as well as a shorter long term survival after development of distant metastasis (Mervic, 2012). However, melanoma incidence in younger women is increasing, and this shift can possibly reflect recent trends in indoor tanning (Little and Eide, 2012). There are also differences related with race and ethnicity, and the lower incidence of melanoma in darker-skinned individuals has been associated with the protective effects of higher melanin densities (Little and Eide, 2012).

Exposure to ultraviolet radiation, personal history of melanoma, genetic predisposition, phenotypic characteristics (fair skin and blond or red hair) and the dysplastic nevus syndrome are known risk factors, although the molecular alterations underlying the development and progression of melanoma are still under investigation (Russo et al., 2009). Other factors, such as socioeconomic status and access to healthcare seem to be relevant to melanoma epidemiology (Little and Eide, 2012).

If diagnosed early, melanoma can be removed by surgery and about 80 % of the cases are successfully dealt with in this way (Gray-Schopfer et al., 2007; Russo et al., 2009). On the contrary, the available treatments for patients with locally advanced or metastatic disease fail to clearly alter the natural history of the disease (Lutzky, 2010; Ko and Fisher, 2011). The resistance of melanoma to most of the available therapies is possibly a consequence of the complex machinery in melanoma onset and progression (Palmieri et al., 2009). Melanomas are highly heterogeneous malignancies, not only between patients, but also within the same tumor, as there are several disease variants, mostly characterized by differences in oncogene and tumor suppressor drivers (Herlyn, 2009). Moreover, within one

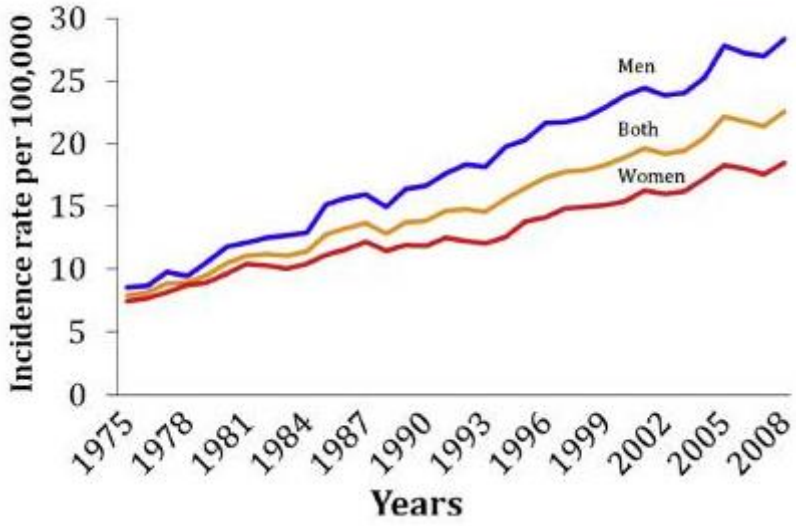


Fig. 1.1 - Age-adjusted melanoma incidence rates from 1975 to 2008 according to the US Surveillance, Epidemiology and End Results program (Little and Eide, 2012).

tumor there are subpopulations of cells that respond differently to therapy as well as multiple signaling pathways constitutively activated (Herlyn, 2009).

Immunotherapy has been extensively evaluated in advanced melanoma with several agents tested over the years, but the only approved immunotherapy regimens were until recently interferon-α and high-dose of interleukin-2 (Lutzky, 2010). Interferon-α is the most widely used adjuvant immunotherapy for melanoma, but it presents limited efficacy in metastatic melanoma. Interferon-α has also been combined with chemotherapy, but without significant improvements in the clinical outcomes (Lutzky, 2010; Ko and Fisher, 2011). Interleukin-2 is approved for metastatic disease, but induces severe toxic effects, such as vascular leak syndrome, with symptoms of hypotension and oliguria, and it is not suitable for most patients, requiring staff expertise and often intensive support (Lutzky, 2010; Ko and Fisher, 2011; Curti and Urba, 2012; Ma and Armstrong, 2013). In 2011, the Food and Drug Administration (FDA) and the European Medicines Agency approved the human monoclonal antibody ipilimumab, which yields significant and durable response rates in 10 % of the patients with metastatic melanoma (Hodi et al., 2010; Curti and Urba, 2012; Wolchok, 2012). However, ipilimumab reactivates immune responses that were silenced, leading to immune-related adverse events, such as rash, diarrhea, colitis with perforation, endocrinopathies, hepatocellular injury, fatigue and pyrexia (Curti and Urba, 2012; Eggermont and Robert, 2012; Ma and Armstrong, 2013).

Systemic chemotherapy has been another therapy of choice for metastatic melanoma. The alkylating agent dacarbazine is the only cytotoxic agent approved by the FDA providing responses in approximately 5-10 % of patients (Lutzky, 2010; Ko and Fisher, 2011). Temozolomide is an orally available analog of dacarbazine that it is preferred in patients with brain metastases due to its better central nervous system (CNS) penetration (Lutzky, 2010). Other chemotherapeutic agents, such as nitrosoureas, taxanes, cisplatin and carboplatin, have similar efficacy profiles in the metastatic setting (Lutzky, 2010; Ko and Fisher, 2011). Several clinical trials have investigated the combination of dacarbazine or temozolomide with other drugs without demonstrating clear benefits in clinically relevant endpoints over single agents (Lutzky, 2010).

In the last years, cellular and molecular studies have significantly increased our knowledge regarding the initiation and progression of melanoma, paving the way for the development of new therapies. The identification of the Ras GTPase/RAF proto-oncogene

serine/threonine-protein kinase (Raf)/mitogen-activated protein kinase kinase (MEK)/ extracellular signal-regulated kinase (ERK) known as the mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide-3-kinase (PI3K) as key signaling pathways in melanoma has provided the opportunity to develop new targeted therapies.

The MAPK pathway is a signal transduction cascade relaying extracellular signals from plasma membrane to the nucleus by consecutive phosphorylation events. Upon cellular stimulation, for instance as consequence of growth factor-mediated activation of receptor tyrosine kinases (RTKs), Ras assumes an activated, guanosine triphosphate-bound state, leading to the recruitment of Raf from the cytosol to the cell membrane where it becomes activated, likely via a Src-family tyrosine kinase. Then, the activated Raf causes the phosphorylation and activation of MEK 1/2, which in turn phosphorylate and activate the ERK. The activated ERK translocates to the nucleus, where it phosphorylates several nuclear transcription factors, stimulating the cellular proliferation, differentiation, and survival.

ERK is constitutively activated in up to 90 % of melanomas, most commonly due to mutations of the genes encoding the GTPase N-Ras (15-30 %) and especially the serine/threonine-protein kinase B-Raf (50-70 %) (Russo et al., 2009). Activating B-Raf mutations occur in approximately 60 % of melanomas and, therefore, are considered the most important target in melanoma (Davies, 2012). The most frequent B-Raf mutation, observed in approximately 90 % of melanomas with alteration of B-Raf, is the T1799A point mutation, in which a T → A transversion converts glutamic acid for valine at the codon 600 in exon 15 (Val600Glu; B-Raf^{V600E}). This mutation introduces a conformational change in the protein structure, leading to its constitutive activation with a substantial increase in the basal kinase activity and, consequently, to the hyperactivity of the MAPK pathway (Russo et al., 2009; Ko and Fisher, 2011). However, the fact that both activating B-Raf and N-Ras mutations are found at high rates in benign nevi suggests that the activation of the MAPK pathway alone does not fully explain the pathogenesis of melanoma and that other pathways may play a critical role as well (Davies, 2012).

Growing evidence implicates the aberrant expression and activity of the PI3K pathway in the onset and progression of melanoma, which frequently occurs with concurrent activation of MAPK signaling. In response to activated growth factor receptors, the PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), activating the protein kinase B, also known as Akt (Russo et al., 2009;

Davies, 2012). Akt is a serine-threonine kinase that is usually present in the cytoplasm in an inactive conformation and has 3 highly homologous isoforms (Akt1, Akt2 and Akt3). Upon activation of PI3K, Akt is recruited to the cell membrane where it is phosphorylated to activate the catalytic activity of Akt (Davies, 2012). The activated Akt, which is one of the major effectors of the PI3K pathway, is then able to phosphorylate the downstream proteins (Russo et al., 2009). The mammalian target of rapamycin (mTOR) is a downstream effector of the PI3K/Akt pathway, that consists of 2 protein complexes mTORC1 and mTORC2 (Ko and Fisher, 2011; Pópulo et al., 2012). The activation of PI3K with subsequent activation of Akt allows mTORC1 to promote cell proliferation via downstream transduction of proliferative signals (LoRusso et al., 2013). Besides the activation by PI3K, mTOR can also be activated by the MAPK pathway, in order to regulate cell proliferation and survival (Ko and Fisher, 2011; Pópulo et al., 2012). The mTORC2 complex regulates the actin cytoskeleton and Akt signaling through phosphorylation (Ko and Fisher, 2011; Pópulo et al., 2012).

A study by Dai *et al* (2005) reported that phosphorylated Akt is expressed in 17 %, 43 %, 49 % and 77 % of the biopsies of normal nevi, dysplastic nevi, primary melanoma and melanoma metastases, respectively, and that a strong phosphorylated Akt expression is inversely correlated with the overall survival of patients with primary melanoma. The predominant active isoform of Akt in melanoma is Akt3, which promotes cell survival and tumor development in 43 to 60 % of nonfamilial melanomas, and its activation increases during tumor progression (Stahl et al., 2004). The activated Akt3 can phosphorylate up to 9 000 substrate proteins and this way regulates several cellular processes (Madhunapantula et al., 2011). Moreover, the inactivation, loss or decreased expression of the phosphatase and tensin homolog (PTEN), a crucial regulator of this pathway that acts through the dephosphorylation of PIP3, is frequently observed in melanoma cell lines and biopsies specimens, and the loss of PTEN was reported in approximately 20-30 % of melanomas (Guldberg et al., 1997; Tsao et al., 1998; Zhou et al., 2000; Madhunapantula et al., 2011). Therefore, considering the key role played by the MAPK and the PI3K pathways in melanoma, several inhibitors of these pathways have been developed.

Vemurafenib is the first and most extensively investigated of selective B-Raf inhibitors (Fig. 1.2). The initial objective response rates to this B-Raf targeted agent are much higher than to any other treatments. In a phase III clinical trial, vemurafenib provided

major tumor responses shortly after the treatment initiation in 50 % of patients (Curti and Urba, 2012; Eggermont and Robert, 2012). However, side effects, including arthralgia, fatigue, and cutaneous manifestations are common in patients taking vemurafenib, and approximately 40 % of patients require a reduction in the recommended dose (Curti and Urba, 2012; Eggermont and Robert, 2012). On the other hand, the degree of tumor reduction varies widely, with less than 10 % of the patients achieving a complete tumor regression (Davies, 2012). Clinical response was only observed in patients whose levels of phosphorylated ERK were reduced in about 80 %, suggesting that an almost complete inhibition of MAPK pathway may be required to achieve a significant tumor response (Bollag et al., 2010). Importantly, the median duration of response is modest and essentially all patients relapse, reflecting the development of resistance, either by MAPK reactivation or by the activation of alternative survival pathways, such as the PI3K pathway (Nazarian et al., 2010; Paraiso et al., 2010; Eggermont and Robert, 2012). It was reported that the PI3K signaling is increased in B-Raf inhibitor-resistant melanoma cells, as well as in a post-relapse tumor sample, and that the simultaneous inhibition of the MAPK and PI3K pathways markedly induces cell death, overcoming the resistance to B-Raf inhibitors (Villanueva et al., 2010; Posch et al., 2013). Thus, the combination of B-Raf and MEK inhibitors or the dual targeting of the MAPK and PI3K pathways have been investigated as an attempt to abrogate drug resistance (Madhunapantula et al., 2011; Shi et al., 2011; Davies, 2012; Eggermont and Robert, 2012).

Multiple inhibitors of PI3K pathway were also developed, including the Akt inhibitor perifosine and the mTOR inhibitors everolimus and temsirolimus (Fig. 1.2), which were tested in patients with metastatic melanoma without providing objective clinical responses (Ernst et al., 2005; Margolin et al., 2005). However, in any of the clinical trials performed to evaluate the benefits of perifosine in melanoma the expression or the activity of Akt was taken into consideration (Madhunapantula et al., 2011). On the other hand, the existence of compensatory signaling can limit the efficacy of PI3K pathway inhibitors, as the inhibition of mTOR by rapamycin or everolimus can lead to the hyperactivation of Akt (Wan et al., 2007; Madhunapantula et al., 2011).

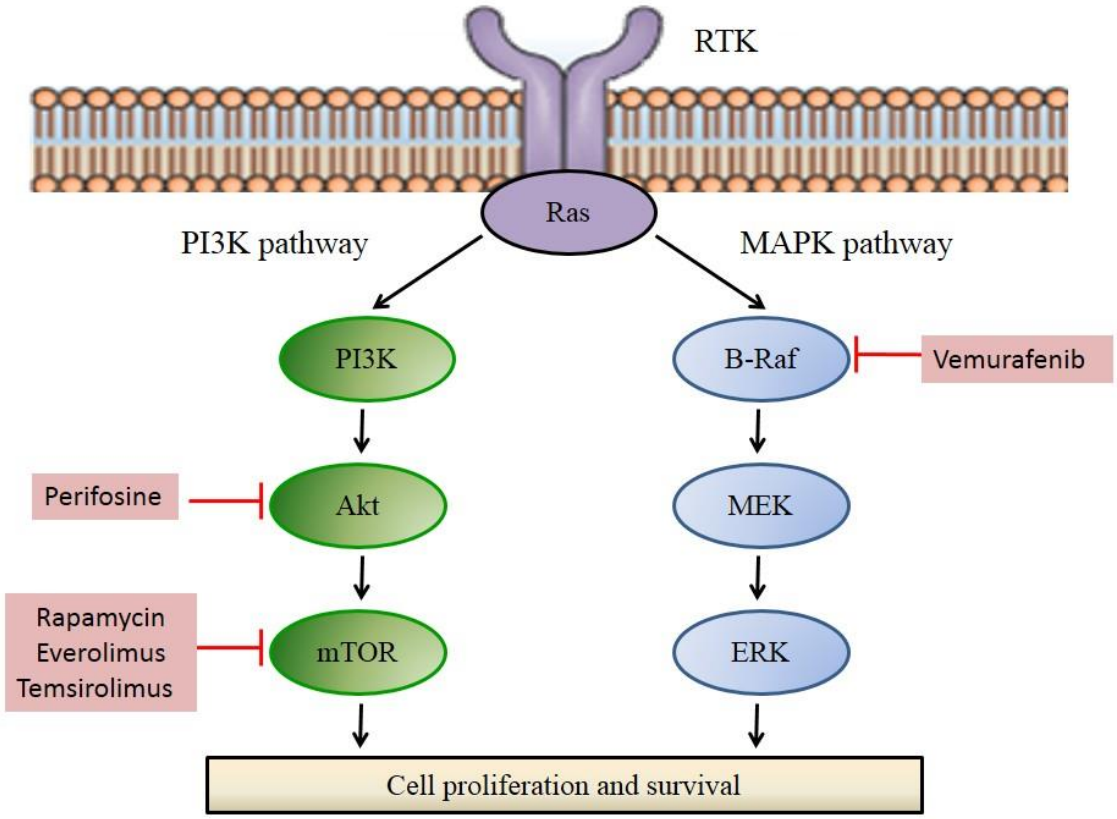


Fig. 1.2 - Major pathways involved in melanoma pathogenesis and point-of-action of targeted therapies. The MAPK and the PI3K pathways are major signaling cascades involved in the development and progression of melanoma. The figure depicts a simplified scheme of both pathways as well as selected agents that target these pathways.

In fact, given the complexity of melanoma, it is unlikely that a single agent can yield a significant antitumor response. To deal effectively with the vast majority of melanomas, it is necessary to develop combinations of drugs that have a strong rationale for single agent therapy, in order to target all cells within a tumor (Herlyn, 2009; Palmieri et al., 2009). Thus, considering the rising incidence of malignant melanoma and its poor prognosis, it is urgent to develop new combinations of drugs that through a synergism of action allow to obtain therapeutic efficacy with lower doses, this way minimizing the toxic effects typical of systemic chemotherapy, and also contributing to avoid the development of resistance to therapy.

1.2 - Use of antiestrogens in malignant melanoma therapy

Introduced in the clinical practice in 1977, the antiestrogen tamoxifen (TAM) remains the standard therapy for women with estrogen receptor (ER)-positive (ER+) breast carcinoma and it is estimated that the lives of half a million women were spared by adjuvant therapy with TAM (Jordan, 2008).

It is widely accepted that the pharmacological action of TAM in breast cancer is mainly mediated by binding to ERα. However, the demonstration that some ER-negative (ER−) breast cancers also respond to TAM, raised the possibility of an antitumor mechanism independent of the ER (Croxtall et al., 1994; Charlier et al., 1995). Since then, the effects of TAM on ovarian cancer, malignant glioma, melanoma, as well as on hepatocellular, pancreatic, and renal cell carcinomas, were investigated in several clinical trials (Goldenberg and Froese, 1982; Gelmann, 1996, 1997).

TAM inhibits melanoma cell proliferation (Gill et al., 1984; Piantelli et al., 1995; Lama et al., 1998; Kanter-Lewensohn et al., 2000), invasion and metastasis (Matsuoka et al., 2009). The mechanisms underlying the antitumor activity of TAM in melanoma are not entirely clarified. Earlier investigations detected the presence of ER in more than 40 % of the patients with malignant melanoma, which had longer disease-free survival than the ER− patients (Fisher et al., 1976; Walker et al., 1987). However, further studies using ER-specific monoclonal antibodies demonstrated that usually melanoma cells do not express ER (Neifeld and Lippman, 1980; Flowers et al., 1987; Cohen et al., 1990; Lecavalier et al., 1990; Kanter-Lewensohn et al., 2000). More recently, methylated ERα were detected in the tumor tissues

and sera of melanoma patients resulting in ERα gene silencing (Mori et al., 2006). These findings have implications not only for prognosis, since the presence of methylated ERα in serum was shown to be an unfavorable prognostic factor, but also for the identification of patients that would benefit from the inclusion of TAM in their therapeutic regimens and the patients for whom an alternative treatment should be sought (Mori et al., 2006; Tanemura et al., 2007).

On the other hand, considerable attention has been given to ER-independent targets, often called nongenomic, because they do not require the interaction of TAM with ER and/or do not directly affect the transcriptional regulatory activities of ER, although these effects may also be important in ER+ tumors (Clarke et al., 2001). One of the primary mechanisms reported for TAM in ER− cancer cells is the inhibition of protein kinase C (PKC) signaling (Gundimeda et al., 1996; Gelmann, 1997; Luo et al., 1997). In melanoma cells, TAM inhibits cell migration, invasion and metastasis through the suppression of PKC/MEK/ERK and PKC/PI3K/Akt activation (Mastuoka et al., 2009). TAM also inactivates the insulin-like growth factor-1 receptor (IGF-1R) in melanoma cells (Kanter-Lewensohn et al., 2000). In addition, TAM stimulates the activity of natural killers, which can also contribute to control the tumor growth (Mandeville et al., 1984; Baral et al., 1995), and inhibits angiogenesis in part by inducing transforming growth factor-β (TGF-β) stimulation (Toma et al., 1999). Moreover, TAM and its active metabolite 4-hydroxytamoxifen (OHTAM) are highly hydrophobic molecules that strongly partition in biomembranes (Custódio et al., 1991) affecting the bilayer dynamics, which is another possible mechanism of the anticancer activity of both drugs (Custódio et al., 1993a, b).

Although TAM individually has provided extremely poor response rates (less than 10 %) in melanoma clinical trials (Rumke et al., 1992; Toma et al., 1999), the synergistic effects reported when TAM was combined with other agents, such as fotemustine (Fischel et al., 1994), cisplatin (McClay et al., 1993; Jones et al., 1997) and interferons (Lindner and Borden, 1997), have led to the investigation of the benefits of the inclusion of TAM in combination regimens. In fact, the initial trials performed in patients with metastatic melanoma provided encouraging results. The combination of TAM with dacarbazine improved the response rate and median survival in comparison with dacarbazine alone, particularly in women (Cocconi et al., 1992), whereas the addition of TAM to dacarbazin and cisplatin induced a small increase in the response rate (Flaherty et al., 1996). However,

subsequent trials failed to show significant improvements in clinically relevant endpoints when TAM was used in combination with several agents in patients with locally advanced and metastatic melanoma. In patients with advanced melanoma, the combination of TAM, carmustine, cisplatin and dacarbazine increased the overall response rate in comparison with dacarbazine alone, but the toxic effects were also enhanced by the combination and the impact on overall survival was very limited (Chiarion-Sileni et al., 2001). Moreover, the addition of TAM to dacarbazine or dacarbazine plus interferon-α or dacarbazine plus carboplatin did not improve the response rate or the overall survival of patients with metastatic melanoma (Falkson et al., 1998; Agarwala et al., 1999). In a phase II trial with patients with metastatic melanoma, the combination of TAM with paclitaxel provided an overall response rate of 24 %, but the contribution of TAM was unclear since plasma levels were not measured (Nathan et al., 2000).

The Dartmouth regimen, which consists of quadruple drug therapy with dacarbazine, cisplatin, carmustine and TAM, has been a widely used combination chemotherapy regimen for metastatic melanoma, since this combination was reported to give an overall response rate of 47 % (McClay and McClay, 1994). However, the value of TAM in the Dartmouth regimen has been questioned and, while in a randomized trial no beneficial effect was reported (Rusthoven et al., 1996), other studies demonstrated advantage in patients treated with the TAM-containing regimen (McClay et al., 1989, 1992).

In a systematic review and meta-analysis of six randomized controlled trials to assess the benefit of TAM addition to various chemotherapy and biochemotherapy regimens, TAM failed to improve the overall response rate, the complete response rate or the survival rate in metastatic disease (Lens, 2003). More recently, Beguerie *et al* (2010) performed a meta-analysis including nine randomized controlled trials. The inclusion of TAM in systemic chemotherapy improved the overall and the partial response of patients with advanced melanoma, with female patients being more likely to respond (Beguerie et al., 2010). However, the incidence of hematologic toxicity was higher in the TAM group, and there was no improvement in 1-year mortality (Beguerie et al., 2010).

Thus, the many clinical trials already performed up to now produced inconclusive results and the benefits of TAM in chemotherapy regimens for melanoma remain highly controversial. The clarification of the mechanisms underlying the effects of antiestrogens on melanoma would certainly shed new lights on the role of antiestrogens in melanoma therapy

and help to identify subgroups of patients who would benefit from an antiestrogen-containing regimen. In fact, recent evidences raise new questions that may contribute to explain the contradictory results obtained in the clinical trials conducted so far. On the one hand, the establishment of an association between TAM metabolism and clinical outcome in breast cancer patients; on the other hand, the identification of a new estrogen receptor, the G protein-coupled receptor 30 (GPR30), also designated G protein coupled estrogen receptor 1 (GPER1).

1.2.1 - Importance of metabolism in the antiestrogenic activity of tamoxifen

TAM is extensively metabolized in the liver by phase I and phase II enzymes, including cytochrome P450 (CYP), sulfotransferases and UDP-glucuronosyltransferases (Kiyotani et al., 2012). The *N*-demethylation of TAM, primarily catalyzed by CYP3A4 and CYP3A5, produces the major metabolite of TAM, *N*-desmethyltamoxifen, which presents steady-state plasma concentrations twice as high as that of its parent drug (Fig. 1.3) (Brauch et al., 2009; Kiyotani et al., 2012). This metabolite, *per se* is not relevant for the biological activity of TAM, as it shows weak affinity for the ERs. The CYP2D6-mediated hydroxylation of *N*-desmethyltamoxifen at the *para* position of the phenyl ring is required to produce the more active metabolite 4-hydroxy-*N*-desmethyltamoxifen, referred as endoxifen (EDX) (Brauch et al., 2009; Kiyotani et al., 2012). Likewise, the other active metabolite of TAM, OHTAM, is formed by CYP2D6-mediated hydroxylation of TAM, also at the *para* position of the phenyl ring of the parent drug (Brauch et al., 2009; Kiyotani et al., 2012). TAM metabolism by CYP enzymes produces other metabolites, but EDX and OHTAM remain the only TAM metabolites with relevant pharmacological activity identified so far (Brauch et al., 2009; Kiyotani et al., 2012).

Since EDX and OHTAM have been considered responsible for the anticancer activity of TAM (Kiyotani et al., 2012), in the last years several studies were performed to clarify the impact of TAM metabolism in the clinical outcomes. The steady-state plasma concentrations of TAM, OHTAM and EDX in patients taking TAM (20 mg/day) are 72-160 µg/L, 1.15-6.4 µg/L and 8.1-20.7 µg/L, respectively (Brauch et al., 2009), but the concentrations reached in tissues are 10- to 60-fold higher (Lien et al., 1991). The wide range

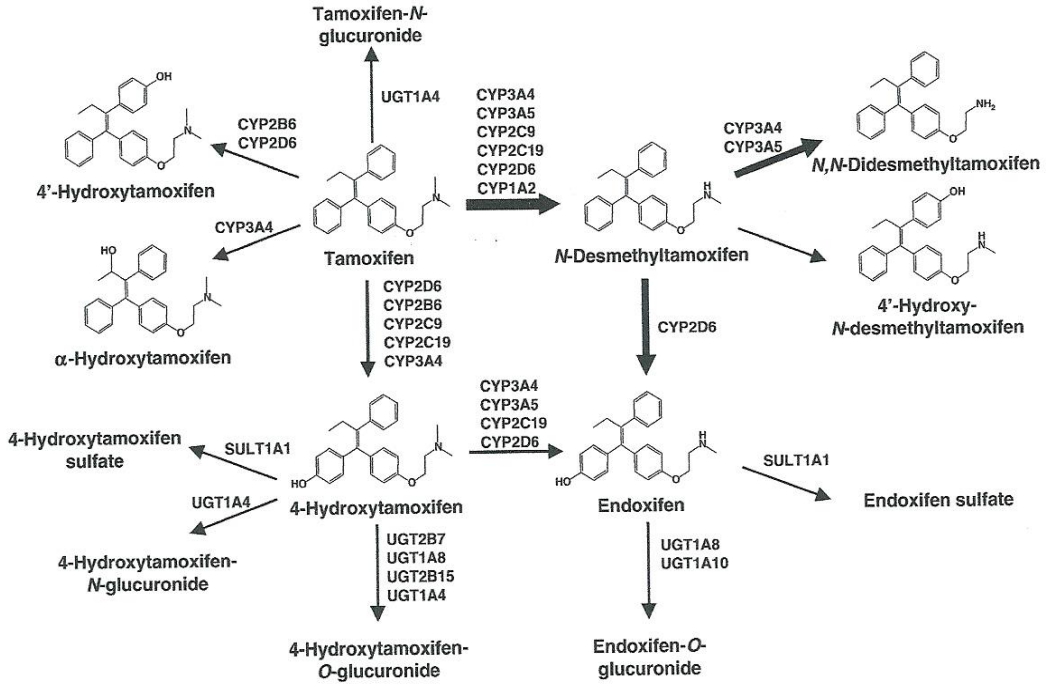


Fig. 1.3 - Major metabolic pathways of TAM in humans (Kiyotani et al., 2012).

of concentrations reported is thought to reflect genetic variants of CYP2D6 that lead to the production of enzymes with different functional activity. Individuals homozygous for alleles that produce enzymes with normal activity are named extensive metabolizers, whereas those carrying multiple copies of CYP2D6 alleles associated with high enzyme activity are termed ultrarapid metabolizers. Individuals with one or two variant alleles with reduced or null activity are designated intermediate or poor metabolizers, respectively (Higgins and Stearns, 2010). Importantly, the available evidence suggests that women treated with TAM who carry nonfunctional or reduced-function CYP2D6 alleles have lower circulating levels of EDX and achieve worse clinical outcomes in comparison with those with normal enzyme activity (Schroth et al., 2009; Higgins and Stearns, 2010; Lammers et al., 2010; Thompson et al., 2011). The incidence of single nucleotide polymorphisms in CYP2D6 varies by race and ethnicity and it estimated that approximately 7 % of caucasian women are homozygous for nonfunctional CYP2D6 alleles (Sachse et al., 1997; Mürdter et al., 2011). These observations may have implications not only in the treatment efficacy, but also in the selection of the optimal dose (Ingle, 2008; Irvin Jr et al., 2011).

Besides the genetic polymorphisms, several drugs can also influence the enzymatic activity of CYP2D6 and this way the biological activity of TAM (Kelly et al., 2010; Lammers et al., 2010; Binkhorst et al., 2012). The simultaneous administration of TAM and CYP2D6 inhibitors was associated with reduced EDX concentrations proportional to the potency of the inhibitor (Jin et al., 2005). Accordingly, a review of medical records showed that breast cancer patients simultaneously treated with TAM and CYP2D6-inhibiting medication had increased risk of recurrence (Aubert et al., 2009).

Considering that the CYP3A4/A5 and the CYP2D6 are involved in the metabolism of 37 % e 15 % of drugs, respectively (Zanger et al., 2008), the coadministration of CYP3A4/A5 and CYP2D6-inhibiting medication should be handled with care. Particularly relevant in the context of breast cancer therapy are the interactions with selective serotonin reuptake inhibitors, frequently prescribed to prevent the hot flushes experienced by up to 45 % of patients taking TAM (Ahmad et al., 2010a; Kelly et al., 2010).

The clinical utilization of TAM active metabolites may, therefore, present strong advantages relatively to the use of the prodrug TAM, as it avoids the intervariability in TAM metabolism, increasing the number of patients who would benefit from the treatment with

antiestrogens, and avoids drug interactions that can compromise the efficacy of the treatment.

In breast cancer therapy, much attention has been given to EDX, which is considered the major active metabolite of TAM, as it presents higher steady-state concentrations than OHTAM (Brauch et al., 2009; Gjerde et al., 2012). EDX has demonstrated an antiestrogenic potency similar to OHTAM that is 30-100-fold more potent than TAM (Lim et al., 2005). Moreover, at concentrations achieved in CYP2D6-extensive metabolizers, EDX also targets ERα degradation by the proteasome in breast cancer cells (Wu et al., 2009). Additionally, EDX is a potent inhibitor of aromatase activity (*Ki* = 4.0 μM), whereas high concentrations of TAM or OHTAM do not significantly affect the enzyme activity (Lu et al., 2012). These data suggest that EDX may present additional mechanisms of action in breast cancer therapy in comparison with TAM and OHTAM, and support the benefits of EDX over TAM. Furthermore, studies performed in healthy individuals demonstrated that EDX is safe and well tolerated and that the oral administration of 4 mg EDX/day allows achieving effective steady-state plasma concentrations (Ahmad et al., 2010b).

In contrast, although relatively well tolerated compared with cytotoxic chemotherapy, TAM induces several adverse effects. TAM treatment is associated with an increased incidence of vaginal bleeding, endometrial polyps, endometrial thickening, and ovarian cysts (Baum, 2002). Moreover, according to several trials performed, hot flushes are reported by approximately 40 % of the women taking TAM (Baum, 2002). Less common is the long-term risk of endometrial cancer and thromboembolic disease (Braithwaite et al., 2003). TAM has been associated with a decreased bone mineral density in premenopausal women, whereas an increase in postmenopausal women is observed (Ramaswamy and Shapiro, 2003). Several cases of hepatotoxicity during treatment with TAM (Labbe et al., 2008), hypertriglyceridemia (Sakhri et al., 2010), as well as hemolytic anemia, which has been related with the disruption of membrane structure (Cruz Silva et al., 2000), were also reported.

To our knowledge the studies performed in melanoma patients have not considered the possibility that the intervariability in TAM metabolism may also have an impact on the outcome of melanoma treatment and the pharmacological activity of TAM metabolites in melanoma has not been studied so far. These are matters deserving further investigation.

1.2.2 - GPR30: an emerging target in cancer therapy

Emerging evidence suggests that G protein-coupled receptors (GPCRs) play a central role in cancer onset, progression, tumor-induced angiogenesis, and metastasis (Lee et al., 2008). Therefore, the interference with GPCRs and their downstream targets has been investigated as an opportunity for the development of mechanism-based strategies for cancer therapy (Lee et al., 2008).

The GPR30 was identified in the late 1990s by four different groups (Owman et al., 1996; Carmeci et al., 1997; Takada et al., 1997; O'Dowd et al., 1998). Following this discovery, other papers have supported the view that the GPR30 is a 17β-estradiol-binding protein, structurally distinct from the classical ERs, which led to a new designation in 2007 – GPER1.

The subcellular localization of GPR30 remains controversial, although it has been reported that it is localized in the plasma membranes (Thomas et al., 2005; Funakoshi et al., 2006; Filardo et al., 2007) or in the endoplasmatic reticulum (Revankar et al., 2005). It has been suggested that the subcellular localization of GPR30 may vary according to the cell requirements, as different locations can possibly produce different biological activity (Prossnitz et al., 2008; Wang et al., 2010). However, according to more recent reports that describe the constitutive internalization of plasma membrane GPR30, the receptor localization seems to be predominantly intracellular (Cheng et al., 2011; Sandén et al., 2011).

The identification of G-1 in 2006 as the first GPR30-selective agonist, which shows no significant activity at concentrations up to 10 μM on classic ERs or on other GPRCRs (Bologa et al., 2006), and the identification of GPR30-selective antagonists G-15 and G-36 (Dennis et al., 2009, 2011) were a step further in the characterization of the GPR30-mediated signaling (Fig. 1.4). In addition to 17β-estradiol and G-1, TAM, OHTAM, fulvestrant and environmental estrogens, such as genistein and bisphenol A, were also found to bind to GPR30 (Fig. 1.4) (Wang et al., 2010). In contrast, estrone and estriol have very low binding affinities to GPR30, whereas 17α-estradiol and other steroid hormones cannot bind to GPR30 at all (Wang et al., 2010).

It is widely accepted that the interaction of 17β-estradiol with the ERs leads to a configurational change that allows the binding to estrogen response elements (ERE) in the promoter regions of target genes and the recruitment of additional transcriptional

coregulators, resulting in the regulation of target protein products. These events usually take hours to days to produce the biological actions characteristic of estrogen, but it can also trigger a variety of rapid signaling events, which occur in seconds to minutes, including the mobilization of intracellular calcium and the activation of multiple intracellular kinase cascades, namely the MAPK, PI3K, protein kinase A (PKA) and PKC pathways (Chen et al., 2008; Wang et al., 2010). Although there is evidence that also relates classic ERs to these nongenomic events, the structure of ERs lacks functional motifs that promote second messenger signaling (Filardo et al., 2000, 2002; Prossnitz et al., 2007). Indeed, around one third of the genes in humans that are regulated by estrogen do not contain ERE-like sequences and are typically regulated via interactions with other transcription factors (Teng et al., 2008). The initiation of these rapid signaling events is commonly associated with the activation of GPCRs and it has led to the investigation of the role of the GPR30 in estrogen-mediated signaling in breast cancer cells. It was reported that ligands, such as 17β-estradiol, TAM, fulvestrant and G-1, cross the plasma membrane and bind to GPR30. Then, through a Gβγ-subunit protein pathway, Src is activated and mediates matrix metalloproteinase (MMP)-dependent cleavage of pro-heparin-bound epidermal growth factor (HB-EGF); the free HB-EGF activates the epidermal growth factor receptor (EGFR), leading to downstream activation of ERK 1/2 (Fig. 1.5) (Prenzel et al., 1999; Filardo et al., 2000). On the other hand, via Gα subunit, 17β-estradiol, fulvestrant or TAM stimulate adenylate cyclase activity and induce cyclic adenosine monophosphate (cAMP)-dependent PKA-mediated suppression of the MAPK pathway, through Raf inactivation (Filardo et al., 2002). Thus, it was suggested that the activation of GPR30 can trigger opposing signaling mechanisms that regulate ERK 1/2 activity (Filardo et al., 2002). On the other hand, 17β-estradiol-mediated activation of GPR30 also stimulates PI3K activation (Revankar et al., 2005) and intracellular calcium mobilization (Fig. 1.5) (Revankar et al., 2005; Ariazi et al., 2010). It is not clear what triggers the increase in calcium. It was proposed that the activated phospholipase C (PLC) produces inositol triphosphate that binds to its receptor and leads to intracellular calcium mobilization (Prossnitz et al., 2008; Wang et al., 2010), but according to Revankar *et al* (2005) PLC does not seem to be involved (Fig. 1.5). Through these rapid actions, GPR30 can also regulate transcriptional activity. The activation of MAPK and PI3K pathways results in the phosphorylation and activation of transcription factors, such as the serum response factor

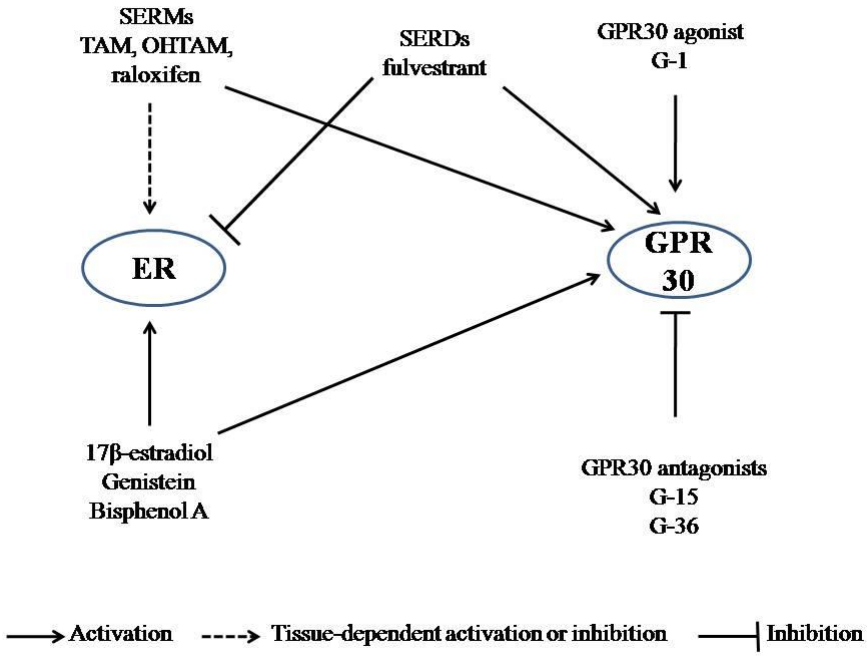


Fig. 1.4 - Agonists and antagonists of ER and GPR30. SERMs, selective estrogen receptors modulators; SERDs, selective estrogen receptors down-regulators (Modified from Barton, 2012).

(SRF) and members of the E26 transformation specific (ETS) family, such as Elk-1, whereas the increase in cAMP elicited by GPR30 signaling could be expected to activate the cAMP-responsive element-binding protein (CREB) (Fig. 1.5) (Prossnitz et al, 2008; Pandey et al., 2009; Wang et al., 2010). These transcription factors then promote the expression of a second wave of transcription factors, as shown in figure 1.5 (Pandey et al. 2009). The gene most strongly induced by 17β-estradiol or OHTAM is the connective tissue growth factor (CTGF), which is involved in cell proliferation and in GPR30-induced stimulation of cell migration (Fig. 1.5) (Pandey et al., 2009).

The GPR30-mediated signaling seems complex, as there may be a variety of cross-talk pathways and both negative and positive feedback loops (Fig. 1.6). For instance, Albanito *et al* (2008) demonstrated that the epidermal growth factor (EGF) up-regulates GPR30 expression through the MAPK pathway and considering that the GPR30 signals through this pathway, it is plausible that the GPR30 itself is part of a positive feedback loop (Maggiolini and Picard, 2010). Moreover, an interplay between ER and GPR30 for the regulation of transcriptional effects was recently demonstrated. Notas *et al* (2012) showed that the majority of the transcription inhibited by G-15 is also inhibited by fulvestrant and only a small number of transcripts is exclusively inhibited by G-15, supporting the existence of specific GPR30 transcriptional activity. Thus, the cross-talk between ER and GPR30 is a matter deserving further investigaton.

So far, the expression of GPR30 was detected in several tissues or cell lines, including heart, liver, lung, ovary, brain, breast, uterus, prostate, arteries and vessels (Mizukami, 2010; Wang et al., 2010) and has been shown to play a role in immunity, in the cardiovascular system and in glucose homeostasis (Wang et al., 2008; Haas et al., 2009; Martensson et al., 2009; Barton, 2012). Futhermore, the expression of GPR30 in osteocytes, osteoclasts, osteoblasts and chondrocytes and the bone preserving effects of 17β-estradiol, selective ER modulators and down-regulators, which act as GPR30 agonists, suggests that in addition to the classic ERs, this receptor may be involved in bone metabolism as well (Prossnitz and Barton, 2012). Emerging data also point to the involvement of GPR30, which is expressed throughout the CNS and peripheral nervous system of male and female rodents, in estradiol-mediated neurological functions (Prossnitz and Barton, 2012).

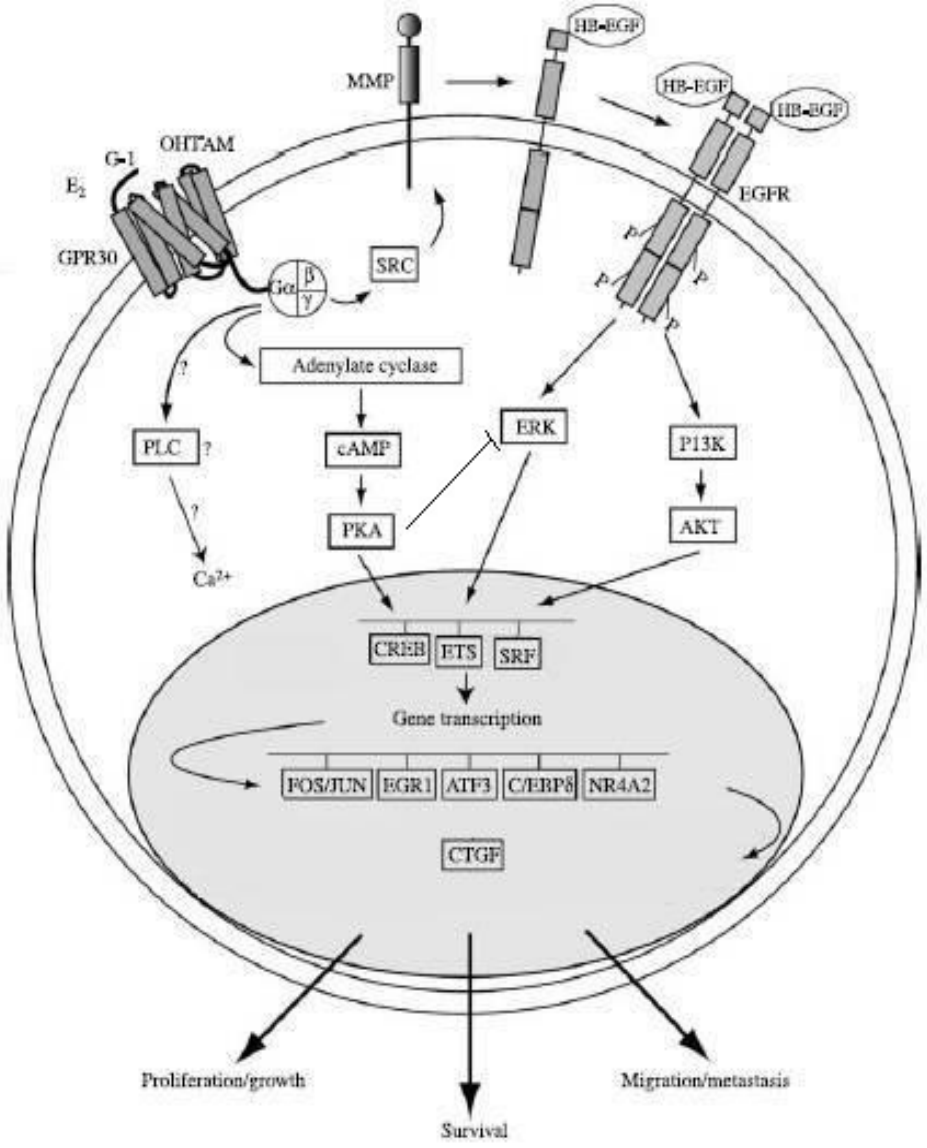


Fig. 1.5 - GPR30-mediated signaling. 17β-estradiol (E2), G-1 or OHTAM activate GPR30, leading to Src-mediated, MMP-dependent cleavage of pro-HB-EGF and release of HB-EGF that transactivates the EGFR, which in turn activates MAPK and PI3K pathways. GPR30 activation also stimulates PKA activity via adenylate cyclase. The final result is the activation of transcription factors, such as SRF, Elk-1 and CREB, which promote the expression of a second wave of transcription factors. CTGF is the gene most strongly induced by E2 or OHTAM. GPR30 activation also induces calcium mobilization, but it is not yet clarified what triggers the increase in calcium. GPR30 is arbitrarily located in the plasma membrane (Modified from Maggiolini and Picard, 2010).

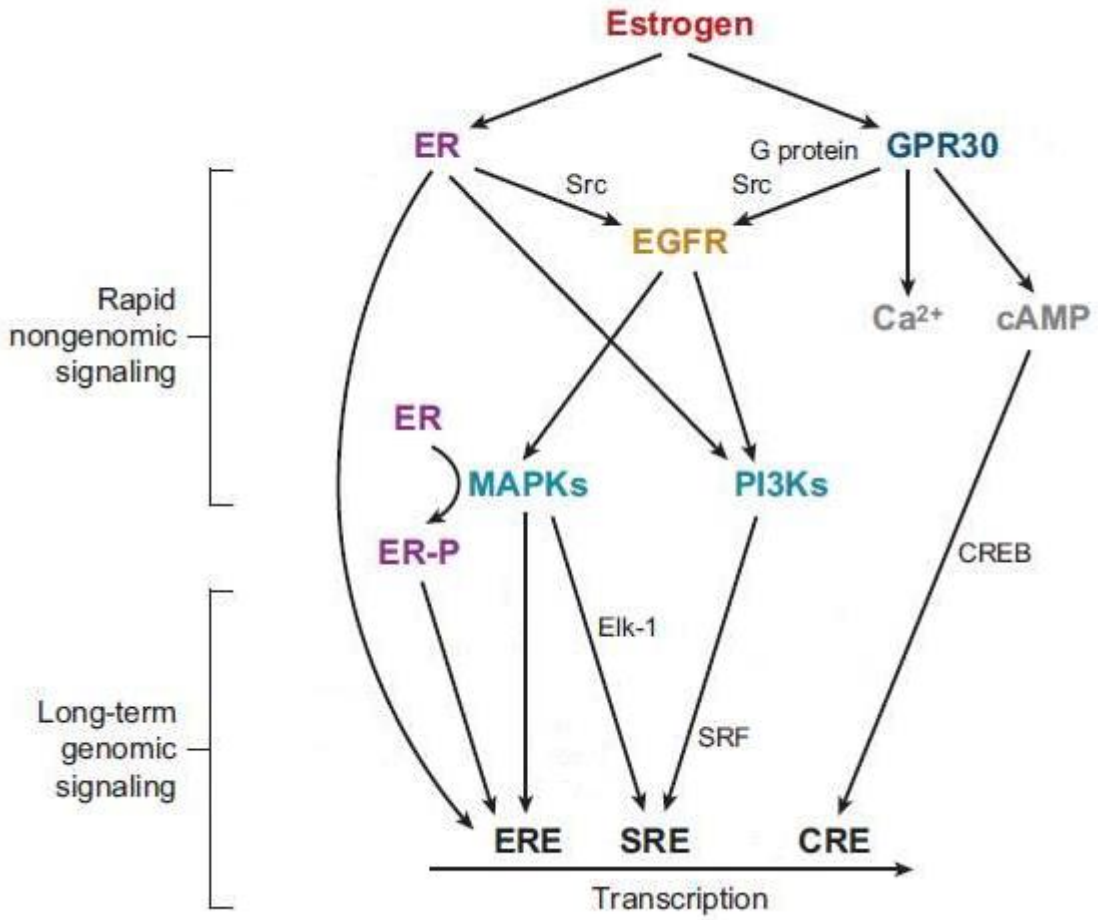


Fig. 1.6 - Interaction between ER- and GPR30-mediated signaling induced by estrogen. Estrogen can activate ERs and directly regulate transcription. Both ERs and GPR30 can activate cellular kinase pathways that can lead to the phosphorylation of ERs. The activation of MAPK and PI3K pathways also results in the phosphorylation and activation of transcription factors, such as Elk-1 and SRF, whereas cAMP production can induce CREB activation. CRE, cAMP response element; SRE, serum response element [Modified from Prossnitz *et al* (2008)].

The role played by GPR30 in cancer has only recently begun to be elucidated. The GPR30 is expressed in cancer cell lines and primary tumors of the breast (Filardo et al., 2000, 2006), endometrium (He et al., 2009; Du et al., 2012), ovaries (Fujiwara et al., 2012), thyroid (Vivacqua et al., 2006a), lung (Jala et al., 2012), prostate (Chan et al., 2010) and testicular germ cells (Chevalier et al., 2012).

As mentioned above, in ER− human breast cancer cells, the activation of GPR30 by 17β-estradiol or by OHTAM leads to the secretion of CTGF, which promotes cell proliferation and migration (Pandey et al., 2009). Accordingly, 17β-estradiol and OHTAM induce the proliferation of triple-negative breast cancer cells (lack ERα, progesterone receptor and do not overexpress human epidermal growth factor receptor 2) and increase the transactivation of EGFR, which is prevented by knockdown of GPR30 expression (Girgert et al., 2012). GPR30 also mediates the increased migration of MCF-7 cells [ERα+/ERβ+/ G protein-coupled receptor 30-positive (GPR30+)] promoted by OHTAM (Li et al., 2013). In SkBr3 breast cancer cells (ERα−/ERβ−/GPR30+), the hypoxia-inducible factor-1α up-regulates the GPR30 and its downstream target CTGF (Recchia et al., 2011). Moreover, it was shown that the apoptotic response to hypoxia is prevented by estrogens through GPR30, indicating that the GPR30 is involved in the adaptation of cells to a low oxygen microenvironment (Recchia et al., 2011). The GPR30 is also required for the proliferation and migration of cancer-associated fibroblasts induced by physiologic concentrations of 17β-estradiol, indicating that the GPR30 has a functional role in the regulation of tumor microenvironment (Madeo and Maggiolini, 2010).

The role of GPR30 in breast cancer response and resistance to hormonotherapy has also been investigated, given that the partial ER antagonists TAM and OHTAM behave as agonists for GPR30 (Wang et al., 2010). In fact, about 25 % of ER+ breast cancer patients do not respond to antiestrogen therapy, suggesting that such tumors remain estrogen responsive in spite of classic ER blockade through another pathway, and the development of resistance to TAM is frequent (Early Breast Cancer Trialists' Collaborative Group, 2005; Wang et al., 2010; Gigoux and Fourmy, 2013). Breast cancer TAM-resistant cells exhibit increased sensitivity to 17β-estradiol and G-1 when compared to the parental cells and in these cells TAM stimulates cell proliferation and MAPK phosphorylation (Ignatov et al., 2010a). 17β-estradiol and fulvestrant inhibit the TGF-β signaling through the activation of the MAPK pathway via GPR30, which is another mechanism known to contribute to

resistance to antiestrogens (Kleuser et al., 2008). Moreover, EGF and transforming growth factor-α (TGF-α) up-regulate GPR30 expression in TAM-resistant breast cancer cells (Vivacqua et al., 2009). As EGFR overexpression is associated with TAM resistance, the activation of EGFR can contribute to explain the failure of TAM therapy by up-regulating GPR30, which facilitates the action of both 17β-estradiol and TAM (Vivacqua et al., 2009). However, the correlation between GPR30 expression and ER expression, tumor characteristics and aggressiveness remains largely controversial (Notas et al., 2012).

While some reports point to a strong association between ER and GPR30 expression in breast cancer (Filardo et al., 2006; Broselid et al., 2013), others reported a low negative correlation (Tu et al., 2009). It was shown that the GPR30 is expressed in approximately 50 % of all breast cancers and correlates with increased tumor size, metastasis, poor outcome (Filardo et al., 2006), and tumor recurrence (Liu et al., 2009). Likewise, Ignatov *et al* (2011) detected GPR30 expression in almost 60 % of the breast cancer specimens, which is associated with shorter relapse-free survival. Noteworthy, GPR30 expression negatively correlates with relapse-free survival only in patients treated with TAM (with or without chemotherapy), but tends to be a favorable factor in patients who did not receive TAM (Ignatov et al., 2011). These results suggest that treatment with antiestrogens might in fact stimulate rather than inhibit a subset of TAM-resistant tumors and therefore breast cancer patients with high GPR30 expression may not benefit from treatment with TAM alone (Ignatov et al., 2011). In contrast, it was reported that the levels of GPR30 mRNA are significantly down-regulated in cancer tissues in comparison with their matched normal tissues and that the expression of GPR30 mRNA is significantly lower in tumor tissues from patients who had lymph node metastasis than in lymph node metastasis-negative patients (Poola et al., 2008). In agreement with these observations, GPR30 mRNA expression is down-regulated in infiltrating ductal carcinoma and a correlation with clinical parameters, including patient survival, was not established (Kuo et al., 2007). Accordingly, the study conducted by Tu *et al* (2009) did not support the existence of an association between GPR30 expression with the size and/or extent of the primary tumor, the spread to nearby lymph nodes or the presence of metastasis (TNM stage). Moreover, in inflammatory breast cancer, an aggressive and commonly hormone-independent form of breast cancer, GPR30 expression was not associated with overall or disease-free survival, but patients with GPR30 overexpression had prolonged disease-free survival although without statistical

significance (Arias-Pulido et al., 2010). In particular, tumors that expressed both GPR30 and ER showed a trend towards better overall or disease-free survival (Arias-Pulido et al., 2010). Moreover, the recent study conducted by Broselid *et al* (2013), supports that the GPR30 is a prognostic indicator of distant disease-free survival. Therefore, these conflicting reports represent a paradox to resolve in future studies, but considering the complexity of these cancers, it is likely that GPR30 may play a different role in different subsets of patients.

More consentual is the possible role played by the GPR30 in the promotion of endometrial and ovarian cancers by 17β-estradiol and antiestrogens. Through the GPR30, TAM, OHTAM, 17β-estradiol and G-1 promote the proliferation and invasive behavior of endometrial cancer cells, and the down-regulation of GPR30 or the blocking of MAPK pathway partly or completely prevented these effects (Vivacqua et al., 2006b; He et al., 2009; Ignatov et al., 2010b; Du et al., 2012). Moreover, there was a significant correlation between GPR30 expression and the TAM-induced endometrial pathology in breast cancer patients (Ignatov et al., 2010b). The expression of GPR30 in endometrial carcinoma was also considered an indicator of poor survival (Smith et al., 2007). In ovarian cancer cells, G-1 increases the phosphorylation of Akt via the EGFR (Fujiwara et al., 2012) and the expression of both GPR30 and ERα along with active EGFR signaling are required for 17β-estradiol or G-1-stimulated cell proliferation (Albanito et al., 2007). However, as pointed by the authors, G-1 induces proliferation in ERα−/GPR30+ breast cancer cells and therefore the requirement for ERα expression in GPR30/EGFR signaling may vary according to the cell type (Albanito et al., 2007). Additionally, 17β-estradiol and G-1 increase ovarian cancer cells motility and invasiveness by increasing the expression and activity of MMP-9 via GPR30 (Yan et al., 2013). The expression of GPR30 (Smith et al., 2009) or of both GPR30 and EGFR are significantly associated with a poor prognosis in patients with ovarian cancer (Fujiwara et al., 2012). On the other hand, Kolkova *et al* (2012) did not detect differences in GPR30 expression at the mRNA or at the protein level in patients with benign and malignant ovarian tumors, and although it was overexpressed in about one third of the malignant tumors, no relation between GPR30 and clinical parameters was found (Kolkova et al., 2012). The involvement of GPR30 in the promotion of cancer cells proliferation induced by 17β-estradiol and OHTAM was also demonstrated in thyroid cancer cells and involved the MAPK pathway (Vivacqua et al., 2006a).

In contrast to the reported promoting action of GPR30 on the proliferation of breast, endometrial, ovarian and thyroid cancer cells, inhibition was also demonstrated. Whereas GPR30 expression induces the growth of SkBr3 cells, G-1 inhibits MCF-7 cell progression stimulated by 17β-estradiol and RNA interference experiments supported the role of GPR30 in the inhibition of proliferation (Ariazi et al., 2010). Recently, it was also reported that the knockdown of the GPR30 decreases either basal or agonist-stimulated proapoptotic receptor signaling in MCF-7 cells (Broselid et al., 2013). Furthermore, it was demonstrated that the combination of G-1 with trastuzumab exhibit an additive effect on the reduction of breast cancer cells proliferation (Lubig et al., 2012). It was also shown that G-1 inhibits the proliferation of prostate cancer cells both *in vitro* and *in vivo* (Chan et al., 2010). The GPR30-mediated inhibition of prostate cancer cell proliferation induced by G-1 involves a sustained activation of ERK 1/2 and cell cycle arrest in the G2 phase (Chan et al., 2010). Moreover, G-1 decreases the proliferation of endothelial cells by blocking cell cycle progression in S and G2 phases (Holm et al., 2011). This antiproliferative effect elicited by G-1 occurred through a pathway other than MAPK, and was reproduced by fulvestrant, suggesting that GPR30 agonists may prevent unwanted formation of new blood vessels, as observed in angiogenesis. Interestingly, it was also reported that the activation of epithelial GPR30 antagonizes 17β-estradiol-induced uterine growth and mediates inhibition of ERK 1/2 and ERα phosphorylation (Gao et al., 2011). Thus, the effects resulting from the activation of GPR30 are complex and may depend on the specific cellular context.

Despite the conflicting reports on the role of GPR30 in cancer, targeting its activity represents an important and innovative strategy in estrogen-sensitive cancers as well as in other diseases in which estrogen signaling is important. The potential contribution of GPR30-mediated signaling to the effects of existing clinically approved drugs, such as TAM, has just begun to be explored. However, the knowledge regarding the expression of GPR30 and its role in malignancies other than endocrine-related cancers remains scarce so far. Since there is increasing evidence supporting the involvement of GPCRs in tumorigenesis and metastatic progression of melanoma, the evaluation of GPR30 function in melanoma requires investigation.

1.3 - Retinoids, retinoic acid receptors and melanoma

Retinoids, the naturally occurring derivatives of vitamin A (all-*trans* retinol), play an important role in a wide range of physiological functions, including vision, immunity and embryonic development (Zusi et al., 2002; Brtko, 2007; Tang and Gudas, 2011). Mammals do not synthesize vitamin A, and obtain it from animal food sources, such as milk products, eggs and fish oils, or from the processing of pro-vitamin β-carotene, which is found in several vegetables (Zusi et al., 2002; Tang and Gudas, 2011). The synthesis of the main biologically active derivative of Vitamin A, all-*trans* retinoic acid (RA), also known as tretinoin, requires the irreversible oxidation of retinol in target cells (Zusi et al., 2002).

The biological activity of retinoids in target cells is mainly mediated through the retinoic acid receptors (RAR) and the retinoid X receptors (RXR), which belong to the steroid/thyroid/vitamin D (VDR)/peroxisome proliferator-activated receptor (PPAR) family of intracellular or nuclear receptors that function as ligand-dependent transcription factors, modulating specific genetic programs (Tang and Gudas, 2011). In vertebrates, there are three isotypes (α, β and γ) for both RAR and RXR with distinct amino- and carboxy-terminal domains, each encoded by a different gene. Two major isoforms generated by alternative splicing or promoter use for RARα (α1 and α2), RARγ (γ1 and γ2), RXRα (α1 and α2), RXRβ (β1 and β2) and RXRγ (γ1 and γ2), as well as four major isoforms for RARβ (β1, β2, β3 and β4), have been identified up to now (Zusi et al., 2002; Brtko, 2007). RAR and RXR have different ligand specificity – whereas RA can only bind and activate RAR, with a similar affinity for all three isotypes, its isomer 9-*cis* can bind and activate all three RAR and RXR, but with different affinities (Zusi et al., 2002; Theodosiou et al., 2010).

The retinoid receptors share the same structure as other nuclear receptors (Fig. 1.7). The amino-terminal A/B-domain contains the activator function 1 (AF-1) that is independent on ligand. The central C-domain is a cysteine-rich region, termed DNA binding domain (DBD), which recognizes specific DNA sequences, the retinoic acid response elements (RAREs). The RARE for RAR/RXR is composed by two or more degenerate copies of the (A/G)G(G/T)TCA or of the more relaxed (A/G)G(G/T)(G/T)(G/C)A half-site motif organized in direct repeats or palindromes normally separated by a nucleotide spacer (Theodosiou et al., 2010). RAR/RXR heterodimers regulate transcription from DR5, DR2 and DR1 elements, which have five, two or one nucleotide spacer, respectively (Theodosiou

et al., 2010). The D-domain is a flexible structure that plays a role as a hinge of the receptor molecule. The carboxy-terminal E-domain harbors the ligand binding domain (LBD), comprising the activatior function 2 (AF-2), which is ligand-dependent, and a major dimerization interface (Zusi et al., 2002; Brtko, 2007; Theodosiou et al., 2010).

The transcriptional activation by RAR depends on the formation of the RAR/RXR heterodimer (Fig. 1.8), which occurs on the DBD interface and on the LBD interface, that stabilizes, without changing, the binding directed by the DBD (Theodosiou et al., 2010). In the absence of RAR ligands, the DNA bound RAR-RXR heterodimers recruit corepressor proteins, including the nuclear receptor corepressor-1 and -2, and associated enzymes, such as histone deacetylases (HDAC) or DNA-methyl transferases, which lead to an inactive condensed chromatin structure hindering the transcription process (Zusi et al., 2002; Brtko, 2007). Upon binding of a ligand, such as the agonist RA, the α-helix 12, which is at the carboxy-terminal end of the RAR LBD, alters the three-dimensional surface of the RAR. The heterodimer with bound ligand interacts with coactivator proteins, such as the steroid receptor coactivator-1, -2 and -3, and proteins with histone acetyltransferases activity, with a higher affinity and the corepressor proteins are released. RAR/RXR complexes also recruit adenosine 5'-triphosphate (ATP)-dependent chromatin remodeling complexes and the mediator complex to promoters. These events enable the communication of RAR-RXR heterodimers with the basal transcriptional machinery to initiate target gene expression (Zusi et al., 2002; Brtko, 2007; Tang and Gudas, 2011). Besides the RAR, the RXR can heterodimerize with many other members of nuclear receptor superfamily and thus play an integrative role in nuclear receptor mediated pathways. However, whereas RAR agonists autonomously activate transcription through RXR/RAR heterodimer, RXR are unable to respond to RXR specific ligands in the absence of a RAR ligand. This phenomenon, referred to as ''RXR subordination'', is due to the inability of RXR ligands to induce the dissociation of corepressor molecule from the RXR/RAR heterodimers (Brtko, 2007; Theodosiou et al., 2010).

Over the past decades, more that 500 genes have been identified as regulatory targets of RA. Although a direct regulation driven by liganded RAR/RXR heterodimers bound to RARE was demonstrated, it seems that in most cases the regulation of the gene target is indirect and occurs through intermediate transcription factors or nonclassical

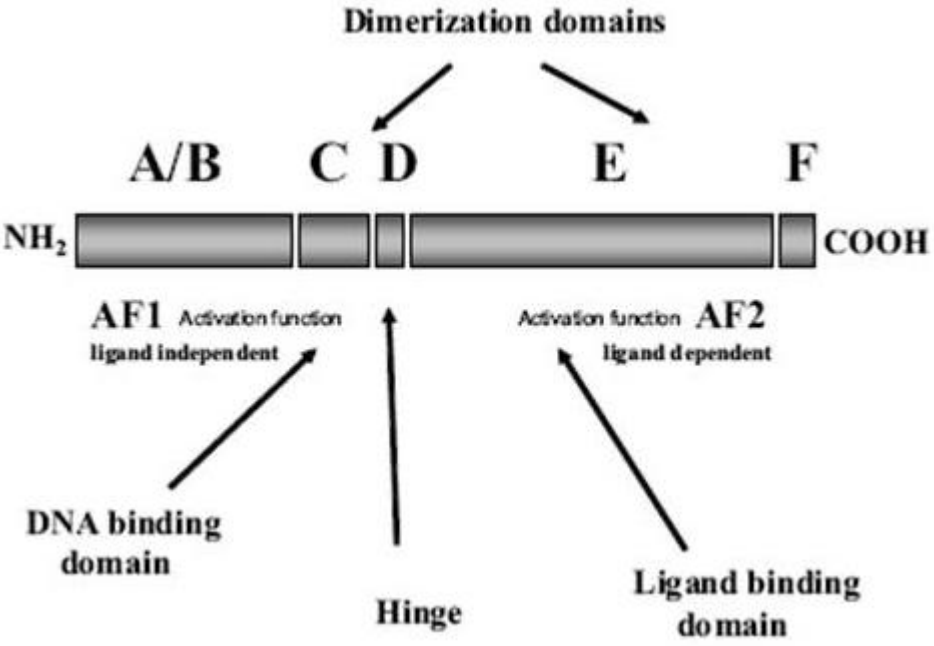


Fig. 1.7 - Structural organization of nuclear receptors (Brtko, 2007).

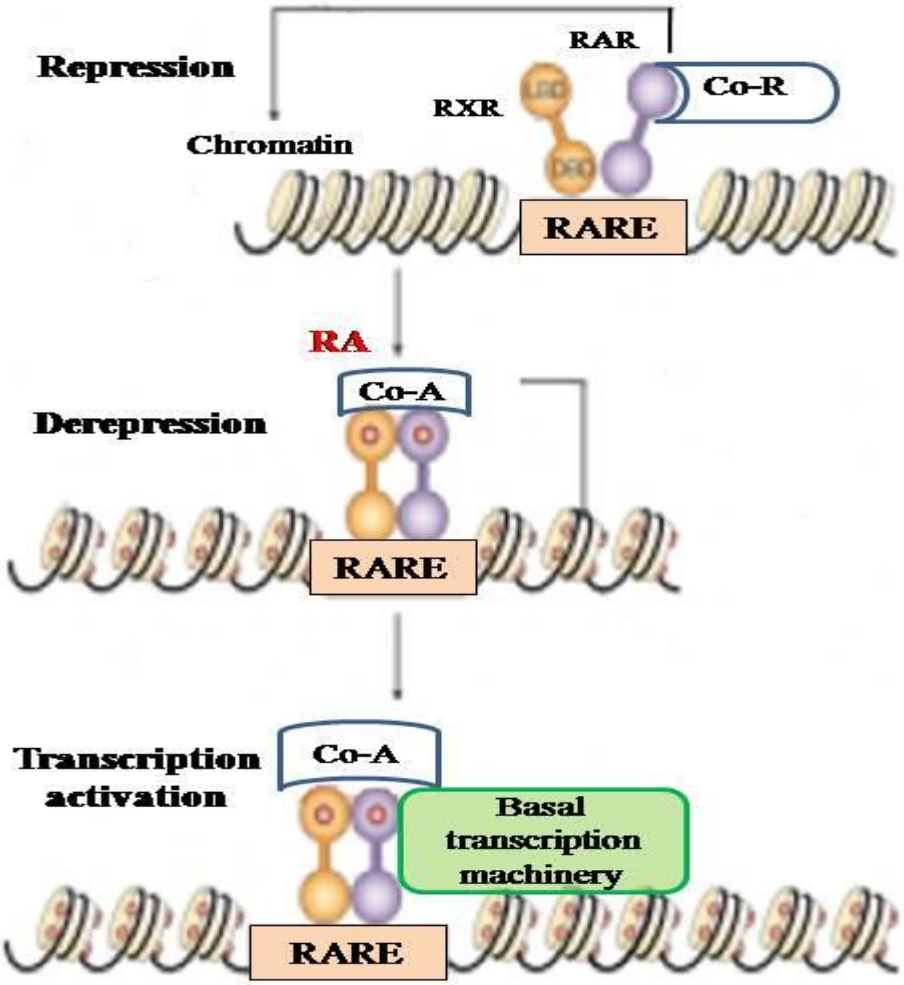


Fig.1.8 - Mechanism of transcription regulation by RAR-RXR heterodimers. In the absence of a RAR ligand, RAR-RXR heterodimers form multiprotein complexes with corepressor (Co-R) proteins, resulting in transcription silencing. The presence of a RAR ligand generates a conformational change that disrupts Co-R binding and induces derepression of the chromatin. The association of coactivator (Co-A) with RAR-RXR enables the recruitment of basal transcription machinery to the promoter and the subsequent activation of various genetic programs (Modified from Altucci and Gronemeyer, 2001).

associations of receptors with other proteins (Theodosiou et al., 2010). Some of the RAR target genes include those encoding RARβ itself, laminin B1, cellular retinol-binding protein, cellular retinoic acid-binding protein, as well as the homeobox genes, which are key players during development (Balmer and Blomhoff, 2002; Theodosiou et al., 2010). Besides the proteins involved in RA metabolism, such as CYP26, RARs also regulate a number of factors involved in metabolism, for example the 17β-hydroxysteroid dehydrogenase, which is implicated in steroidogenesis, or the liver bile acid transporter sodium-taurocholate cotransporting polypeptide (Theodosiou et al., 2010).

In addition to the effects promoted by binding to their receptors, retinoids also exert their effects via a transcription independent pathway (Bushue and Wan, 2010; Theodosiou et al., 2010). The activation of the PI3K signaling pathway is required for RA-induced differentiation of SH-SY5Y neuroblastoma cells (Masiá et al., 2007). Retinoids also inhibit the activation of activator protein 1 (AP-1), a heterodimeric transcription factor, which usually mediates cell proliferation signals (Fanjul et al., 1994; Benkoussa et al., 2002). Since transcriptional activation is not required for RAR-mediated AP-1 inhibition, the effects of retinoids on AP-1 involve the RAR-dependent control of transcription factors and cofactor assembly on AP-1-regulated promoters (Fanjul et al., 1994; Benkoussa et al., 2002). The activity of the nuclear factor-κB (NF-κB) is also regulated nongenomically by RA, probably in a RAR-dependent manner, suggesting that the modulation of immune and inflammatory responses by retinoids involves NF-κB signaling (Austenaa et al., 2004; Theodosiou et al., 2010). In the absence of retinoid receptors, RA can also induce the rapid activation of CREB protein, which involves PKC, ERK 1/2 and p90 ribosomal S6 kinase activity, as demonstrated in human bronchial epithelial cells (Aggarwal et al., 2006). Importantly, it has been reported the existence of a specific RA-binding site on PKC and the interaction between RA and PKCα decreases PKC activity (Radominska-Pandya et al., 2000). The fact that RA treatment modulates PKC activity might be useful to alter malignant cells functions regulated by PKC isozymes, such as proliferation, differentiation or apoptosis.

RA and its isomers 13-*cis*-retinoic acid and 9-*cis*-retinoic acid are widely used in the treatment of skin conditions, including acne, psoriasis and photoaging (Germain et al., 2006). On the other hand, retinoid signaling is often compromised early in carcinogenesis and epidemiological studies have demonstrated that lower vitamin A intake results in a higher risk of developing cancer (Sun and Lotan, 2002). Several preclinical and clinical

studies have evaluated the use of RA and other synthetic retinoids, alone or in combination therapy, in the treatment of breast, ovarian, renal, head and neck, melanoma and prostate cancers (Tang and Gudas, 2011). However, it is not yet clarified whether retinoids induce cell proliferation arrest, cell death, cell differentiation, or all of these mechanisms, but positive therapeutic effects were reported (Tang and Gudas, 2011).

In 1995, the FDA approved RA for the treatment of acute promyelocytic leukemia (APL), which remains the most studied and effective clinical use of RA up to now. APL is characterized by selected expansion of immature myeloid precursors or malignant myeloid cells blocked at the promyelocytic stage of hemopoietic development (Bushue and Wan, 2010). Molecular studies identified the translocation of RARα gene on chromosome 17 and fusion to partner genes as the crucial event in APL pathogenesis. The fusion partners include the promyelocytic leukemia zinc finger (PLZF) gene, the nucleophosmin gene, the nuclear mitotic apparatus gene, and the signal transducer and activator of transcription 5B gene. However, the most common fusion partner is the promyelocytic leukemia protein (PML) gene on chromosome 15 yielding the PML-RARα chimeric gene t(15;17) (Zusi et al., 2002; Brtko, 2007; Bushue and Wan, 2010). The PML-RARα can form a homodimer through the coiled coil motif of PML, inhibiting RARα binding to RARE and the activation of downstream target genes (Brtko, 2007; Bushue and Wan, 2010). The higher affinity of PML-RARα for HDAC leads to a 'super-repression' of RARα signaling pathways in APL blasts and to the disruption of PML function as a transcriptional coactivator of the tumor suppressor p53 (Zusi et al., 2002).

The introduction of RA into the therapy of APL radically changed the management and the outcome of this disease, which was previously associated with a significant morbidity and mortality. RA induces the differentiation of cells by causing the degradation of the mutant PML-RARα and by inducing the dissociation of its corepressors (Brtko, 2007; Bushue and Wan, 2010). In addition, RA inhibits cell proliferation by blocking cell cycle progression in G1 and high concentrations of RA induce post-maturation apoptosis of APL blasts (Brtko, 2007; Bushue and Wan, 2010).

Currently, RA is included in every phase of the treatment of pediatric APL in combination with anthracyclines, leading to a complete remission in 90-95 % of the cases (Masetti et al., 2012). Noteworthy, some RA-resistant human APL cells present impaired degradation of PML/RARα, while others show mutations in the LBD of RARα of PML-

RARα (Zhou et al., 2002). A rare variant of APL, characterized by a chromosomal translocation of the RARα and the PLZF genes, causes expression of a PLZF-RARα fusion protein that has a second binding site for a nuclear corepressor complex with HDAC activity leading to a very stable association between PLZF-RARα and the corepressor complex. This leukemia is nonresponsive to RA, but the combination with HDAC inhibitors can lead to remission in animal models or blast differentiation *in vitro* (He et al., 2001; Petti et al., 2002). Thus, combination treatments can possibly overcome RA resistance in other types of tumors as well.

The successful application of RA for the management of APL renewed the interest in the use of retinoids for the treatment of a variety of established malignancies due to their differentiation, antiproliferative, proapoptotic and antioxidant effects (Bushue and Wan, 2010). Since the late 1970's, the use of retinoids in malignant melanoma prevention and treatment has been extensively evaluated. Both normal human melanocytes and melanoma cells exhibit mRNA for RARs (α, β and γ) and RXRα (Rosdahl et al., 1997). Accordingly, the study of Zhang *et al* (2003) demonstrates the presence of RARs and RXRα in eleven primary and matched metastatic cutaneous melanoma cell lines, and no differences were detected between the primary and metastatic tumors.

RA decreases the proliferation of melanoma cell lines S91, B16 and K1735-M2 (Lotan et al., 1978; Helige et al., 1993). It has been proposed that the inhibition of the proliferation of primary and metastatic melanoma cells involves mitochondrial dysfunction, given that low concentrations of RA decrease the viability of melanoma cells mitochondria in a concentration-dependent manner (Zhang et al., 2003). Alterations of the cell cycle distribution and, at higher concentrations, apoptotic cell death were also reported (Zhang et al., 2003; Baroni et al., 2003). Noteworthy, the dysfunction of mitochondria and the induction of apoptosis are more pronounced in the primary tumor cells than in the metastatic cells from the same patients, suggesting that an early stage melanoma may have better response to retinoid adjuvant therapy than the late stage melanoma (Zhang et al., 2003). The antiproliferative effects reported *in vitro* are further supported by studies in animal models of melanoma. Retinal, retinol and RA decreased melanoma cell growth *in vitro* and *in vivo*, and RA was the most effective retinoid (Drewa and Schachtschabel, 1985). In another mouse model of melanoma, 13-*cis* retinoic acid treatment does not significantly affect the tumor

incidence, but decreases the tumor growth and the number of metastatic lesions (Schleicher et al., 1988).

However, other studies provided conflicting results. In the study conducted by Lotan (1979), RA decreases the proliferation of two melanoma cell lines, but the remaining four cell lines tested do not respond to RA, indicating that human cells derived from tumors of similar histopathological type may differ in their responsiveness to RA. Several explanations may contribute to this discrepancy. Boehm *et al* (2004) demonstrate that RARβ and RXRα are present in normal skin, but RXRα is not detected in melanocytic tumors, neither in nevi, nor in melanomas, whereas RARβ is also absent from melanoma cells, but it is present in nevus cells (Boehm et al., 2004). In fact, melanoma progression seems to be characterized by a simultaneous loss or decrease in the expression of various RAR and RXR receptors, which result in deficient RAR/RXR heterodimers (Chakravarti et al., 2007). This decreased retinoid receptor expression may render retinoids unable to turn on normal cellular programs and contributes to retinoid resistance (Chakravarti et al., 2007). The patients with simultaneous loss of cytoplasmic staining for RARα and RXRα have decreased overall survival, indicating that the loss of retinoid receptor expression correlates with melanoma progression (Chakravarti et al., 2007). However, those with RARα−/RXRα+ phenotype have better prognosis suggesting that in patients lacking expression of RARs, RXRα can contribute to inhibit cell proliferation via a different pathway (Chakravarti et al., 2007).

Considering that ERK is constitutively activated in up to 90 % of melanomas (Russo et al., 2009), and that the turnover of retinoid receptors is regulated by their phosphorylation status, it is possible that aberrant MAPK pathway signaling may contribute to decrease the expression of retinoid receptors (Chakravarti et al., 2007). The elevated levels of corepressors and/or the loss of expression of coactivator proteins, in addition to the increased activity of the Akt pathway, have also been proposed as possible explanations for the diminution of RAR-regulated signaling in tumor cells (Tang and Gudas, 2011). On the other hand, the study of Demary *et al* (2001) supports that RA resistance in A375 cells involves repressed RAR activity through an epigenetic mechanism. RA-resistant melanoma A375 cells present substantially higher intracellular reactive oxygen species (ROS) than S91 cells and the reduction of oxidative stress is able to resensitize cells to RA (Demary et al., 2001). As the functional activity of several important transcription factors is regulated by the cellular redox state and reducing conditions promote DNA binding, the redox state of

melanoma cells provides for an epigenetic control mechanism of RAR activity and RA resistance (Demary et al., 2001). Moreover, hypermethylation of RARβ2 was found in 70 % of both primary and metastatic melanomas and the mRNA transcripts could be re-expressed following treatment with 5'-aza 2'-deoxycytidine, supporting that promoter methylation is responsible for silencing of RARβ2 in some melanoma cells (Hoon et al., 2004; Fan et al., 2010). Importantly, it was demonstrated that the antiproliferative activity of RA correlates with the expression of RARβ2 (Fan et al., 2010).

In addition to its antiproliferative activity, RA can also exhibit an anti-invasive effect on melanoma cells. RA inhibits B16a and K1735-M2 melanoma cells migration and invasion *in vitro* and *in vivo*, which adds to the decreased proliferation observed in these cells (McGarvey and Persky, 1989; McGarvey et al., 1990). N-(4-hydrophenyl) retinamide, a derivative of RA, also suppresses melanoma cells migration and invasion through reconstituted basement membrane (Wu et al., 2005). Tumor invasion is a complex process that involves changes in cellular adhesion, production of degradative enzymes that lead to host tissue degradation, alterations in the actin cytoskeleton and increased motility, and RA affects all of these processes in melanoma cells.

Treatment of M14 melanoma cells with RA down-regulates the expression of vitronectin receptor ($\alpha_v\beta_3$), a member of the integrin superfamily, which is thought to play an important role in regulating tumor cell invasion, and closely correlate with the RA-dependent inhibition of actin polymerization, cell adhesion and migration (Baroni et al., 2003). On the other hand, melanoma cell lines treated with RA express decreased amounts of gp78, a cell surface receptor for motility factor implicated in invasion and metastasis (Hendrix et al., 1990; Lotan et al., 1992). In addition, the carbohydrate sulfotransferase 10 is a RARγ target gene in S91 melanoma cells, where it functions as a suppressor of invasiveness (Zhao et al., 2009).

RA at 1 μM only slightly decreases the directional migration of melanoma cells, an indicator of cell motility (Helige et al., 1993). The study conducted by Situ *et al* (1993) also demonstrates that RA treatment blocks adhesion and motility of mouse and human melanoma cells; the malignant cells are less sensitive to RA than normal melanocytes in the adhesion assay, although equally sensitive in the motility assay.

Furthermore, A375 human melanoma cells treated with RA secrete lower levels of collagenolytic enzymes and decrease in a dose- and time-dependent manner the ability of

these cells to penetrate Matrigel-coated filters (Hendrix et al., 1990). Moreover, the evaluation of host tissue degradation induced by K1735-M2 melanoma cells invasion, revealed that after treatment with RA the stromal component is more intact (Helige et al., 1993). RA also regulates the expression of MMP-1 and the tissue inhibitor of metalloproteinases-2 in a panel of melanoma cell lines (Jacob et al., 1998). Altogether, these data support that RA can decrease melanoma cell invasion through the modulation of several cellular properties.

The differentiation process has been associated with reorganization of actin (Lehtonen et al., 1983) and retinoids are known to have profound effects on cellular differentiation (Bushue and Wan, 2010). Accordingly, studies using rhodamine-labeled phalloidin demonstrate that K1735-M2 melanoma cells treated with RA exhibit alterations of the actin cytoskeleton, exhibiting numerous filament bundles (Helige et al., 1993).

Recent studies also suggest that retinoids may be useful as antiangiogenic agents. RA has demonstrated anti-inflammatory activity in an animal model of carrageenan-induced acute inflammation and a formaldehyde-induced chronic inflammation (Siddikuzzaman and Grace, 2013). In addition, it inhibited in about 56 % the tumor-associated capillary formation in mice induced by the highly metastatic B16F10 melanoma cells (Siddikuzzaman and Grace, 2013). Accordingly, 13-*cis*-retinoic acid inhibited vascular endothelial cell proliferation, migration and tube formation and regulated the levels of proinflammatory cytokines during the onset of angiogenesis (Guruvayoorappan and Kuttan, 2008).

Regarding the use of retinoids in the preventive setting, both acitretin and 13-*cis*-retinoic acid are effective for the prevention of non-melanoma skin cancer, but further studies are required to establish the most suitable retinoid, the dosage and the duration of the preventive treatment and how to manage side effects in the case of long-lasting treatment (Bettoli et al., 2013). Moreover, a recent large prospective cohort supported that supplemental intake of retinol is associated with a reduction in risk of melanoma (Asgari et al., 2012). Whereas the risk reduction in females is pronounced, the risk reduction in males is not statistically significant, which may be related with gender differences in melanoma outcomes (Asgari et al., 2012).

In spite of the strong rationale supporting the inclusion of retinoids in melanoma prevention and therapeutic regimens, their use in clinical practice has been hampered by their adverse effects. Vitamin A excess is toxic to several organs and tissues, including the

liver, CNS, musculoskeletal system, internal organs and skin and can lead to reduced mineral bone density and to malformations during embryonic development (Theodosiou et al., 2010). Retinoids are also known teratogens and inhibit chondrogenesis and induce mucocutaneous cytotoxicity (Zusi et al., 2002). In phase I/II studies, RA induced cheilitis, skin reactions, headache, nausea and vomiting, as well as transient hypertriglyceridemia and elevations of liver enzymes (Lee et al., 1993; Budd et al., 1998). Skin toxicities and headaches were considered to be the dose-limiting toxicity, and the maximum tolerated dose established is in the 150-190 mg/m^2/day range (Lee et al., 1993; Budd et al., 1998). RA syndrome is a life-threatening complication seen in patients treated with RA, characterized by dyspnea, fever, weight gain, hypotension, edema, pleural or pericardial effusions and, occasionally, renal failure and increased leukocyte counts (Zusi et al., 2002; Bushue and Wan, 2010).

A possible strategy to decrease the retinoid associated toxicity is the combination with other chemotherapeutic agents. Besides minimizing the development of resistance, the combination of drugs with complementary mechanisms of action and different toxicity profiles allows to obtain equivalent or even superior efficacy, while substantially reducing toxicity, through the use of lower doses.

The interconnection between retinoid and estrogen signaling in breast cancer has garnered significant attention in the last years. Whereas retinoids decrease the proliferation of ERα+ breast cancer cells, ERα− cells are usually resistant, but can be turned into retinoid-responsive when transfected with ER (Sheikh et al., 1993; Rubin et al., 1994; Rosenauer et al., 1998). Noteworthy, the effects of 9-*cis* retinoic acid are similar to those of RA, suggesting that the ability to bind both RAR and RXR does not enhance the growth inhibition induced by retinoids and that RARs are the key players regarding the antiproliferative effects of retinoids in breast cancer cells (Rubin et al., 1994). Furthermore, an additive/synergism of action was reported for the combination of RA with either TAM or OHTAM in breast cancer cells (Wetherall and Taylor, 1986; Wang et al., 2007; Koay et al., 2010). Moreover, in a phase I/II trial in patients with potentially hormone responsive advanced breast cancer, significant responses were observed in patients taking the combination of RA with TAM who had previously shown evidence of TAM resistance (Budd et al., 1998). These observations support the existence of a cross-talk between the RAR and ERα pathways in human breast cancer and suggest a possible combination therapy in ERα+/RAR+ breast

cancers. Several studies conducted to clarify the mechanism underlying this interplay have supported this hypothesis.

Both 9-*cis* retinoic acid and RA induce down-regulation of the expression of ER itself or estrogen-responsive genes in MCF-7 cells (Rubin et al., 1994). Rousseau *et al* (2003) demonstrated that in ERα+ cells the basal expression of the RARE RARβ2 is suppressed but can be strongly induced in the presence of RA, while in the ERα− cells the transcription is only weakly induced by RA (Rousseau et al., 2003). The induction of RARβ2 transcriptional activity when ERα is expressed involves the N-terminal AF-1-containing region, including the DBD, and it is independent of the C-terminal LBD (Rousseau et al., 2003). Accordingly, OHTAM, which inhibits AF-2-mediated transcriptional activity of ERα, while allowing the activity from the AF-1, had no significant effect on βRARE activity, but the full ER antagonist fulvestrant, which blocks both the AF-1 and AF-2 activities of ERα, was able to relieve the repression of basal βRARE activity (Rousseau et al., 2003). The fact that ERα does not alter the transcription mediated by the VDR or thyroid hormone receptors, which also heterodimerize with the RXR, indicates that the effect of ERα is specific for RAR-mediated transcription (Rousseau et al., 2003). More recently, using chromatin immunoprecipitation, Hua *et al* (2009) demonstrated that RARα/RARγ binding throughout the genome is highly coincident with ERα binding in MCF-7 human breast cancer cells, often leading to competitive binding activity to the same element or nearby *cis*-regulatory elements. The subsequent transcriptional analysis revealed an antagonistic regulation of breast cancer-associated genes by RA and estrogen (Hua et al., 2009). Importantly, the putative direct targets of ERα and RAR identified in MCF-7 cells were found to be highly expressed in luminal type breast tumors, indicating that their antagonistic effects may be relevant for primary ER+ tumors. On the other hand, considering that estrogen is the predominant hormone in breast cancer biology, Ross-Innes *et al* (2010) studied the interaction between ER and RARα in the presence of estrogen. Under these circumstances, RARα and ERα can co-occupy regulatory regions together within the chromatin (Ross-Innes et al., 2010). In fact, RARα is part of the ER transcription complex, where it plays an essential role in the regulation of estrogen-mediated gene expression by maintaining ER-cofactor interactions (Ross-Innes et al., 2010). The authors proposed that RARα can have two independent functions: a classic role as a heterodimeric partner of RXR and another one, as an ER-associated protein (Ross-Innes et al., 2010). The presence of RA ligands promotes

the classic RARα role, leading to activation of the classic RARα pathways; that way RARα is depleted from its role as a component of the estrogen-ER pathway, sacrificing estrogen-mediated gene expression (Ross-Innes et al., 2010). This occurs either when RARα agonists or antagonists are used, explaining why both can decrease breast cancer cell proliferation (Dawson et al., 1995; Toma et al., 1998). Thus, the balance between the classic and the novel pathways of RARα is an important regulator of ER function in breast cancer cells.

Considering that estrogen-independent ER signaling has been implicated in the development of resistance to adjuvant therapy, a recent study conducted by Salazar *et al* (2011) evaluated the interplay between the apo- forms (ligand free) of ER and RAR and its impact on basal proliferation, under the conditions of hormonal adjuvant therapy of hormone depletion or antiestrogen antagonism. The basal cellular proliferation persists in estrogen-sensitive breast cancer cells grown in hormone depleted conditioned media and it is inhibited by down-regulating ER (Salazar et al., 2011). Remarkably, the action of apo-ER is also insensitive to OHTAM at a dose that was clinically relevant (Salazar et al., 2011). According to this study, a major mechanism by which ER supports the cell cycle in hormone-sensitive cells depleted of hormone or treated with OHTAM is by promoting the basal expression of RARα1, through a direct regulation of the RARα and by additional post-transcriptional mechanisms (Salazar et al., 2011). Another important observation is that most of the common target genes of apo-ER and apo-RARα1 are insensitive to RA, suggesting that the apo-RARα1 acts by nonclassical mechanisms and that the hormone-sensitive breast cancer cells may not be sensitive to conventional RAR ligands, but specific inactivators or down-regulators of RARα1 would be useful instead (Salazar et al., 2011). Thus, there is also an interaction between the ligand-free ER and RARα that contributes to the persistence of a small fraction of cells in S-phase during hormonal adjuvant therapy in breast cancer, contributing to the development and progression of resistant tumors (Salazar et al., 2011).

Altogether, the studies performed up to now support that the combinations of antiestrogens and retinoids may be useful in the treatment of breast cancer, due to an interaction between retinoid and estrogen signaling in these cells. However, it is not known whether these combinations may be useful in other types of cancer cells as well. Given that the use of retinoids in melanoma therapy has been hindered by their adverse effects, a possible synergistic action with antiestrogens would largely contribute to turn retinoids into more suitable drugs for melanoma therapy.

1.4 - Glutamate receptor antagonists and cancer therapy

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and exerts its biological activity through a variety of receptors. Glutamate receptors (GluRs) are divided into two major classes on the basis of the mechanism by which they relay their signal: the ionotropic glutamate receptors (iGluRs), which are ligand-gated cation channels, and the metabotropic glutamate receptors (mGluRs) that are G protein-coupled receptors (Teh and Chen, 2012). The iGluRs are further subdivided into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate, KA) receptors, according to the agonists that were originally identified to activate them selectively (Fig. 1.9) (Kew and Kemp, 2005). Eight subtypes of mGluRs have been identified so far and classified into three groups according to their sequence homologies, pharmacological responses and intracellular second messengers (Fig. 1.9). The group I (mGluR1 and mGluR5) upon activation stimulates PLC, whereas group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) are negatively coupled to adenylate cyclase (Kew and Kemp, 2005; Niswender and Conn, 2010; Teh and Chen, 2012).

The NMDA receptors (NMDARs) are composed of two obligatory GluN1 subunits, which impart on heteromeric NMDAR channels ion permeability, GluN2 subunits A, B, C, and D, as well as GluN3 subunits A and B, which determine the electrophysiological properties of the channel (Cull-Candy et al., 2001; Kew and Kemp, 2005). The NMDAR requires two obligatory coagonists binding at the glycine (or D-serine) and glutamate binding sites localized on the GluN1 and GluN2 subunits, respectively (Traynelis et al., 2010). Thus, it seems likely that the NMDAR is a tetrameric structure composed of a GluN1 dimer in combination with a GluN2 dimer; in the receptors containing GluN1, GluN2 and GluN3 subunits, the latter possibly substitutes one of the GluN2 subunits, creating a functional receptor that is not affected by glutamate (Cull-Candy et al. 2001; Kew and Kemp, 2005). The opening of the NMDAR requires simultaneous activation by glutamate and depolarization to abrogate the magnesium block, allowing the entry of calcium and sodium into the cell and the efflux of potassium. Among the iGluRs, the NMDARs are

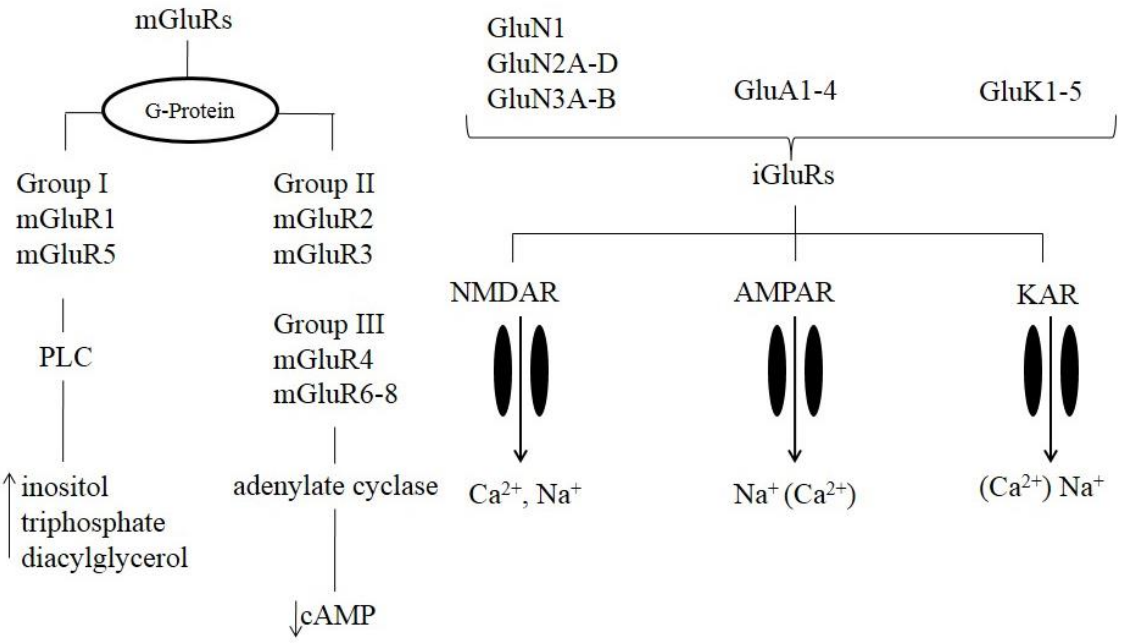


Fig. 1.9 - Classification of glutamate receptors (GluRs) according to the mechanism by which they relay their signal.

generally the most permeable to calcium (Chen and Lipton, 2006). AMPA receptors (AMPARs) are homo or heterotetramers composed of GluA1-GluA4 subunits (Palmer et al., 2005). AMPAR gate Na^+ (and Ca^{2+} in the absence of GluA2 subunit) in response to ligand binding, triggering a fast excitatory postsynaptic response in neurons and activating second messenger pathways. The conductance and kinetic properties of the AMPAR channel depend on the subunit composition. AMPAR containing the GluA2 subunit are impermeable for calcium as the genomic DNA of the GluA2 subunit contains a glutamine (Q) residue at amino acid 607, that is replaced by arginine (R) in the majority of neuronal cells during the process of nuclear RNA editing (Kew and Kemp, 2005; Palmer et al., 2005). KA receptors (KARs) are composed of GluK1-GluK5 that form tetrameric combinations (Kew and Kemp, 2005). GluK4 and GluK5 subunits do not form functional homomeric receptors, but combine in heteromeric assemblies with GluK1-GluK3 to form functional receptors; on the contrary, the GluK1-GluK3 subunits can form functional homomeric receptors (Kew and Kemp, 2005). Similarly to GluA2, GluK1 and GluK2 undergo glutamine/arginine editing that decreases the calcium permeability of these receptors (Lerma et al., 2001). In addition to RNA editing, iGluRs subunits undergo alternative splicing introducing additional heterogeneity (Dingledine et al., 1999; Kew and Kemp, 2005).

Glutamate plays a key role in several physiological functions, including interneuronal signaling, sensory perception, memory, learning, and synaptic plasticity (Cavalheiro and Olney, 2001). On the other hand, abnormal glutamate signaling is involved in the pathogenesis of acute brain injury conditions, such as stroke, epilepsy and head trauma, and in chronic CNS diseases, including Parkinson's and Alzheimer's disease (Cavalheiro and Olney, 2001; Chen and Lipton, 2006).

Several GluR antagonists have been developed to prevent excessive glutamate signaling. NMDAR channel blockers, like MK-801 or memantine, are drugs that enter the receptor-associated ion channel when it opens, thus preventing the flux of ions, and are considered uncompetitive antagonists (Chen and Lipton, 2006). These drugs require prior activation of the receptor and therefore lead to channel blockade in the presence of excessive levels of glutamate and little blockade at relatively lower levels (Chen and Lipton, 2006). Therefore, NMDAR channel blockers have been preferred over the competitive agonists, such as D-(-)-2-amino-5-phosphonopentanoic acid (APV), which compete with glutamate or glycine at the agonist binding sites (Chen and Lipton, 2006). The quinoxalinedione

compounds, including 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline (NBQX), are competitive antagonists that block AMPAR and KAR with very little selectivity between both receptors (Kew and Kemp, 2005). CNQX also has activity at the glycine binding site on NMDAR, whereas NBQX, which has improved AMPAR selectivity with respect to CNQX, is used as the antagonist of choice in many *in vitro* models, in spite of the absence of *in vivo* activity following systemic administration (Kew and Kemp, 2005; Catarzi et al., 2007). GYKI52466 and CMF-2 are noncompetitive AMPAR antagonists, selective for AMPAR over KAR and NMDAR (Kew and Kemp, 2005).

Although excessive glutamate signaling is related to cell death, glutamate has an important trophic function in the development of the CNS, regulating the proliferation and migration of neuronal progenitors (Cavalheiro and Olney, 2001). In fact, whereas the blockade of NMDAR in the developing rodent brain by NMDAR antagonists leads to apoptotic neuronal death (Ikonomidou et al., 1999; Hansen et al., 2004), the stimulation of AMPAR and NMDAR in neurons has been linked to activation of the MAPK and PI3K pathways, resulting in increased cell proliferation and migration (Perkinton et al., 1999; Platenik et al., 2000; Mao et al., 2004). Similarly, iGluRs are involved in the proliferation of tumor cells derived from neuronal tissue, such as glioma, glioblastoma, medulloblastoma and neuroblastoma, which have been shown to express iGluRs subunits (Yoshioka et al., 1996; Aronica et al., 2001; Ishiuchi et al., 2002). Glutamate plays a central role in the malignant phenotype of gliomas via two main mechanisms: on the one hand, the glutamate produced and released from glioma cells causes excitotoxicity which contributes to the expansion of tumor cells; on the other hand, glutamate also activates iGluR and mGluR on glioma cells (de Groot and Sontheimer, 2010). The activation of AMPAR by glutamate induces intracellular calcium oscillations (Lyons et al., 2007) that seem to play a crucial role in the growth and motility of glioblastoma, the most aggressive malignant primary brain tumor in humans. Noteworthy, glioblastoma samples present higher levels of GluA1 expression in comparison with low-grade tumors and silencing of GluA1 inhibits AMPAR-mediated activation of MAPK pathway and decreases glioma cells proliferation (de Groot et al., 2008). Moreover, the AMPAR GluA2 subunit, which confers calcium impermeability, is absent in high-grade glioma specimens, but is expressed in low-grade gliomas (Beretta et al., 2009). Accordingly, Ishiuchi *et al* (2002) reported that overexpression of calcium-

permeable AMPAR facilitates migration and proliferation of glioblastoma cells and that the conversion of calcium-permeable to calcium-impermeable AMPAR inhibits cell locomotion and induces apoptosis. Similar findings were reported by Beretta *et al* (2009) who demonstrated that GluA2 overexpression inhibits glioblastoma cells proliferation by inactivating the MAPK pathway and induces apoptosis. On the other hand, it was demonstrated that AMPAR-regulated proliferation of glioblastoma cells involves the Akt pathway (Ishiuchi et al., 2007; Schunemann et al., 2010). In addition, differentiated SK-N-SH neuroblastoma cells express functional NMDAR (Pizzi et al., 2002). In the study conducted by Korczak *et al* (1995), mRNA for AMPAR and KAR subunits were detected in four neuroblastoma cell lines, but immunoblot analysis and electrophysiological recordings failed to confirm the expression of proteins and the formation of functional AMPAR or KAR channels (Korczak et al., 1995). Likewise, Yoshioka *et al* (1996) demonstrated the presence of mRNA encoding NMDAR, AMPAR and KAR subunits, but the treatment of the cells with L-glutamate, KA, AMPA or NMDA did not cause cell damage or increases in calcium influx. These observations are indicative of the existence of transcriptional and post-transcriptional regulation of iGluRs expression.

In the last decade, evidence has emerged implicating glutamate as a signal mediator in non-neuronal tissues and similarly to its trophic role in the CNS, glutamate stimulates the proliferation of non-neuronal tumor cells (Rzeski et al., 2001; Skerry and Genever, 2001; Hinoi et al., 2004). mRNA for iGluR subunits are differentially expressed in thyroid, lung and breast carcinomas, multiple myeloma, glioma, colon adenocarcinoma, T-cell leukemia (Stepulak et al., 2009), larynx (Stepulak et al., 2011), gastric cancer cells (Watanabe et al., 2008) and osteosarcoma (Kalariti et al., 2005). For the majority of tumors, mRNA levels of GluA4, GluK2 and GluK5 are lower compared to human brain tissue and NMDAR subunits are much lower (Stepulak et al., 2009). Interestingly, Stepulak *et al* (2009) detected mRNA for GluN2D subunit in all the analyzed cancer cell lines. GluN2D subunits are usually present at high levels prenatally, but decrease postnatally, which may suggest that the re-expression of GluN2D subunits in cancer cells may correlate with their proliferative phenotype (Stepulak et al., 2009). It was also reported the expression of GluR subunits at the protein level in prostate (Abdul and Hoosein, 2005), breast (North et al., 2010a) and lung (North et al., 2010b) cancers.

The expression of GluR subunits can also be associated with prognosis-related factors, as the GluN1 subunit expression was associated with tumor size, presence of lymph node metastases and poorer survival in patients with oral squamous cell carcinoma (Choi et al., 2004). Moreover, positive staining for the GluN1 subunit was detected in samples of high-grade invasive ductal and lobular breast cancer (North et al., 2010a).

The knockdown of selected GluR subunits has supported the relevance of the iGluR-mediated signaling in cancer cells proliferation and invasion. Reduced expression of GluA4 in human rhabdomyosarcoma/medulloblastoma cells markedly increased the propensity of cancer cells to proliferate, as well as their mobility, as a result of an up-regulation of the expression of key genes that encode proteins involved in signal transduction (γ-synuclein and myc proto-oncogene protein), in invasion and metastasis (MMP-2 and metastasis-associated protein MTA-2) and adhesion proteins (integrin beta-3) (Luksch et al., 2011). In agreement with these observations, the blockade of AMPAR with GYKI52466 significantly decreased rhabdomyosarcoma/medulloblastoma cells proliferation and migration (Rzeski et al., 2001). Furthermore, in a human gastric cancer cell line, silencing of the GluN2A subunit increased the proportion of cells in the G1 phase of the cell cycle, indicative that the GluN2A subunit promotes cell proliferation by accelerating the cell cycle (Watanabe et al., 2008).

It was reported that NMDAR and AMPAR antagonists limit the proliferation of several non-neuronal cancer cells, including colon, larynx, breast, prostate, lung and thyroid, in addition to glial and neuronal tumors (Rzeski et al., 2001; Abdul and Hoosein, 2005; North et al., 2010a, b). Importantly, NMDAR antagonists memantine and MK-801 reduce the viability of the NCI H82 variant cell line, which is a model of recurrent and drug-resistant small-cell lung cancer (North et al., 2010b).

The antiproliferative effect of GluR antagonists was reported to be calcium-dependent, since tumor cells grown under calcium deprivation are resistant to the antiproliferative effects of NMDAR and AMPAR antagonists (Rzeski et al., 2001). In fact, it was proposed that glutamate-dependent receptor/ion channel complexes on tumor cells contribute to regulate their proliferation and migration by modulating calcium fluxes; glutamate can depolarize membranes and induces an elevation of intracellular calcium (Rzeski et al., 2001) which stimulates tumor growth (Celli et al., 1999), regulates protein trafficking through the nuclear membrane (Stehno-Bittel et al., 1995) and cell migration

(Marks and Maxfield, 1990; Clapham, 1995; Lawson and Maxfield, 1995; Gomez and Spitzer, 1999). In accordance with this hypothesis, the resting membrane potential in tumor cells varies between −30 and −50 mV (Iwata et al., 1999) and at such membrane potentials magnesium block of NMDAR channels in neurons is reduced whereas AMPAR channels are permeable to cations (Burnashev et al., 1995; Rzeski et al., 2001). Moreover, calcium-permeable AMPAR are required for the agonist-induced proliferation of glioma cells (Ishiuchi et al., 2002).

However, further studies have challenged this hypothesis. Experiments carried out in lung carcinoma and rhabdomyosarcoma/medulloblastoma cells show that the expression of mRNA of the GluR subunits and the receptor-mediated evoked currents are low compared to the brain, questioning the functional significance of iGluR as ion channels in cancer cells (Stepulak et al., 2009). In addition, lung cancer cells express mRNA of the GluA2 subunit, and therefore it is not plausible that the antiproliferative effect of AMPAR antagonists is related to blockade of calcium entry within the cell (Stepulak et al., 2007). Thus, Stepulak *et al* (2009) suggest that GluR may activate intracellular signaling pathways that modulate the survival and proliferation of cancer cells.

The mechanisms underlying the antiproliferative effects of GluRs antagonists are not yet entirely clarified, but it seems that the inhibition of the MAPK pathway plays a key role. The activation of NMDAR in neurons results in the phosphorylation of ERK 1/2 and CREB (Kemp and McKernan, 2002; Hardingham and Bading, 2003). Accordingly, AMPAR antagonists and NMDAR antagonists inhibit the growth of lung cancer cells promoted by EGF and insulin-like growth factor (IGF) and at higher concentrations decrease the ERK 1/2 phosphorylation (Stepulak et al., 2005, 2007). Moreover, AMPAR and NMDAR antagonists, by inhibiting the MAPK pathway, reduce the phosphorylation of CREB and the expression of CREB-regulated genes, including cyclin D1, and up-regulate the cell cycle regulators and tumor suppressor proteins p21 and p53 (Stepulak et al., 2005, 2007). The decreased expression of cyclin D1, that promotes the progression through the G1-S phase of the cell cycle (Fu et al., 2004), as well as the increased expression of p21 and p53, provides a link to the cell cycle machinery, explaining the decreased number of lung adenocarcinoma cells in G2 and S phases of the cell cycle after treatment with NMDAR and AMPAR antagonists (Stepulak et al., 2005, 2007). The inhibition of the MAPK pathway by AMPAR

and NMDAR antagonists was also demonstrated in laryngeal cancer cells (Stepulak et al., 2011).

In addition to their antiproliferative activity, it was also demonstrated that GluR antagonists alter the morphology and decrease the motility of cancer cells. Thyroid carcinoma cells exhibit marked membrane ruffling and numerous pseudopodia characteristic of an invasive phenotype, whereas cells treated with NMDAR antagonist MK-801 or the AMPAR antagonist GYKI52466 for 96 h present fewer pseudopodia protrusions (Rzeski et al., 2001). The motility of lung carcinoma, rhabdomyosarcoma/medulloblastoma and thyroid carcinoma cells is also decreased after 96 h of treatment with MK-801 or GYKI52466 (Rzeski et al., 2001).

The evidences obtained *in vitro* were further supported by the promising results obtained *in vivo*. MK-801 at a daily dose of 0.1 mg/kg body weight doubles the survival of mice with metastatic lung adenocarcinoma and it is well tolerated by the animals (Stepulak et al., 2005). Accordingly, the administration of escalating doses of MK-801 (ranging from 0.1 up to 0.3 mg/kg body weight) for ten days reduces in approximately 50 % the tumor growth rate of animal models of small-cell lung cancer (North et al., 2010b). The escalating dose range was selected by the authors to produce maximal effects without inducing significant adverse behavioral changes and MK-801 seemed well tolerated by the animals (North et al., 2010b). Furthermore, MK-801 (0.3 mg/kg body weight twice a day) slows the growth of neuroblastoma/rhabdomyosarcoma in mice, but using this dose, mild increases in the locomotor activity for up to 1 h after administration were reported (Stepulak et al., 2005). Daily treatments with MK-801 over 5 days (two daily doses of 0.3 mg/kg body weight) also arrested the growth of mice bearing breast cancer without inducing apparent negative effects on the health of animals (North et al., 2010a).

Importantly, the GluR antagonists may be useful agents in therapeutic association. MK-801 and GYKI52466 enhance the antiproliferative effect of cyclophosphamide and thiotepa in rhabdomyosarcoma/medulloblastoma, neuroblastoma, lung carcinoma and astrocytoma cells due to both increased tumor cell death and to diminished cell division (Rzeski et al., 2001).

In the last years, several studies have implicated mGluR signaling in the onset and progression of melanoma. mGluR1 is expressed in several human melanoma biopsies and cell lines, but not in benign nevi and melanocytes (Pollock et al., 2003), and its expression

is required for the development and growth of melanoma *in vivo* (Pollock et al., 2003; Ohtani et al., 2008). In fact, treatment of human melanoma cells expressing mGluR1 with a mGluR1 antagonist or with the glutamate release inhibitor riluzole decreases cell proliferation and the levels of extracellular glutamate (Namkoong et al., 2007). Likewise, a selective noncompetitive mGluR1 antagonist significantly inhibits the proliferation of human melanoma cell lines (Haas et al., 2007). Interestingly, inhibitors of the PLC, PKC, calcium release, calmodulin and MAPK inhibit melanoma development in mGluR1 transgenic mice, but only the MAPK inhibitor is able to inhibit melanoma growth once it develops (Abdel-Daim et al., 2010). The results reported by Abdel-Daim *et al* (2010) suggest that the activation of every signaling pathway from mGluR1 is required for the development of melanoma and that the MAPK pathway plays a key role in its progression. Recently, Choi *et al* (2011) demonstrated that overexpression of mGluR5 also induces melanoma in transgenic mouse lines and implicated ERK 1/2 as a key downstream effector of mGluR5 (Choi et al., 2011). Another mGluR, mGluR3, is frequently mutated in melanoma and by regulating the phosphorylation of MEK leads to increased growth and migration (Prickett et al., 2011). On the other hand, a mGluR1 antagonist significantly enhances the effects of docetaxel, suggesting that GluRs antagonists may potentiate the anticancer activity of existing therapies of melanoma (Haas et al., 2007).

Although these studies support the importance of mGluR signaling in melanoma, the information regarding the role played by iGluR remains scarce. Hoogduijn *et al* (2006) demonstrated that GluA2 and GluN2A subunits are expressed in melanocyte cultures; since the stimulation with AMPA or NMDA elevated intracellular calcium concentrations, the authors concluded that melanocytes present functional AMPAR and NMDAR. Moreover, treatment with AMPAR and NMDAR antagonists (CFM-2 and MK-801) changes melanocyte morphology and induces alterations in the actin cytoskeleton (Hoogduijn et al., 2006). Importantly, CFM-2 markedly decreases the expression of the microphthalmia-associated transcription factor (MiTF), a master regulator of melanocyte differentiation and proliferation (Hoogduijn et al., 2006). More recently, it was reported that melanoma cells WM451 express functional NMDAR, as shown by the increase in intracellular calcium concentrations induced by NMDA, which are decreased by MK-801 (Song et al., 2012). The treatment of melanoma cells with MK-801 induces changes in the cellular morphology that resemble that of normal melanocytes and decrease cell motility, as well as proliferation

(Song et al., 2012). Furthermore, MK-801 (0.6 mg/kg every three days) decreases tumor volume and growth in mouse models inoculated with melanoma cells (Song et al., 2012).

Therefore, further studies devoted to the investigation of the effects of iGluR antagonists on melanoma proliferation either individually or in combination with other anticancer agents are required.

1.5 - Mitochondria as drug targets

Mitochondria are dynamic cytoplasmatic organelles, which present varying shape, size, number and location within the cell (Campello and Scorrano, 2010). Mitochondria possess an outer membrane (OMM) that is rich in cholesterol and essentially permeable to ions and solutes up to 14 kDa, similarly to other cell membranes (Wallace and Starkov, 2000). The intermembrane space (IMS) is located between the OMM and the inner mitochondrial membrane (IMM), and contains proteins such as the adenylate kinase and the creatine kinase (CK), as well as the cytochrome *c* and the apoptosis-inducing factor (AIF), which translocate to the cytoplasm during the apoptotic process, and other protein involved in cellular metabolism. The IMM is folded, forming the cristae, and in contrast to the OMM, is rich in cardiolipin and impermeable to ions and polar molecules. The IMM possesses transporters for ATP/adenosine 5'-diphosphate (ADP), aspartate/malate, among others that regulate the movements of molecules across the IMM, and also the five multisubunit complexes (Complex I, II, III, IV, and V) involved in the oxidative phosphorylation. The space enclosed by the IMM is the matrix, which contains the proteins involved in the Krebs cycle, in the fatty acid oxidation, as well as in the synthesis of the heme and steroids (Scatena et al., 2007; Pereira et al., 2009; Nadanaciva and Will, 2009; Cohen, 2010). The mitochondrial matrix also contains the mitochondrial DNA (mtDNA), maternally inherited, which is circular and lacks histones, resembling the bacterial DNA. The mtDNA encodes 13 polypeptides that are the subunits of the complexes I, III, IV and V that are synthesized in mitochondrial ribosomes. The majority of the other mitochondrial proteins, including the subunits of complex II, are encoded by nuclear DNA, and imported into mitochondria after synthesis on cytosolic ribosomes (Wallace, 2005; Pereira et al., 2009; Cohen, 2010; Nadanaciva and Will, 2011a). Therefore, mitochondria are not only responsible for the synthesis of most of the ATP produced in the cell, but they are multifunctional organelles

with diverse roles, including the fatty acid oxidation, the synthesis of the heme and steroids, calcium regulation and cell death. Importantly, the electron leak during the transfer of electrons along the electron transport chain to oxygen, the ultimate acceptor of electrons, turns mitochondria into the major source of ROS. Although the formation of ROS can be damaging to the organelle, ROS generation is also required to maintain the redox balance, which is critical to regulate the functions that assist the normal cellular physiology (Cohen, 2010).

Considering the key role played by mitochondria in the cell survival and death, mitochondria are often implicated in disease and xenobiotic-induced toxicity (Pereira et al., 2009; Cohen, 2010; Nadanaciva and Will, 2011a, b).

The mtDNA is not protected by histones and due to the proximity to the sites where ROS are routinely generated and the less efficient DNA repair processes, the frequency of mtDNA mutation is much higher than that of nuclear DNA (Scatena et al., 2007). The mutations of mtDNA can lead to mitochondrial diseases, including the mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy associated with ragged-red fibres (MERRF) and Leber's hereditary optic neuropathy (LHON) (Pereira et al., 2009; Nadanaciva and Will, 2011a). Additionally, a pathogenic role for mitochondrial dysfunction has also been invoked in neurodegenerative disease, cancer, type 2 diabetes and aging (Wallace, 2005).

In the least years, the evaluation of drug-induced mitochondrial damage has received considerable attention, as several classes of drugs have been shown to compromise mitochondrial functions (Nadanaciva and Will, 2009, 2011a). For instance, cerivastatin, troglitazone, tolcapone and nefazodone, which have been implicated in mitochondrial dysfunction were withdrawn from the market by the FDA due to safety concerns (Nadanaciva and Will, 2009). Thus, the study of the effects of drugs on mitochondria allows for a better understanding of the pharmacological and toxicological mechanisms underlying the mode of action of drugs. Moreover, the use of isolated mitochondria fractions to predict drug safety can also decrease the number of laboratory animals and the costs of preclinical studies (Pereira et al., 2009).

Mitochondrial dysfunction is more pronounced in tissues with high energetic demands and that rely heavily on oxidative phosphorylation (heart, kidney and CNS). On

the other hand, considering the exposure to high concentrations of drugs, the liver is often another target of mitochondrial toxicity (Nadanaciva and Will, 2009).

Drugs can compromise mitochondrial functions in a multitude of ways. The table 1 shows examples of drugs that affect mitochondrial functions by different mechanisms.

Drugs can damage hepatic mitochondria in some individuals but not in others, and our current knowledge does not allow to predict the idiosyncratic liver injury related with drug-induced mitochondrial dysfunction. It seems that genetic, metabolic and environmental factors that impair mitochondrial function can add their effects to those of mitochondria-targeting drugs, compromising mitochondrial function to an extent where hepatic manifestations start to occur (Labbe et al., 2008). In addition, drug-induced mitochondrial dysfunction can also trigger diverse extrahepatic or general manifestations. The syndromes associated to mitochondrial toxicity are not uncommon and include lactic acidosis, myopathy, peripheral neuropathy, rhabdomyelosis and pancreatitis (Scatena et al., 2007; Labbe et al., 2008; Nadanaciva and Will, 2009, 2011b).

However, the interaction of drugs with mitochondria may not always be negative, and the therapeutic effects obtained from the pharmacological modulation of mitochondrial activity are being investigated, particularly in cancer therapy (Scatena et al., 2007; Fulda et al., 2010).

1.5.1 - Bioenergetic functions

Mitochondria are considered the powerhouses of the cell, since they generate most of the ATP produced (Nadanaciva and Will, 2009). In the IMM are located the five complexes involved in the oxidative phosphorylation. The nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) generated in glycolysis, fatty acid oxidation and Krebs cycle are oxidized by Complex I (NADH ubiquinone reductase) and Complex II (succinate dehydrogenase), respectively, and the electrons are passed to ubiquinone (coenzyme Q), a mobile electron carrier within the IMM that shuttles electrons to Complex III (ubiquinol:cytochrome *c* reductase) (Fig. 1.10). The complex III oxidizes the reduced ubiquinol and reduces the cytochrome *c*, a small protein that rotates freely in the cytosolic side of the IMM. The cytochrome *c* shuttles the electrons to complex

Target	Effect on mitochondria	Examples of drugs
Bioenergetic functions	Inhibitors of electron transport	Tamoxifen, fenofibrate, metformin
	Uncouplers	Diclofenac, chlorpromazine
Mitochondrial permeability transition	Inducers	Retinoic acid, nimesulide
	Inhibitors	Tamoxifen
mtDNA	Inhibitors of mtDNA replication	Stavudine, lamivudine
	Inhibitors of mtDNA-encoded protein synthesis	Linezolid, chloramphenicol
	Oxidative stress	Doxorrubicin
Lipid metabolism	Inhibitors of fatty acid oxidation	Valproic acid

Table 1 - Examples of mechanisms by which drugs can impair mitochondrial function (Modified from Nadanaciva and Will, 2009).

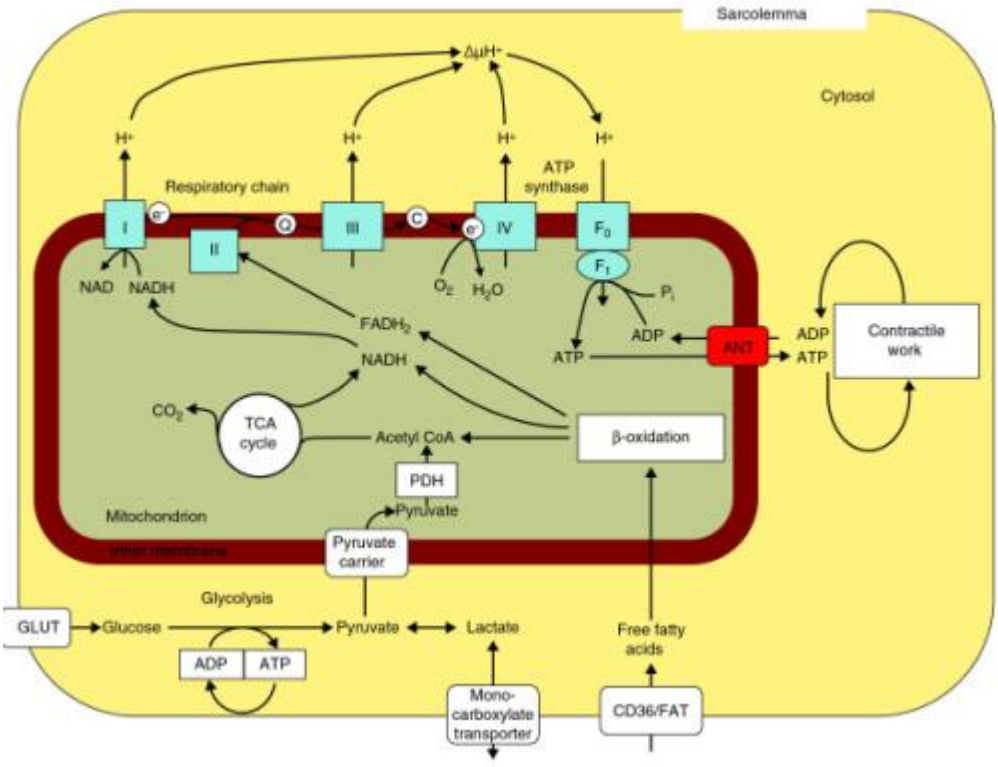


Fig. 1.10 - Schematic representation of the oxidative phosphorylation. The electrons donated from NADH and $FADH_2$ to Complex I and Complex II are passed to ubiquinone (Q), a mobile electron carrier that shuttles protons to the complex III, which oxidizes the reduced ubiquinol and reduces the cytochrome *c*. The cytochrome *c* then shuttles the electrons to complex IV, where molecular oxygen is reduced to water. In parallel to the electron transfer, the complexes I, III and IV pump protons from the matrix to the IMS generating an electrochemical gradient, which is used by Complex V to synthesize ATP from ADP and inorganic phosphate (Murray, 2009).

IV (cytochrome *c* oxidase) which directly reduces molecular oxygen to water (Wallace and Starkov, 2000; Pereira et al., 2009). Complexes I, III and IV are redox-driven proton pumps that use the energy derived from oxidation-reduction reactions to pump protons from the matrix to the IMS, in parallel to the electron transfer, generating an electrochemical proton gradient (proton motive force) ($\Delta\mu H^+$), which is comprised of two components: a pH gradient (ΔpH) and a membrane potential ($\Delta\Psi$) (Fig. 1.10).

The energy contained in the $\Delta\mu H^+$ is used by Complex V (ATP synthase) to synthesize ATP from ADP and inorganic phosphate (Fig. 1.10). The complex V consists of the F1 subunit, a soluble portion situated in the mitochondrial matrix, and the Fo subunit, bound to the IMM. When cells need energy, the levels of ADP within the mitochondria rise and induce the re-entry of protons into the matrix through the Fo portion of complex V, partially dissipating the $\Delta\Psi$. The energy released is then used by the F1 subunit to phosphorylate ADP into ATP. The transient decrease in $\Delta\Psi$ promotes the electron transfer along the electron transport chain and the coupled ejection of protons towards the IMS, restoring the $\Delta\Psi$ (Labbe et al., 2008; Jonckheere et al., 2012). Oxidative phosphorylation requires the presence of NADH or $FADH_2$, oxygen, ADP and phosphate. However, as discussed above, the levels of ADP are the most critical factor regulating the rate of phosphorylation. Thus, the oxidative phosphorylation is coupled to the ATP requirements and the electron flow along the electron transport chain only occurs when the synthesis of ATP is required. The impermeability of the IMM to protons is crucial for ensuring that the electron transfer along the electron transport chain is coupled to ATP synthesis by Complex V, so that there is no respiration without proton pumping and *vice-versa* (Wallace and Starkov, 2000; Nadanaciva and Will, 2009; Pereira et al., 2009).

However, and particularly at complexes I and III, some of these electrons escape and directly react with oxygen (Labbe et al., 2008). This process generates the superoxide anion radical which is then dismutated by the mitochondrial manganese superoxide dismutase into hydrogen peroxide; the latter is detoxified into water by the mitochondrial glutathione peroxidase and hence, in normal circumstances, most of the ROS generated by the electron transport chain are neutralized by the mitochondrial antioxidant defences (Labbe et al., 2008).

Xenobiotics can compromise mitochondrial bioenergetics either by interfering with the generation of $\Delta\Psi$ or by causing its dissipation (Wallace and Starkov, 2000). The

inhibition of the electron transport chain has great impact on tissue metabolism by compromising the reoxidation of NADH and $FADH_2$ into NAD^+ and FAD, respectively, required for the activity of several dehydrogenases of the Krebs cycle and the mitochondrial β-oxidation (Begriche et al., 2006; Rolo et al., 2012). Furthermore, the damaged electron transport chain will increase the levels of oxidative stress that promote several deleterious effects (Begriche et al., 2006; Rolo et al., 2012). On the other hand, drugs that increase the membrane permeability to ions, such as channel-forming proteins, ionophores, uncouplers of oxidative phosphorylation and inducers of the mitochondrial permeability transition (MPT) dissipate the proton gradient between the IMS and the matrix without ATP generation, resulting in the generation of heat (Wallace and Starkov, 2000; Scatena et al., 2007; Nadanaciva and Will, 2009).

The antiestrogen TAM depresses the phosphorylation efficiency and the mitochondrial levels of ATP in a concentration-dependent manner, possibly due to a decrease in the active adenine nucleotide transporter (ANT) content and a partial inhibition of the phosphate carrier (Cardoso et al., 2001, 2004). At high concentrations, TAM uncouples the mitochondrial respiration (Tuquet el al., 2000; Cardoso et al., 2001). Considering that TAM is a highly hydrophobic molecule that strong partitions in biomembranes (Custódio et al., 1991), it is possible that TAM amine group may bind and shuttle protons across the IMM like a classic protonophore (Cardoso et al., 2001). Indeed, amphiphilic drugs like TAM are protonated within the acidic IMS and 'pulled' into the mitochondrial matrix, which is basic and negatively charged, where the protonated molecule releases its proton; this abnormal entry of protons bypasses the Fo fraction of Complex V, thus uncoupling the oxidative phosphorylation (Labbe et al., 2008). On the other hand, high concentrations of TAM can also affect the IMM integrity (Custódio et al., 1996; Chen et al., 1999), enhancing the proton leak (Cardoso et al., 2001). Thus, depending on the concentrations used, TAM can induce mitochondrial uncoupling by a proton shuttling mechanism or by disrupting the structure of the IMM.

The stimulation of mitochondrial respiration is then followed by its inhibition, as the TAM accumulated eventually reaches concentrations capable of compromising the electron transfer along the electron transport chain (Tuquet el al., 2000; Cardoso et al., 2001). Further studies conducted by Moreira *et al* (2006) revealed that TAM at 25 µM significantly affects complex I and identified the flavin mononucleotide site of complex I as the target of

TAM. However, the inhibition of respiration induced by high concentrations of TAM can also be related to the disruption of the IMM (Cardoso et al., 2001). The rigidifying effect of TAM on membrane fluidity (Custódio et al., 1993a) may also compromise the diffusional mobility of membrane proteins, hampering the electron transfer along the electron transport chain (Cardoso et al., 2001). In addition to the inhibition of the electron transport chain as a consequence of the impaired electron flow demonstrated *in vitro* (Cardoso et al., 2001; Moreira et al., 2006), TAM also significantly depletes hepatic mtDNA in mice treated with 0.5 mmol/kg for more than 12 days (Larosche et al., 2007), which further contributes to inhibit mitochondrial electron transport chain activity.

The inhibition of the electron transport chain secondarily inhibits mitochondrial β-oxidation through the decreased regeneration of the oxidized cofactors (NAD^+ and FAD) required for β-oxidation, and can also lead to the depletion of ATP within the hepatocytes and trigger cell death (Labbe et al., 2008). The slight and prolonged inhibition of mitochondrial β-oxidation induced by TAM can cause macrovacuolar steatosis, a benign liver lesion in the short term that is characterized by the presence of a single, large lipid vacuole in the cytoplasm of the hepatocytes (Labbe et al., 2008). However, macrovacuolar steatosis can progress in some patients towards steatohepatitis after several years (Labbe et al., 2008).

Another consequence of the inhibition of the electron transport chain is the accumulation of electrons in the electron transport chain complexes that can escape and directly react with oxygen to form the superoxide anion radical (Labbe et al., 2008). Thus, the impairment of mitochondrial electron transport chain activity is also associated with enhanced ROS production and increased lipid peroxidation that is favored by lipid accumulation. Hence, the augmented ROS production is possibly a key event in the pathogenesis of drug-induced steatohepatitis (Labbe et al., 2008).

Importantly, according to the study performed by Cardoso *et al* (2002a), the TAM active metabolite OHTAM is less toxic to mitochondrial functions than the prodrug. OHTAM slightly affects the phosphorylation efficiency of rat liver mitochondria in comparison with TAM, and only at concentrations above those reached in tissues (Cardoso et al., 2002a). Like TAM, OHTAM increases the state 4 of respiration and decreases the $\Delta\Psi$ in a concentration-dependent manner, but the effects are much less pronounced than those of TAM (Cardoso et al., 2002a). The authors proposed that OHTAM increases the membrane

permeability to protons by acting as a proton shuttle, whereas the same concentrations of TAM induce a much more potent proton leak, possibly related with the disruption of mitochondrial membrane (Cardoso et al., 2001; Cardoso et al., 2002a).

RA has been shown to affect mitochondrial bioenergetics as well. RA above 0.25 nmol/mg protein induces uncoupling of mitochondria, which is related to the ability of retinol and RA to increase IMM permeability (Stillwell et al., 1982; Stillwell and Nahmias, 1983). On the other hand, a recent study conducted by Xun *et al* (2012) demonstrated that during RA-induced differentiation of neuroblastoma cells the oxygen consumption rate is increased and that this effect of RA on mitochondria may be beneficial in cancer therapy. Thus, further studies are required to clarify the effects of RA on mitochondrial bioenergetics.

1.5.2 - Mitochondrial permeability transition

MPT can be defined as a sudden increase of IMM permeability to solutes with molecular masses up to 1 500 Da, due to the opening of a voltage and calcium-dependent, cyclosporine A (CyA)-sensitive channel (Rasola and Bernardi, 2007). For several years, it was considered that the MPT pore was composed by cyclophilin-D (CypD) in the matrix, the ANT in the IMM and the voltage-dependent anion channel (VDAC) in the OMM (Fig. 1.11) (Halestrap, 2009; Zorov et al., 2009). Additional proteins, such as the CK, hexokinase II (HKII) and the peripheral benzodiazepine receptor (PBR) were also proposed to take part in the MPT pore (Halestrap, 2009; Zorov et al., 2009). However, emerging evidences have challenged this model. The fact that the loss of CypD does not prevent MPT pore opening, and only increases the calcium load required, suggests that MPT pore opening involves a conformational change in a membrane protein, which is triggered by calcium and facilitated by CypD, but can occur even in the absence of CypD if sufficient stimulus is given (Basso et al., 2005; Halestrap, 2009). Recent studies have also eliminated the VDAC as an essential component of the MPT pore complex and the ANT is considered to play a regulatory role rather than a structural one (Fig. 1.11) (Javadov et al., 2009). Recently, the mitochondrial phosphate carrier was also proposed as a possible key component of the MPT pore complex (Leung et al., 2008). The evidence regarding the involvement of the other proteins remains largely circumstantial (Halestrap, 2009).

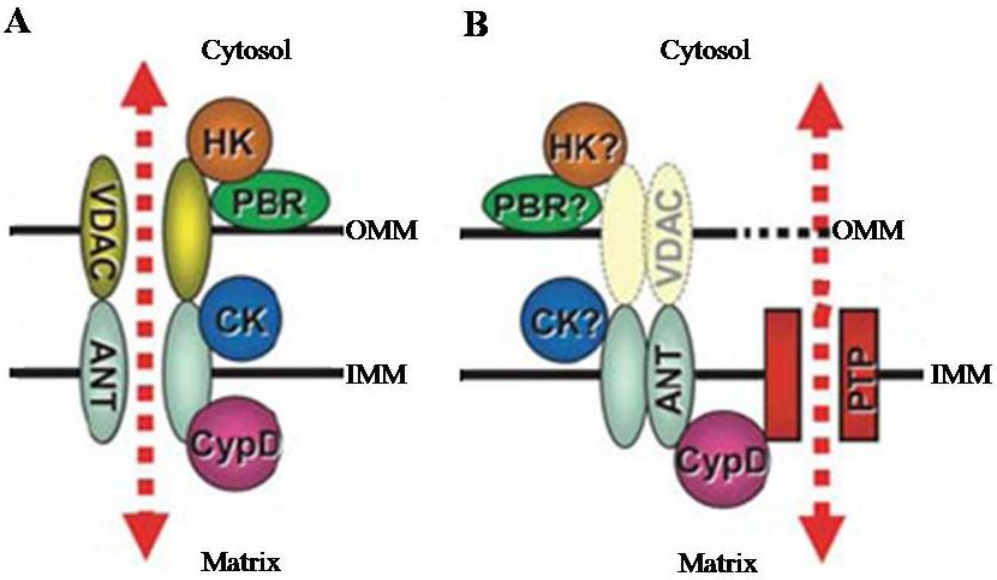


Fig. 1.11 - Classical (A) and current (B) views of the MPT pore complex. (A) The MPT pore complex is formed by the VDAC, the ANT and the CypD complex. HKII, CK and PBR were considered possible regulatory components. (B) The CypD and the ANT are now considered key regulators of the MPT, whereas the VDAC is no longer seen as an essential or even regulator MPT pore component. The involvement of HKII, CK and PBR remains controversial (Zorov et al., 2009).

Although the MPT pore opening is intimately related with the calcium concentration in the mitochondrial matrix, the levels required are largely dependent on other factors. Oxidative stress, depletion of ADP and ATP and high concentrations of phosphate increase the sensitivity of MPT to calcium, while a low pH inhibits MPT pore opening (Halestrap, 2009). The $\Delta\Psi$ is another critical factor regulating MPT – a high (negative) $\Delta\Psi$ inhibits MPT pore opening, but if mitochondria are depolarized before the addition of calcium, the calcium accumulation is prevented and MPT pore opening requires a much higher concentration of calcium (Halestrap, 2009).

As a consequence of MPT pore opening, the IMM loses its impermeability to protons, leading to depolarization of $\Delta\Psi$ and the dissipation of ΔpH, resulting in oxidative phosphorylation uncoupling. Moreover, the proton-translocating ATPase goes into reverse and hydrolyses the ATP generated by glycolysis further contributing to ATP depletion and bioenergetic failure (Helestrap, 2009). Another consequence of MPT pore opening is the increased permeability of the IMM to solutes with molecular weight up to 1 500 Da, allowing their movement and equilibration between the matrix and the cytosol whilst proteins are retained within their respective compartments. Since the protein concentration is higher in the matrix than in the cytosol, a high osmotic pressure is generated and leads to mitochondrial swelling and eventually to the rupture of OMM. In this scenario, cytochrome *c* and other proapoptotic proteins are released to the cytosol and may initiate the apoptotic cell death (Halestrap, 2009; Kinnally et al., 2011). The AIF leads to the fragmentation of nuclear DNA, whereas the second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) inactivates the inhibitor of apoptosis proteins (Fig. 1.12) (Labbe et al., 2008). The released cytochrome *c* binds to the apoptotic protease-activating factor 1 to activate caspase-9, and since it is a key component of the electron transport chain, its leakage from the mitochondria eventually impairs electron transfer within the electron transport chainand enhances ROS generation (Labbe et al., 2008). If there are no sufficient levels of ATP, necrotic death may predominate over apoptosis (Baines et al., 2009).

In addition, MPT plays a relevant role in pathological situations, contributing to cell death. That is the case of ischemia-reperfusion in cardiac myocytes, where mitochondrial ROS generation after reperfusion promotes MPT onset and subsequent myocyte death,

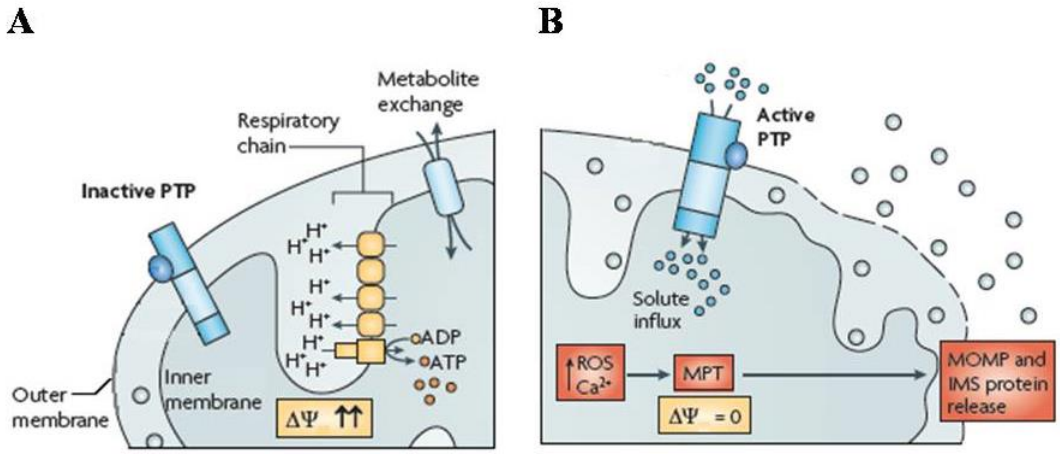


Fig. 1.12 - Mitochondrial permeability transition (MPT). (A) In physiological conditions, the permeability transition pore (PTP) is inactive and mitochondria exhibit a high ΔΨ, which is generated by the electron transport chain and used for ATP generation. In these conditions, mitochondrial solute carriers regulate the exchange of small metabolites between the cytosol and the mitochondrial matrix. (B) In pathological situations or in response to some drugs, the opening of the PTP allows the deregulated entry of small solutes into the mitochondrial matrix driven by electrochemical forces. MPT leads to the dissipation of the ΔΨ and to the osmotic swelling of the mitochondrial matrix, which eventually results in mitochondrial outer membrane permeabilization (MOMP). Consequently, proteins normally confined within the IMS, such as cytochrome *c* and Smac/DIABLO, as well as caspase-independent cell death effectors, like AIF and endonuclease G are released into the cytosol [Modified from Fulda *et al* (2010)].

turning the inhibition of MPT pore opening a possible approach for cardioprotection (Javadov et al., 2009; Lemasters et al., 2009).

MPT can also be induced by xenobiotics, either by an interference with the proteins that regulate the MPT pore opening or through the modulation of critical factors to the onset of MPT (Pereira et al., 2009). Importantly, the induction of MPT in liver mitochondria can trigger apoptosis and/or necrosis, and cause 'cytolytic' hepatitis leading occasionally to fulminant hepatic failure (Labbe et al., 2008). Therefore, the induction of MPT is recognized as a mechanism by which drugs can induce hepatotoxicity (Labbe et al., 2008) and several hepatotoxic drugs have been shown to cause MPT pore opening, such as diclofenac, nimesulide, troglitazone and gemfibrozil (Nadanaciva and Will, 2009). On the other hand, the induction of MPT has been investigated as a therapeutic strategy, particularly in cancer therapy, due to its ability to activate the cell death machinery. It is thought that this strategy may bypass the resistance mechanisms related with conventional chemotherapy that do not target mitochondria directly, but interfere with signaling pathways which lie upstream of mitochondria that are frequently deregulated in cancer (Fulda et al., 2010). However, further studies are required to clarify the differences between mitochondria in normal and cancer cells to increase the level of selectivity of mitochondria-targeted anticancer agents and turn the triggering of MPT into a possible therapeutic strategy for cancer therapy (Fulda et al., 2010).

The ability of antiestrogens TAM and OHTAM to inhibit MPT pore opening is well documented. TAM prevents and reverses the MPT induced by calcium and phosphate (Custódio et al., 1998), by pro-oxidants (Cardoso et al., 2004), by carboxyatractyloside (CATR) (Hernández-Esquivel et al., 2011a) and by mercury (Hernández-Esquivel et al., 2011b). Likewise, OHTAM also prevents and reverses the MPT induced by calcium and phosphate (Cardoso et al., 2002b). The mechanisms underlying the inhibition of MPT by TAM and OHTAM are not entirely clarified. Both TAM and OHTAM have been shown to scavange peroxyl radicals (Custódio et al., 1994). Considering that oxidative stress is one of the mechanisms regulating the MPT pore opening (Halestrap, 2009), the antioxidant activity of antiestrogens could possibly account for the inhibition of MPT (Cardoso et al., 2004; Hernández-Esquivel et al., 2011b). However, Custódio *et al* (1998) suggest that the protective effect afforded by TAM is due to some factor other than its antioxidant action, as no change in the oxidation state of mitochondrial glutathione was detected during the course

of calcium and phosphate-induced MPT. In fact, TAM inhibits the MPT induced by agents that do not increase the levels of oxidative stress, such as CATR (Hernández-Esquivel et al., 2011a). TAM and OHTAM are highly hydrophobic molecules that strongly partition in biomembranes (Custódio et al., 1991, 1993a, b; Kazanci and Severcan, 2007), affecting the dynamic properties of lipids. The ability of TAM to prevent the MPT induced by CATR is substantially decreased when experiments are carried out at higher temperatures, suggesting that this effect may be related with the interference with membrane fluidity (Hernández-Esquivel et al., 2011a); this could lead to a configurational change of the ANT and explain the effects of TAM and OHTAM on MPT. On the other hand, TAM and OHTAM decrease the content of active ANT and inhibit the phosphate carrier (Cardoso et al., 2003), but the concentrations required are possibly too high to explain the inhibition of MPT. It is also not possible to exclude that the antiestrogens bind to CypD, and inhibit the MPT in a similar way to CyA (Zoratti and Szabo, 1995). Therefore, the MPT inhibition by antiestrogens may be related with different effects exerted by these drugs on mitochondria.

On the other hand, retinoids (retinol, RA and its isomers 9-*cis* and 13-*cis*) induce mitochondrial swelling and decrease the $\Delta\Psi$ of rat liver mitochondria. Their action is stimulated by calcium and inhibited by CyA, suggesting that retinoids are inducers of MPT pore opening (Rigobello et al., 1999; Notario et al., 2003; Klamt et al., 2005). The fact that RA binds to the ANT, decreasing its activity (Notario et al, 2003), can possibly explain the ability of retinoids to induce MPT.

As discussed above, mitochondria are targets of RA and antiestrogens actions. It is plausible that the effects of both classes of compounds on mitochondrial bioenergetics and calcium homeostasis are closely related with the cases of hepatotoxicity observed in patients treated with these drugs, since mitochondrial dysfunction is widely recognized as a major mechanism underlying drug-induced liver injury (Labbe et al., 2008; Nadanaciva and Will, 2009; Russmann et al., 2009).

In fact, relatively high concentrations of TAM and its metabolites were detected in the liver (Lien et al., 1991), and patients with breast cancer treated with TAM had elevated plasma concentrations of aspartate aminotransferase, alanine aminotransferase or both (Liu et al., 2006). Moreover, hypertriglyceridemia and fatty liver were reported in approximately 33 % of patients who took TAM (Liu and Yang, 2003; Sakhri et al., 2010), whereas nonalcoholic steatohepatitis is present in 2.2 % of the patients with breast cancer treated with

TAM (Saphner et al., 2009). Although TAM is a drug capable of inducing hepatotoxicity caused by mitochondrial dysfunction, it was not withdrawn from the market considering the favorable benefit-risk ratio, but it has received a Black Box warning from drug agencies (Labbe et al., 2008). It was also reported that retinoids induce hepatotoxicity, either during dietary supplementation (Stickel et al., 2011) or treatment of APL patients (de-Medeiros et al., 1998) and hypertriglyceridemia (Standeven et al., 1996). Moreover, vitamin A supplementation in rats induced slight enlargement of mitochondria (Leo et al., 1982), hepatic oxidative insult and mitochondrial dysfunction (de Oliveira et al., 2009), supporting the view that mitochondria are a target of retinoid toxicity.

As the clinical utilization RA has been limited by its adverse effects (Zusi et al., 2002), the development of combinations of drugs that can enhance RA therapeutic activity, while minimizing its toxic effects is highly desirable. The combination of RA with TAM has shown synergistic antitumor activity in breast cancer cells (Danforth, 2004; Wang et al., 2007; Searovic et al., 2009; Koay et al., 2010). However, the combination of drugs, which have both been shown to be hepatotoxic, such as RA and TAM, can be a problem. For instance, retinol has been shown to potentiate acetaminophen-induced heptatoxicity in mouse models (Bray and Rosengren, 2001). Thus, the future use of RA in combination with antiestrogenic compounds should be carefully evaluated, and the study of the effects of these combinations on liver mitochondrial functions can help to predict the toxicity and, therefore, the value of this therapeutic association.

1.6 - Aims and structure of the dissertation

Malignant melanoma is the most aggressive form of skin cancer and its incidence is rapidly increasing. Given the poor prognosis for locally advanced and metastatic disease and the severe side effects induced by the currently available therapies, more effective and less toxic therapeutic strategies are urgently required.

The antiestrogen TAM is widely used in the treatment of ER+ breast carcinoma, but has also been used in the management of malignant melanoma individually or, more commonly, in therapeutic association. However, TAM induces multiple effects on mitochondrial functions that may account for the hepatotoxicity and the alterations in lipid metabolism frequently observed in patients treated with TAM. Meanwhile, the benefits of

the inclusion of TAM in therapeutic regimens and its mechanism of action in melanoma remain unclear. Recent studies demonstrate that the efficacy of TAM in breast cancer is limited by CYP2D6 polymorphisms or by the coadministration of medication acting as a CYP2D6 inhibitor or inducer, suggesting that the use of an active metabolite instead of the prodrug may present strong therapeutic advantages. On the other hand, considering the heterogeneity and the plasticity of melanoma, it is unlikely that a single agent can provide a significant response in most patients. The combination of drugs can possibly allow to achieve therapeutic efficacy in melanoma with lower concentrations, this way minimizing the adverse effects typical of systemic chemotherapy. Therefore, in the present dissertation we aimed to investigate whether EDX, a key active metabolite of TAM, is a less toxic drug in cancer therapy and a more effective alternative to TAM in the management of melanoma, either individually or in combination with the retinoid RA or with the NMDAR antagonist MK-801.

Since the study of the effects of drugs on mitochondrial function has gained recognition as an important part of the preclinical evaluation of drug safety, the effects of EDX on rat liver mitochondria were evaluated, either individually or in combination with RA.

The effects of EDX on mitochondrial bioenergetics, MPT and oxidative stress were monitored and compared to those promoted by TAM and OHTAM. Our results demonstrate that EDX only compromises mitochondrial bioenergetics functions at concentrations much higher than those reached in patient tissues, suggesting that EDX is less toxic to mitochondria than TAM. Moreover, similarly to TAM and OHTAM, EDX prevents the MPT pore opening induced by calcium and phosphate. These results were published in *Toxicol. Appl. Pharmacol.* 267, 104-112 and are presented in chapter II.

It has been reported that mitochondria are implicated in the pharmacological actions of RA, namely in the inhibition of the proliferation of melanoma cells, as well as in the differentiation and suppression of the proliferation of neuroblastoma cells, and the induction of MPT has also considered a possible strategy to trigger cancer cell death. On the other hand, the utilization of RA is limited due to its several side effects, including hepatotoxicity and hypertriglyceridemia, which are related with mitochondrial dysfunction.

Since the combinations of RA with TAM demonstrated a synergism of action in breast cancer cells and our results demonstrate that EDX promotes less deleterious effects

on mitochondria, we evaluated the effects of EDX in combination with RA on mitochondrial bioenergetic functions, MPT and oxidative stress, providing relevant mechanistic information regarding the pharmacological action and the potential toxicity of this combination. The effects of EDX in combination with RA were also compared to those promoted by the association of RA with TAM and OHTAM. Our results show that RA compromises mitochondrial bioenergetics in a concentration-dependent manner, but its effects are not enhanced by EDX. Moreover, EDX similarly to the other antiestrogens prevents the MPT induced by RA. These results were published in *Life Sci*. 93, 96-107 and are presented in chapter III.

As EDX at the concentrations reached in patient tissues does not significantly affect mitochondrial functions and decreases the toxicity of RA through the inhibition of MPT, we monitored the effects of EDX in combination with RA on melanoma cell proliferation and migration and compared to those promoted by the combination with TAM. Furthermore, the effects of these combinations on endothelial cells were also evaluated to provide the first clues on the potential toxicity for non-neoplasic cells of the combinations of RA with antiestrogenic compounds. The data obtained demonstrate that EDX is more effective than TAM in the inhibition of melanoma cell biomass. The combination of this metabolite with RA enhances the antiproliferative effect induced by each drug individually, by blocking cell cycle progression in G1 phase, and decreases the migration of melanoma cells without significant toxicity in normal cells. The results were published in the *Eur. J. Pharmacol*. 715, 354-362 and are presented in chapter IV.

There are strong evidences suggesting that glutamate plays an important role in the regulation of tumor cells proliferation. Accordingly, NMDAR antagonists limit the proliferation of several non-neuronal cancers, including melanoma, and enhance the effects of cytostatic drugs. Thus, the effects of iGluR antagonists on melanoma cells proliferation were monitored and the NMDAR antagonist MK-801 was selected for further studies in combination with antiestrogenic compounds. The antiestrogens EDX, TAM and OHTAM combined with MK-801 potentiate the antiproliferative effect induced by the compounds individually and the combinations with EDX or OHTAM were able to block cell cycle progression in G1. These results were submitted for publication in *Exp. Dermatol.* and are presented in chapter V.

In spite of the several studies conducted up to now, the inclusion of TAM in melanoma therapeutic regimens remains controversial. Our results demonstrate that EDX is a more powerful cytostatic in melanoma than TAM, suggesting that the variability in TAM metabolism can affect the treatment outcome in melanoma, as demonstrated in breast cancer. However, the mechanisms of action of antiestrogens in melanoma remain poorly defined after several years of research and it is possible that certain patients, according to the specific traits of their lesions, can benefit from the treatment with antiestrogens, while others do not. Therefore, it is a matter of crucial importance the identification of the action mechanisms of antiestrogens in melanoma and the clarification for the superior cytostatic activity of EDX over TAM. The identification of the GPR30 as a new ER, which can be activated by TAM and OHTAM, led us to investigate the expression and the role played by GPR30 in melanoma cells. Our study demonstrates that K1735-M2 melanoma cells express GPR30 and its activation by the selective agonist G-1 markedly decreases melanoma cell proliferation. Therefore, this work identifies GPR30 as a new target in melanoma therapy that is possibly involved in the antiproliferative effects promoted by antiestrogens. On the other hand, considering the intracellular signaling pathways commonly involved in cell proliferation, EDX was the only compound that was able to induce a significant decrease in ERK 1/2 activation in our experimental setting, which can contribute to explain the higher efficacy of EDX in the reduction of melanoma cell proliferation when compared with TAM. These results are shown in chapter VI and are presented as a manuscript for future publication.

Thus, EDX may represent an advantageous therapeutic alternative to TAM in the management of malignant melanoma, either individually or in association, which is a new indication for this drug that is already being tested in humans.

CHAPTER II

THE ANTIESTROGEN ENDOXIFEN PROTECTS RAT LIVER MITOCHONDRIA FROM PERMEABILITY TRANSITION PORE OPENING AND OXIDATIVE STRESS AT CONCENTRATIONS THAT DO NOT AFFECT THE PHOSPHORYLATION EFFICIENCY

[Ribeiro, M.P., Silva, F.S., Santos, A.E., Santos, M.S. and Custódio, J.B. (2013) The antiestrogen endoxifen protects rat liver mitochondria from permeability transition pore opening and oxidative stress at concentrations that do not affect the phosphorylation efficiency. *Toxicol. Appl. Pharmacol.* 267, 104-112]

Abstract

Endoxifen (EDX) is a key active metabolite of tamoxifen (TAM) with higher affinity and specificity to estrogen receptors that also inhibits aromatase activity. It is safe and well tolerated by healthy humans, but its use requires toxicological characterization. In this study, the effects of EDX on mitochondria, the primary targets for xenobiotic-induced toxicity, were monitored to clarify its potential side effects. EDX up to 30 nmol/mg protein did not affect the mitochondrial oxidative phosphorylation. At 50 nmol EDX/mg protein, EDX decreased the ADP phosphorylation rate and a partial collapse of mitochondrial membrane potential ($\Delta\Psi$), that parallels a state 4 stimulation, was observed. As the stimulation of state 4 was not inhibited by oligomycin and 50 nmol EDX/mg protein caused a slight decrease in the light scattering of mitochondria, these data suggest that EDX promotes membrane permeabilization to protons, whereas TAM at the same concentration induced mitochondrial membrane disruption. Moreover, EDX at 10 nmol/mg protein prevented and reversed the Ca^{2+}-induced depolarization of $\Delta\Psi$ and the release of mitochondrial Ca^{2+}, similarly to cyclosporine A, indicating that EDX did not affect Ca^{2+} uptake, but directly interfered with the proteins of the mitochondrial permeability transition (MPT) megacomplex, inhibiting MPT induction. At this concentration, EDX exhibited antioxidant activity that may account for the protective effect against MPT pore opening. In conclusion, EDX within the range of concentrations reached in tissues did not significantly damage the bioenergetic functions of mitochondria, contrarily to the prodrug TAM, and prevented the MPT pore opening and the oxidative stress in mitochondria, supporting that EDX may be a less toxic drug for women with breast carcinoma.

2.1 - Introduction

Introduced in the market 30 years ago, tamoxifen (TAM) remains the standard endocrine therapy for the treatment of estrogen receptor (ER)-positive breast cancer. However, TAM utilization has been questioned due to its carcinogenic action on uterus and endometrium and many women experience hot flushes (Henry et al., 2009), muscle aches and other symptoms that limit their compliance with treatment, resulting in increased rates of breast cancer recurrence (Thompson et al., 2011).

TAM is a prodrug that is extensively metabolised by cytochrome P450 (CYP) enzymes, namely CYP3A4 and CYP2D6, generating two active metabolites, 4-hydroxytamoxifen (OHTAM) and endoxifen (EDX) (Kiyotani et al., 2012). In the last years, it was raised the possibility that CYP3A4 and CYP2D6 polymorphisms could explain the large variations among patients in both the therapeutic efficacy and side effects (Kiyotani et al., 2012). Recent studies confirmed an association between CYP2D6 variation and clinical outcome in women treated with TAM and indicated that coadministration of CYP2D6-inhibiting medication diminishes the treatment efficacy of TAM (Kelly et al., 2010; Lammers et al., 2010). In fact, after 6 weeks of therapy with TAM, the steady-state levels are in the range of 24-317 ng/mL (Ingle et al., 1999). This important patient intervariability suggests that the same dose of TAM may not be suitable for all patients and that TAM may not be the appropriate drug for some patients (Ingle, 2008). On the other hand, TAM has been associated with cases of idiosyncratic liver injury (DeLeve, 2007) and different patterns of elevated liver enzymes have been observed in both human and animal models (Ching et al., 1992; Oien et al., 1999; Moreira et al., 2007). Moreover, approximately 33 % of patients who took TAM developed fatty liver and nonalcoholic steatohepatitis was present in 2.2 % of the patients with breast cancer treated with TAM (Saphner et al., 2009). Some patients also develop hepatic fibrosis, cirrhosis and hepatic necrosis (Storen et al., 2000; Farrel, 2002). In addition, the damaging effects on the lipid metabolism may also account for the hypertriglyceridemia observed in some patients treated with TAM (Liu and Yang, 2003; Sakhri et al., 2010).

It is plausible that the hepatotoxicity induced by TAM may be related with its effects on mitochondrial functions, as previous studies have described the multiple effects of TAM on liver mitochondria and emerging evidence points to an important role played by

mitochondrial dysfunction in drug-induced liver injury (Labbe et al., 2008). TAM has been shown to inhibit the mitochondrial permeability transition (MPT) promoted by different inducers (Custódio et al., 1998; Hernández-Esquivel et al., 2011a) and to affect the mitochondrial complex I, the respiration rate and the phosphorylation efficiency (Cardoso et al., 2001; Moreira et al., 2006). The impairment of the respiratory chain compromises the reoxidation of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) into NAD^+ and FAD, respectively, which are required for the activity of several dehydrogenases of the Krebs cycle and the mitochondrial β-oxidation, leading to the inhibition of fatty acid oxidation and lipid metabolism alterations (Begriche et al., 2006; Rolo et al., 2012). Furthermore, the damaged respiratory chain will increase the mitochondrial reactive oxygen species (ROS) formation, which will induce lipid peroxidation and the release of aldehydic derivatives that promote deleterious effects on hepatocytes and other hepatic cells (Begriche et al., 2006; Rolo et al., 2012).

EDX, a key active metabolite of TAM, presents a plasma concentration over 6-fold higher than that of OHTAM in breast cancer patients (Lee et al., 2003). Moreover, EDX has demonstrated to be 100 times more potent than TAM, and has a potency similar to that of OHTAM with respect to ER binding affinity, in suppression of estrogen-dependent cell growth and gene expression (Kiyotani et al., 2012). It has been reported that EDX targets ERα for degradation by the proteosome in breast cancer cells (Wu et al., 2009) and inhibits aromatase activity, which are mechanisms that may contribute to its efficacy in the treatment of breast cancer (Lu et al., 2012). Importantly, EDX demonstrated the ability to inhibit the growth of human mammary tumor xenografts in female mice (Ahmad et al., 2010a). Additionally, in healthy human subjects orally administered EDX was safe and well tolerated (Ahmad et al., 2010b). According to the pharmacokinetic studies performed by Ahmad *et al* (2010b), EDX reaches systemically effective levels in human subjects and the authors propose that multiple daily doses of 2.0-4.0 mg of EDX would result in EDX exposures that would be similar to those found in patients with normal CYP2D6 function who are administered TAM at 20 mg/day. Altogether, these data suggest that the use of EDX would be a possible strategy to overcome TAM limitations. However, the available information regarding the potential toxic effects of EDX on mitochondria is very limited at the moment.

Therefore, the aim of this study was to investigate the effects of EDX on mitochondrial bioenergetic functions, MPT and oxidative stress, in order to understand the

potential toxic effects induced by EDX in relation to the effects promoted by TAM and OHTAM, which will provide new evidences that can contribute to the safe use of EDX in breast cancer therapy. To achieve our goal, we have evaluated several mitochondrial parameters from the respiratory chain (states 3 and 4 of respiration, respiratory control ratio (RCR) and ADP/O index) and oxidative phosphorylation system [transmembranar potential ($\Delta\Psi$), depolarization induced by ADP, lag phase and active adenine nucleotide translocase (ANT) content]. Moreover, we have studied the susceptibility of mitochondria to MPT pore opening and the effects of EDX on the oxidative stress evaluated by oxygen consumption and thiobarbituric acid reactive substances (TBARS) levels.

2.2 - Materials and methods

Animals

Wistar rats (250-350g), of either sex, were maintained at 22 ± 2 ºC under artificial light for 12 h light/dark cycle and with access to water and food *ad libitum*. The experiments reported here were carried out in accordance with the National Requirements for Vertebrate Animal Research and in accordance with the European Convention for the Protection of Animals used for Experimental and other Scientific Purposes.

Isolation of rat liver mitochondria

Mitochondria were isolated from the liver of Wistar rats as previously described (Custódio et al., 1998). Briefly, liver was quickly removed from decapitated rats, finely minced and homogenized in an ice-cold homogenization medium containing 250 mM sucrose, 10 mM 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA), and 0.1 % (w/v) bovine serum albumin (BSA). The homogenate was centrifuged at 800×*g* for 10 min and mitochondria were recovered from the supernatant by centrifugation at 10 000×*g* for 10 min. The mitochondrial pellet obtained was suspended twice in the final washing medium, i.e., homogenization medium adjusted to pH 7.2, in the absence of EGTA and BSA. All the procedures were performed at 0-4 °C. Mitochondrial protein concentration was determined by the Biuret method using BSA as the protein standard.

Mitochondrial membrane potential (ΔΨ)

Mitochondrial membrane potential (ΔΨ) was assessed by measuring the movements of the lipophilic cation tetraphenylphosphonium (TPP^+) across the mitochondrial membrane with a TPP^+ selective electrode and using a $Ag/AgCl_2$ electrode as reference (model MI 402; Microelectrodes, Inc., Bedford, NH). The reactions were carried out in an open vessel with magnetic stirring in 2 mL of the standard respiratory medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 5 mM KH_2PO_4, 5 mM HEPES, pH 7.4, supplemented with 3 µM TPP^+ and glutamate/malate (5 mM/2.5 mM), at 37 °C. The assays were initiated by adding 1 mg of mitochondrial protein and the potential difference between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously with a suitable recorder as described elsewhere (Moreira et al., 2007). After a steady-state distribution of TPP^+ had been reached (ca. 1 min of recording), different concentrations of EDX were added to the reaction medium and allowed to incubate for 3 min prior the addition of ADP (250 nmol/mg protein). The voltage response of the TPP^+ electrode to log $[TPP^+]$ was linear with a slope of 59 ± 1, in a good agreement with the Nernst equation. The ΔΨ was estimated by the following equation: $\Delta\Psi = 59 \times \log(v/V) - 59 \times \log(10^{\Delta E/59} - 1)$, where v, V, and ΔE stand for inner mitochondrial volume, incubation medium volume and deflection of the electrode potential from the baseline, respectively. A matrix volume of 1.1 µl/mg of protein was assumed.

Mitochondrial respiration

Oxygen consumption of isolated liver mitochondria was assessed polarographically with a Clark-type oxygen electrode (YSI model 5331, Yellow Spring Inst.) connected to a suitable recorder in a 1 mL thermostatted water-jacket closed chamber with magnetic stirring (Moreira et al., 2006). Liver mitochondria (1 mg) were suspended in 1 mL of standard respiratory medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 5 mM KH_2PO4, 5 mM HEPES, and pH 7.4, at 37 °C. Respiration was initiated upon addition of 10 mM glutamate/5 mM malate (state 2) and 250 nmol/mg protein of ADP was added to induce state 3 respiration. After phosphorylation of all the added ADP, the oxygen consumption rate decreased and state 4 respiration was resumed. The state 4 in the presence of oligomycin (state 4 olig) was induced by the addition of oligomycin (1 µg/mg protein) to evaluate the proton leak through the Fo fraction of complex V. The mitochondrial suspension

was incubated in the absence (control) and in the presence of different concentrations of EDX for 3 min before starting the reactions. Respiration rates were calculated assuming an oxygen concentration of 240 nmol O_2/mL. The RCR was calculated as the ratio between state 3 (consumption of oxygen in the presence of substrate and ADP) and state 4 (consumption of oxygen after ADP phosphorylation), and it is considered an indicator of mitochondrial membrane integrity. The ADP/O ratio is the number of ADP molecules added to the medium per oxygen atom consumed during phosphorylation, and it is an index of oxidative phosphorylation efficiency.

For monitoring the active adenine nucleotide translocase (ANT) content, mitochondria were titrated with carboxyatractyloside (CATR) (Vignais, 1976). Mitochondria (0.5 mg) were incubated in 1 mL of standard reaction medium (200 mM sucrose, 5 mM KH_2PO4, 20 mM Tris-HCl, pH 7.4). The active ANT content was determined by titrating the rate of state 3 respiration with increasing concentrations of CATR (Vignais, 1976). Oxygen consumption rate was evaluated at 25 °C using a closed chamber fitted with a Clark-type oxygen electrode as described above. Mitochondria were incubated in the absence or presence of EDX and 3 min later CATR was added to mitochondria. After the incubation period, the reactions were started by the addition of 10 mM glutamate/5 mM malate and state 3 respiration was initiated by adding 0.2 mM ADP. Plots of O_2 consumption *versus* CATR concentration are biphasic, with an increasing inhibitory effect followed by a steady-state effect which corresponds to the complete inhibition of state 3 respiration. The amount of CATR corresponding to complete inhibition of state 3 respiration was used to estimate the active ANT content assuming a 1:1 binding stoichiometry. The active ANT content was expressed as CATR amount per mg of mitochondrial protein (pmol CATR/mg protein) (Cardoso et al., 2003).

Mitochondrial swelling

Mitochondrial osmotic volume changes were followed by monitoring the decrease in absorbance (light scattering) at 540 nm with a Perkin Elmer, Lambda 45 UV/VIS spectrometer. The reactions were conducted at 25 °C with 1 mg mitochondrial protein in 2 mL of standard MPT reaction medium containing 200 mM sucrose, 10 mM Tris-3-[N-Morpholino]propanesulfonic acid (Mops), 1 mM KH_2PO_4, 10 μM EGTA, 3 μM rotenone and 3 μM TPP^+, pH 7.4, in the presence of substrate (5 mM glutamate/2.5 mM malate).

Different concentrations of EDX, TAM (50 nmol/mg protein), OHTAM (50 nmol/mg protein) and Triton X-100 (0.01 %) were added to mitochondrial suspension after 3 minutes of recording (Cardoso et al., 2002a).

Measurements of mitochondrial permeability transition pore (MPT)

The Ca^{2+}-dependent MPT was characterized by the evaluation of $\Delta\Psi$ dissipation and Ca^{2+} fluxes as previously described (Custódio et al., 1998). For the $\Delta\Psi$ determination, mitochondria (0.5 mg/mL) were suspended in 2 mL of standard MPT reaction medium containing 200 mM sucrose, 10 mM Tris-Mops, 1 mM KH_2PO_4, 10 μM EGTA, 3 μM rotenone and 3 μM TPP^+, pH 7.4, at 30 °C and energized with 5 mM succinate. After reaching a steady-state distribution of TPP^+, Ca^{2+} was added (90-120 nmol/mg protein, depending on mitochondrial preparation) and $\Delta\Psi$ was measured as described above. EDX and cyclosporine A (CyA) were incubated for 3 min with mitochondria before the addition of succinate or after mitochondrial loading with Ca^{2+}, as indicated in the figure legends.

The mitochondrial Ca^{2+} uptake and release was monitored using the fluorescence probe Calcium Green 5-N (Moreira et al., 2007). Fluorescence was recorded continuously at 30 °C, using a Perkin Elmer LS 50B luminescence spectrometer with excitation and emission wavelengths of 506 and 531 nm, respectively. Liver mitochondria (0.5 mg protein) were suspended in 2 mL of standard MPT reaction medium supplemented with 3 μM rotenone and 100 nM calcium green. Mitochondria were energized with 5 mM succinate after 1 min of Ca^{2+} addition (90-120 nmol/mg protein), in the absence (control) or in the presence of EDX and CyA. In other experiments, EDX and CyA were added after Ca^{2+} and succinate energization, as indicated in the figure legends.

Evaluation of oxidative stress

Oxidative stress was evaluated by oxygen consumption monitored polarographically at 30 °C using a Clark-type oxygen electrode (YSI Model 5331, Yellow Springs Inst) placed in a glass chamber equipped with magnetic stirring (Moreira et al., 2007). Reactions were carried out in 1 mL of reaction medium containing 175 mM KCl, 10 mM Tris, pH 7.4 and 1 mg of liver mitochondria. The reactions were started by the addition of ADP/Fe^{2+} (1 mM/0.1 mM) and the changes in oxygen tension calculated assuming a concentration of 240 nmol O_2/mL in the experimental medium at 30 °C. EDX was incubated

with mitochondria for 3 min before the addition of ADP/Fe^{2+}. In parallel, the extent of lipid peroxidation was determined by measuring TBARS, using the thiobarbituric acid assay, according to a modified procedure as described elsewhere (Santos et al., 2001). The amount of TBARS formed was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as percentage of control.

Statistical analysis

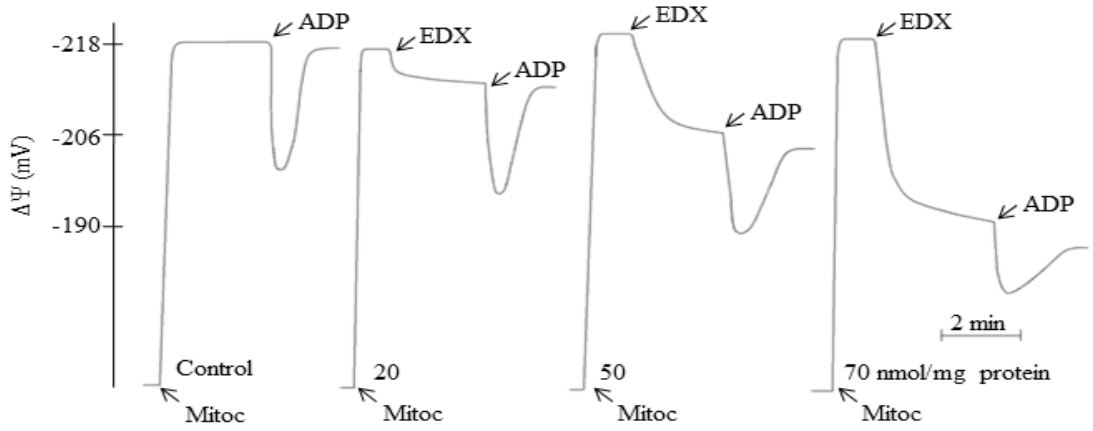
The results are presented as mean ± S.E.M. of the indicated number of experiments. Statistical significance between the different assays was determined using the one-way analysis of variance (ANOVA), followed by the Tukey post-test, for multiple comparisons. A p value <0.05 was considered statistically significant.

2.3 - Results

To assess whether EDX affects mitochondrial bioenergetic functions, we evaluated the ΔΨ fluctuations associated with the phosphorylation cycle induced by ADP, using glutamate/malate as substrate. Additionally, the effects induced by EDX were also compared to those induced by the prodrug, TAM, and the other active metabolite, OHTAM. The range of EDX concentrations used allows for a full characterization of the compound from the absence to the maximum effects. The figure 2.1 represents a typical recording of the phosphorylation cycle induced by ADP, and the table underneath shows the mean ± S.E.M. of the indicated parameters. As observed in figure 2.1, mitochondria energized with glutamate/malate, in the absence of EDX (control), developed a ΔΨ of 219.9 ± 1.3 mV (negative inside) and the addition of ADP dropped the ΔΨ in 21.2 ± 1.2 mV (ADP depolarization). The time required for the phosphorylation of ADP (lag phase) was 71.7 ± 3.6 s and after ADP phosphorylation, the ΔΨ returned to −217.7 ± 1.5 mV (repolarization ΔΨ), which is close to its initial value. In the range of 5-30 nmol/mg protein, EDX did not significantly affect the phosphorylation cycle induced by ADP (Fig. 2.1). At 50 nmol/mg protein, EDX significantly increased the phosphorylation lag phase and decreased the ΔΨ developed after substrate energization, as well as the repolarization ΔΨ, but the ADP depolarization was not significantly affected (Fig. 2.1). At 70 nmol/mg protein, EDX markedly decreased both the ΔΨ developed after substrate energization and the

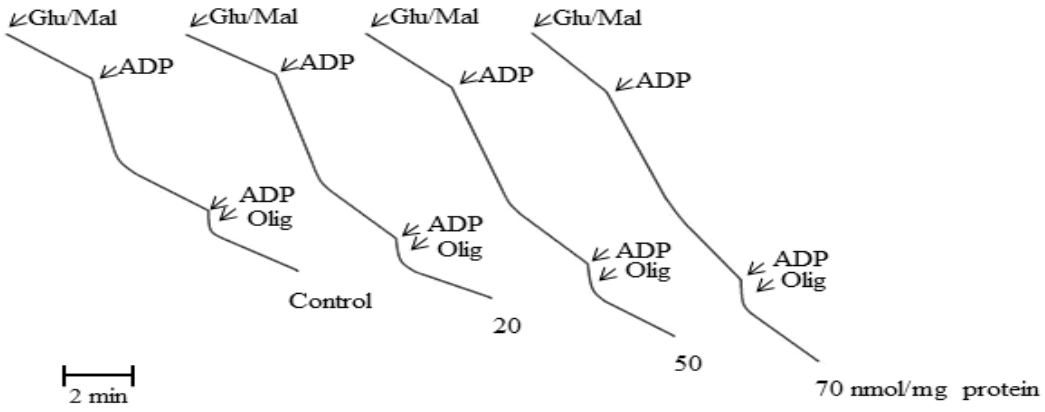
repolarization ΔΨ, whereas the phosphorylation lag phase increased (Fig. 2.1). The effects induced by 20 nmol/mg protein of EDX were then compared to those induced by the same concentration of TAM and OHTAM (Fig. 2.1). OHTAM at 20 nmol/mg protein did not present significant effects on the phosphorylation cycle induced by ADP, similarly to EDX (Fig. 2.1). In contrast, TAM at 20 nmol/mg protein significantly depressed the ΔΨ developed after substrate energization and the repolarization ΔΨ, and increased the phosphorylation lag phase (Fig. 2.1).

The effects of EDX on mitochondrial bioenergetics were also investigated by the analysis of several respiration parameters (Fig. 2.2). As shown figure 2.2, respiration was initiated by adding glutamate/malate (state 2 respiration). When 250 nmol/mg protein of ADP was added, the rate of oxygen consumption increased (state 3 respiration) and after the phosphorylation of all the added ADP, the oxygen consumption rate decreased and state 4 respiration was resumed. The state 4 respiration in the presence of oligomycin (state 4 olig) was induced by the addition of oligomycin, in order to assess the proton leak through the Fo fraction of complex V. EDX between 5 and 30 nmol/mg protein did not significantly affect the respiration parameters, except the RCR, which is decreased as a consequence of both the stimulation of state 4 and the inhibition of state 3 respiration (Fig. 2.2). At 50 nmol/mg protein, EDX significantly decreased the state 3 and stimulated the state 4 respiration, significantly decreasing the RCR ratio (Fig. 2.2), in agreement with the results obtained in the ΔΨ associated to mitochondrial respiration and the phosphorylation lag phase (Fig. 2.1). We have also observed that the stimulation of state 4 was not inhibited in the presence of oligomycin (Fig. 2.2). The stimulation of state 4 is followed by a pronounced decrease in the states 3 and 4 respiration at 70 nmol/mg protein (Fig. 2.2). The effects induced by 20 nmol/mg protein of EDX on respiration parameters were also compared to those induced by the same concentration of TAM and OHTAM (Fig. 2.2). OHTAM at 20 nmol/mg protein did not present significant effects on the respiration parameters, except for the RCR, similarly to EDX (Fig. 2.2). In contrast, at 20 nmol/mg protein, TAM significantly stimulated the state 4 respiration and diminished the state 3, the ADP/O ratio and the RCR (Fig. 2.2). Is it noteworthy that the RCR of TAM and its metabolites was significantly depressed above 20 nmol/mg protein, but as expected, TAM induced a more pronounced decrease of the RCR, reflecting the more intense effects on both state 3 and state 4 respiration (Fig. 2.2).



(nmol/mg protein)	ΔΨ (-mV)			Phosphorylation lag phase (s)
	Glu/Mal Energization	ADP Depolarization	Repolarization	
Control	219.1 ± 1.3	21.2 ± 1.2	217.7 ±1.5	71.7 ± 3.6
5 EDX	219.2 ± 3.5	22.7 ± 1.7	218.2 ± 3.4	84.7 ± 6.0
10 EDX	218.2 ± 3.8	22.4 ± 1.9	216.6 ± 3.8	89.58 ± 5.8
20 EDX	212.7 ± 1.6	22.5 ± 1.3	210.5 ± 1.6	88.97 ± 3.8
30 EDX	210.5 ± 2.6	22.4 ± 1.7	207.8 ± 2.2	93.50 ± 5.8
50 EDX	201.5 ± 2.8***	23.1 ± 1.4	196.7 ± 3.4***	116.0 ± 4.0***
70 EDX	181.2 ± 4.3***	20.4 ± 1.6	176.6 ± 7.6***	135.0 ± 3.0***
20 TAM	205.5 ± 4.6*	20.9 ± 3.3	204.3 ± 4.6*	102.0 ± 0.0*
20 OHTAM	210.2 ± 2.1	20.9 ± 1.5	207.8 ± 1.9	82.00 ± 5.0

Fig. 2.1 - Effect of EDX on the mitochondrial transmembrane potential (ΔΨ). The reactions were started by adding 1 mg of mitochondrial protein (Mitoc) to 2 mL of the standard respiratory medium, supplemented with 3 µM TPP^+ and glutamate/malate (5 mM/2.5 mM). After a steady-state distribution of TPP^+ had been reached (ca. 1 min of recording), different concentrations of EDX were added to the reaction medium and allowed to incubate for 3 min prior the addition of ADP (250 nmol/mg protein). The traces are typical recordings representative of several experiments obtained in the absence of drugs (control), and in the presence of different EDX concentrations (nmol/mg protein) indicated by the numbers adjacent to traces. The table underneath shows the mean ± S.E.M. of membrane potential and phosphorylation time at the indicated concentrations of EDX, TAM and OHTAM. *** $p<0.001$, * $p<0.05$ when compared with control. The results are the mean ± S.E.M. of four independent experiments.



(nmol/mg protein)	State 3	State 4	State Olig	RCR	ADP/O
Control	115.9 ± 4.844	20.8 ± 2.2	14.4 ± 1.9	6.2 ± 0.7	2.3 ± 0.1
5 EDX	102.1 ± 5.0	23.3 ± 3.5	15.4 ± 2.3	5.4 ± 1.2	2.1 ± 0.2
10 EDX	102.7 ± 4.8	26.5 ± 3.5	17.5 ± 3.5	3.6 ± 0.7	2.0 ± 0.2
20 EDX	104.2 ± 4.8	29.8 ± 2.7	19.7 ± 2.7	4.2 ± 0.5	1.9 ± 0.1
30 EDX	100.5 ± 5.7	35.1 ± 4.6	22.0 ± 3.7	3.7 ± 0.8	1.8 ± 0.1
50 EDX	90.5 ± 5.2*	38.9 ± 5.4**	22.6 ± 4.0	2.3 ± 0.2*	1.7 ± 0.1
70 EDX	41.8 ± 4.6***	31.4 ± 3.3	16.9 ± 2.1	1.5 ± 0.1**	1.6 ± 0.2
20 TAM	88.4 ± 7.4*	41.9 ± 5.4 ***	26.0 ± 3.3	2.7 ± 0.2**	1.6 ± 0.1*
20 OHTAM	97.3 ± 7.4	31.6 ± 3.3	28.0 ± 3.5	3.4 ± 0.4	1.8 ± 0.1

Fig. 2.2 - Effect of EDX on respiration parameters of rat liver mitochondria. Mitochondria (1 mg) in 1 mL of standard respiratory medium were energized with 10 mM glutamate/5 mM malate (Glu/Mal). ADP (250 nmol/mg protein) was added to induce state 3 respiration. Inhibition of state 3 respiration was induced by adding 2 µg/mL oligomycin (Olig). The mitochondrial suspension was incubated in the absence (control) and in the presence of different concentrations of EDX for 3 min before starting the reactions at 37 ºC. The traces are typical recordings representative of several experiments obtained in the absence of drugs (control), and in the presence of different EDX concentrations (nmol/mg protein) indicated by the numbers adjacent to traces. The table underneath shows the mean ± S.E.M. of respiratory parameters at the indicated concentrations of EDX, TAM and OHTAM. State 3, state 4, state olig are expressed in nmol O_2/mg protein/min. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ when compared with control. The results are the mean ± S.E.M. of six independent experiments.

In order to clarify the effects on ΔΨ and respiration rates induced by high concentrations of EDX, the mitochondrial membrane integrity was evaluated by following the decrease in light scattering at 540 nm of mitochondria in the presence of substrate (Fig. 2.3). In control condition, the light scattering was sustained over 25 min (Fig. 2.3, trace a). At 25-50 nmol/mg protein (Fig. 2.3, traces b and c, respectively), EDX causes a small dose-dependent decrease in light scattering, reflecting a slight direct effect of this drug on mitochondrial membrane integrity. This effect becomes more pronounced at 70 nmol/mg protein (Fig. 2.3, trace d), suggesting that high concentrations of this drug may compromise the integrity of mitochondrial membranes, interfering with their permeability. When we compare the effects on mitochondrial swelling induced by 50 nmol/mg protein of EDX, with the same concentration of OHTAM or TAM, it is possible to observe that EDX (Fig. 2.3, trace c) was the compound that least decreased the light scattering, followed by OHTAM (Fig. 2.3, trace e). Both metabolites presented significantly less effects than TAM (Fig. 2.3, trace f), which at 50 nmol/mg protein caused an extensive swelling of mitochondria, similar to that induced by triton X-100 (Fig. 2.3, trace g).

The effect of EDX on Ca^{2+}-induced MPT of rat liver mitochondria was also evaluated by monitoring Ca^{2+} release (Fig. 2.4) and ΔΨ dissipation (Fig. 2.5). As shown in figure 2.4A, mitochondria energized with succinate in the presence of Ca^{2+} (90-120 nmol/mg protein), rapidly release the accumulated Ca^{2+} to the reaction medium (Fig. 2.4A, control) as indicated by the increase in the fluorescence intensity. The coincubation with EDX (10 nmol/mg protein) enables mitochondria to accumulate and sustain Ca^{2+}, similarly to CyA, a specific and potent inhibitor of MPT (Fig. 2.4A). Moreover, EDX or CyA, when added after energization of mitochondria with succinate and loading with Ca^{2+}, reverses the release of Ca^{2+} to the reaction medium, which is accumulated by mitochondria once again (Fig. 2.4B).

To provide further evidence regarding the effects of EDX on Ca^{2+} homeostasis, we evaluated the effects of EDX on ΔΨ fluctuations associated with MPT induction (Fig. 2.5). Under succinate energization, mitochondria built up a potential of about −220 mV. In control conditions, upon addition of Ca^{2+} (90-120 nmol/mg protein), the potential drops, since the Ca^{2+} uniport consumes membrane potential to transport Ca^{2+} into mitochondria and returns to nearly its initial value, which is followed by an irreversible depolarization of mitochondria over the next 10 min (Fig. 2.5A). Mitochondria preincubated with either EDX (10 nmol/mg

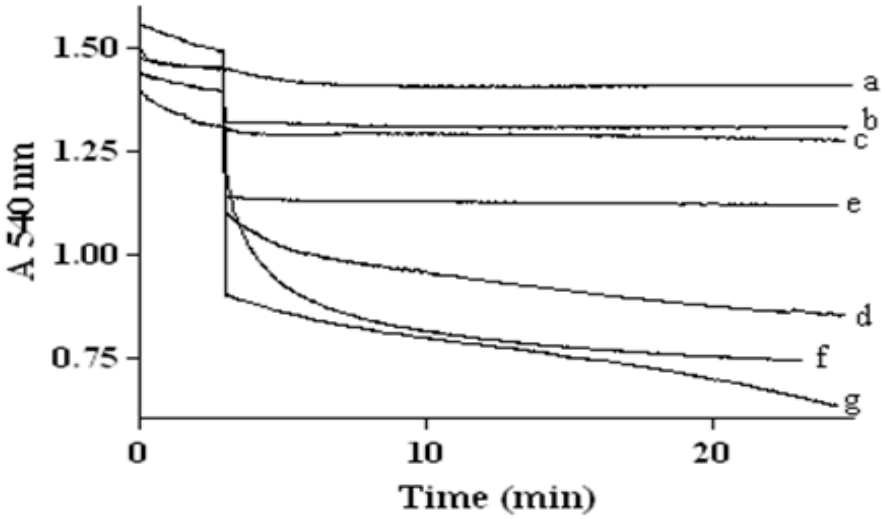


Fig. 2.3 - Effects of EDX on mitochondrial swelling. Mitochondria (1 mg) were suspended in 2 mL of standard MPT reaction medium, at 25 ºC, energized with glutamate/malate (5 mM/2.5 mM) and incubated in the absence (control, a) or in the presence of EDX at 25 (b), 50 (c) and 70 (d) nmol/mg protein, added after 3 min of recording. Experiments with OHTAM (e) and TAM (f) at 50 nmol/mg protein were also performed. An experiment using Triton X-100 (g) was performed. The traces, obtained by following the light scattering at 540 nm for 25 min, are typical of three separate experiments with different mitochondrial preparations.

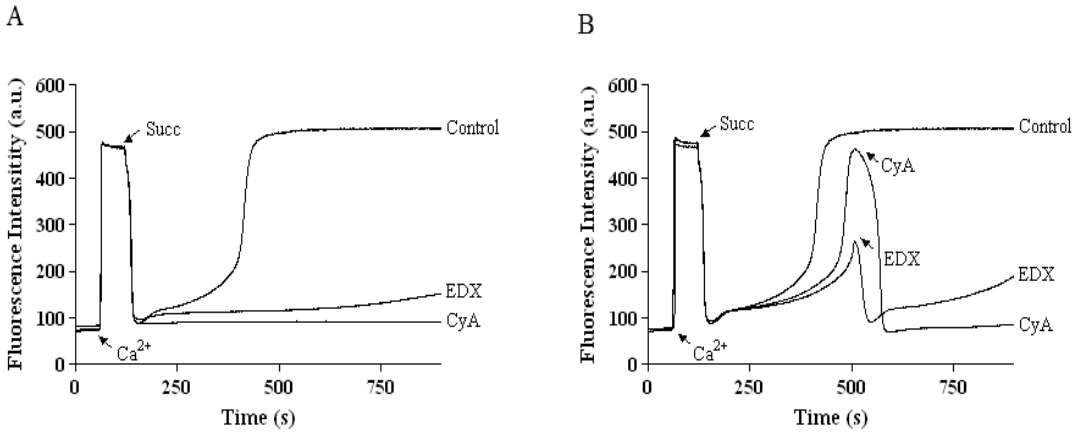


Fig. 2.4 - Inhibitory effect of EDX on mitochondrial Ca^{2+} release associated with MPT induction. Freshly isolated mitochondria (0.5 mg protein) were suspended in 2 mL of standard MPT reaction medium, supplemented with 3 µM rotenone and 100 nM Calcium Green. Fluorescence was recorded continuously at 30 ^{0}C, with excitation and emission wavelengths of 506 and 531 nm, respectively. After a stable baseline was obtained, Ca^{2+} (90-120 nmol/mg protein) was added to the reaction medium, prior the energization with 5 mM succinate (Succ). EDX (10 nmol/mg protein) and CyA (1 µM) were preincubated with mitochondria for 3 min before starting the record (A) or added after mitochondria Ca^{2+} loading and energization with succinate, during the course of MPT, as indicated by the correspondingly labeled arrows (B). The traces are typical of three different mitochondrial preparations.

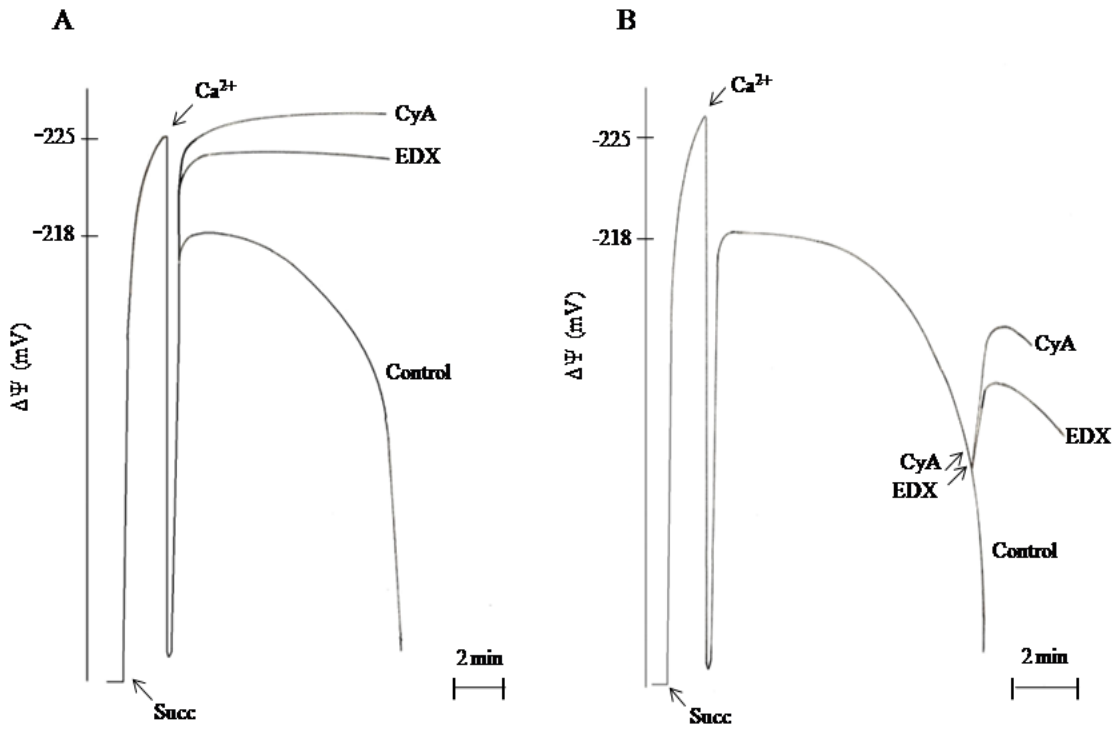


Fig. 2.5 - Inhibitory effect of EDX on Ca^{2+}-induced mitochondrial membrane depolarization. Mitochondria (1 mg) were suspended in 2 mL of the standard MPT reaction medium, supplemented with 3 µM TPP^+ and 3 µM rotenone. The reactions were started with 5 mM succinate (Succ) and Ca^{2+} (90-120 nmol/mg protein) was added after a steady-state distribution of TPP^+ had been reached. Mitochondria were incubated for 3 min, at 37 ºC, in the absence (control) or in the presence of EDX (10 nmol/mg protein) or CyA (1 µM) (A). EDX (10 nmol/mg protein) or CyA (1 µM) was also added after Ca^{2+}-induced membrane depolarization, where indicated by arrows (B). The traces are typical of three independent experiments with different mitochondrial preparations.

protein) or CyA are able to sustain the potential, as shown in Fig. 2.5A. Moreover, and in agreement with the effects on extramitochondrial Ca^{2+} movements (Fig. 2.4B), EDX reverses the Ca^{2+}-induced membrane depolarization if added during depolarization of mitochondria (Fig. 2.5B).

To clarify the mechanism underlying the ability of EDX to prevent the MPT induced by Ca^{2+} and phosphate, experiments that evaluate the effects of EDX on ANT activity (Fig. 2.6) and oxidative stress (Fig. 2.7) were carried out. The active ANT content in mitochondria can be estimated through titrations with CATR, an inhibitor of the mitochondrial ANT, which reacts with the ANT in a 1:1 stoichiometry (Vignais, 1976). The mitochondrial content of active ANT is determined by the amount of CATR required to reduce state 3 respiration to the state 4 respiratory rate, when CATR presumably saturates the translocase sites (Vignais, 1976). As shown in figure 2.6, in control conditions, the addition of 169.0 ± 8.6 pmol CATR/mg protein inhibited the ANT, i.e., mitochondria lost the ADP phosphorylative capacity and it was only observed the state 4 respiratory rate. When mitochondria were incubated with 20 nmol EDX /mg protein, ANT sites were saturated by the addition of 164.6 ± 8.3 pmol CATR/mg protein, similarly to control. In the presence of 50 nmol EDX/mg protein the active ANT content was decreased to 131.0 ± 18.33 pmol CATR/mg protein. Consequently, the active ANT content was decreased by 50 nmol EDX/mg protein to approximately 78 % relatively to control (Fig. 2.6). Therefore, although EDX decreased the active ANT content of mitochondria in a concentration-dependent manner, this decrease was not significant at concentrations up to 50 nmol/mg protein and less drastic that those induced by TAM (Cardoso et al., 2003).

The effects of EDX on oxidative stress induced by the pro-oxidant pair ADP/Fe^{2+} were evaluated by oxygen consumption (Fig. 2.7A) and TBARS formation (Fig. 2.7B). In the absence of EDX, after the addition of the pro-oxidant pair, it is possible to observe an initial phase characterized by slow oxygen consumption (lag phase). This lag phase probably reflects the time required for the generation of sufficient perferryl ion complex which is thought to initiate lipid peroxidation. The phase of slow oxygen consumption was followed by a rapid oxygen consumption phase that is probably related with the oxidation of the polyunsaturated fatty acid acyl chain of membrane phospholipids by ROS and, consequently, to the propagation of lipid peroxidation. EDX at concentrations up to 25 nmol/mg protein

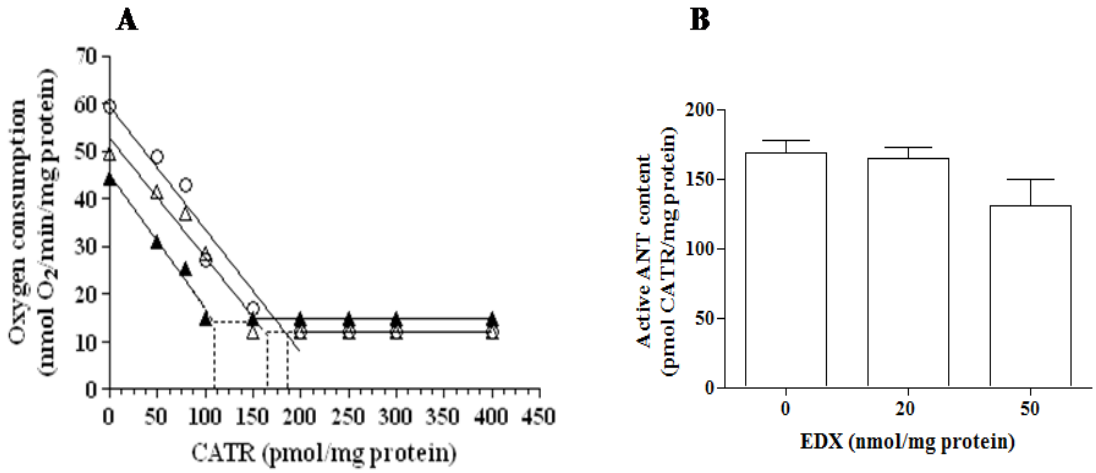


Fig. 2.6 - Determination of mitochondrial active ANT content by titration of active respiration with carboxyatractiloside (CATR). A representative pattern of titration curves shows the method of estimation of the active ANT content (A). Mitochondria (0.5 mg) in 1 mL of the standard reaction medium (200 mM sucrose, 5 mM KH_2PO4, 20 mM Tris-HCl, pH 7.4) were preincubated with different concentrations of CATR during 2 min, after a previous incubation for 3 min, in the absence (○) or in the presence of 20 (Δ) and 50 nmol EDX/mg protein (▲). The reaction was started by the addition of 0.2 mM ADP to initiate state 3 respiration and mitochondrial respiration rates were determined by oxygen consumption with a Clark-type electrode. The individual active ANT contents (B) were determined from the types of experiments as shown in (A) and are expressed as pmol CATR/mg mitochondrial protein. The values represent the mean ± S.E.M. of four mitochondrial preparations.

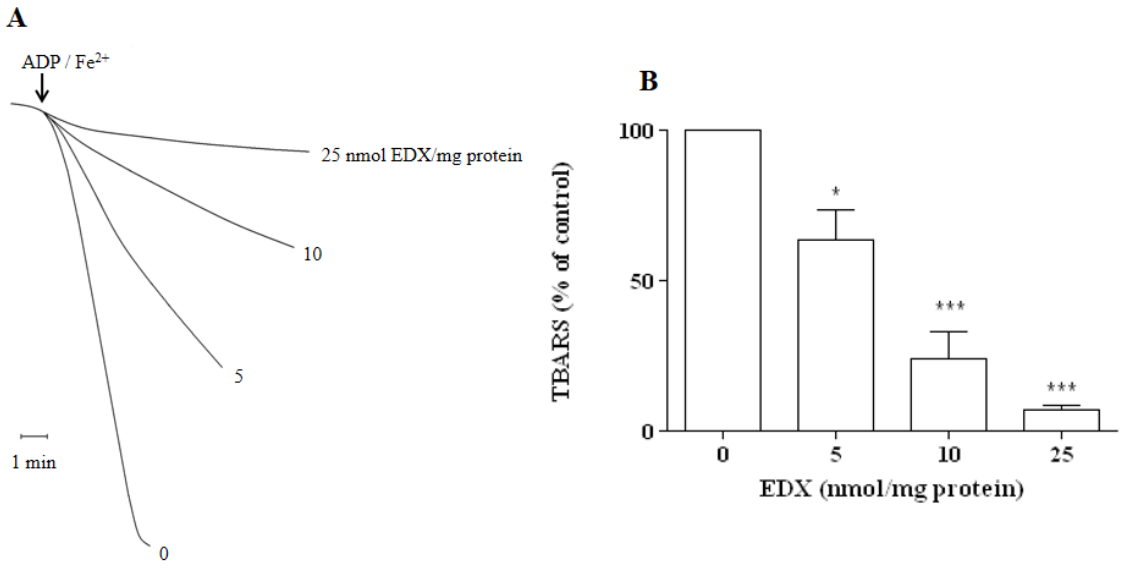


Fig. 2.7 - Protective effect of EDX on oxidative stress of rat liver mitochondria induced by the pro-oxidant pair ADP/Fe^{2+}. Oxidative stress was evaluated by oxygen consumption (A) and TBARS assay (B). Mitochondria (1 mg) were suspended in 1 mL of medium containing 175 mM KCl, 10 mM Tris, pH 7.4, supplemented with 3 µM rotenone and preincubated for 3 min, at 37 ºC, in the absence (control) or in the presence of different EDX concentrations (nmol/mg protein), before initiating the reaction by adding 1 mM ADP/0.1 mM Fe^{2+}. The traces in (A) represent typical direct recordings representative of four experiments obtained from different mitochondrial preparations. Ten min after the addition of ADP/Fe^{2+}, aliquots of 0.5 mL of mitochondrial suspensions were collected and the amount of TBARS (B) was determined as described under Materials and methods section. The data, expressed as percentage of control, correspond to the mean ± S.E.M. of four independent experiments. *** $p<0.001$, * $p<0.05$ when compared with control.

enlarged the lag phase of slow oxygen consumption before oxygen uptake burst induced by the complex ADP/Fe^{2+}, and decreased the rate of the rapid oxygen consumption phase, in a concentration-dependent manner, with a total inhibition of lipid peroxidation at 25 nmol/mg protein (Fig. 2.7A). In parallel to oxygen consumption experiments, the effects of EDX on lipid peroxidation were also monitored by TBA assay (Fig. 2.7B). Accordingly to oxygen consumption, 5 nmol EDX/mg protein diminished TBARS formation to approximately 63.9 % of control (Fig. 2.7B). This effect of EDX was much more pronounced at 10 and 25 nmol/mg protein that induced a decrease in TBARS levels to about 24.1 % and 7.2 % of control, respectively (Fig. 2.7B).

2.4 - Discussion

The disruption of mitochondrial bioenergetics plays an important role in the lethal cell injury induced by xenobiotics (Wallace and Starkov, 2000), and may account for the hepatotoxicity and the alterations in lipid metabolism observed in patients under therapy with TAM. As previously reported for TAM and OHTAM (Cardoso et al., 2001, 2002a), EDX increases the time required for ADP phosphorylation (Fig. 2.1) and decreases the state 3 respiration and the RCR ratio (Fig. 2.2). However, the effects on the phosphorylation lag phase and on the state 3 respiration only become significant at 50 nmol/mg protein of EDX, indicating that at concentrations above those reached in tissues (Lien et al., 1991; Borges et al., 2006) EDX depresses the phosphorylation capacity of mitochondria. Additionally, EDX at 50 nmol/mg protein induces a partial collapse of $\Delta\Psi$ (Fig. 2.1) that parallels with an increase of state 4 respiration (Fig. 2.2). As the stimulatory effect on state 4 is not inhibited in the presence of oligomycin (Fig. 2.2), the effects on state 4 and on $\Delta\Psi$ are not related with a proton leak through the Fo fraction of complex V. Considering that EDX at 50 nmol/mg protein decreases the light scattering of mitochondria, our data suggest that high concentrations of EDX promote membrane permeabilization to protons (Fig. 2.3). At 70 nmol EDX/mg protein, the state 4 respiration (Fig. 2.2) and the $\Delta\Psi$ developed after substrate energization, as well as the repolarization of $\Delta\Psi$ (Fig. 2.1), are markedly diminished. Since a pronounced swelling of mitochondria is observed at this concentration (Fig. 2.3), the decrease in state 4 respiration and in $\Delta\Psi$ is probably related with the disruption of mitochondrial membrane. However, TAM at 50 nmol/mg protein already promotes a much

more potent proton leak than that induced by EDX at 70 nmol/mg protein, indicating that EDX, as reported for OHTAM (Cardoso et al., 2002a), mildly affects membrane integrity, in comparison with TAM. This piece of information may be relevant when one considers that TAM disrupts unspecifically the structure of model membranes and that this lack of specificity may be responsible for several side effects. In fact, the hemolytic anemia caused by TAM was associated with the disruption of erythrocyte membrane (Cruz Silva et al., 2000), while high concentrations of OHTAM induced hemolysis in a smaller extent than TAM (Cruz Silva et al., 2001). Therefore, the fact that EDX only compromises membrane integrity at higher concentrations than those observed with TAM suggests that the therapy with EDX may present less unspecific effects than the treatment with TAM.

Deregulation of Ca^{2+} homeostasis has long been implicated in cell injury (Bernardi and von Stockum, 2012). This work demonstrates that EDX prevents the release of the mitochondrial accumulated Ca^{2+} (Fig. 2.4A) and the Ca^{2+}-dependent membrane depolarization (Fig. 2.5A), similarly to CyA, a specific and potent inhibitor of the MPT, suggesting that EDX is an inhibitor of Ca^{2+}-induced MPT. Furthermore, the protection against Ca^{2+}-dependent MPT afforded by EDX is observed either when the drug is added before (Figs. 2.4A and 2.5A) or after (Figs. 2.4B and 2.5B) energization of mitochondria with succinate and loading with Ca^{2+}. These evidences suggest a direct effect on the proteins that form the MPT complex, and exclude a possible interference with Ca^{2+} uptake across the uniport or other mitochondrial effects. Similar effects on MPT inhibition have already been reported for TAM (Custódio et al., 1998) and the other TAM active metabolite, OHTAM (Cardoso et al., 2002b).

In spite of the extensive research carried out in the last years, the composition of the MPT pore remains controversial. It is now considered that the cyclophilin D (CypD) is the only essential component of the MTP pore complex, and that the ANT plays a regulatory role (Javadov et al., 2009). Recently, the mitochondrial phosphate carrier has also been proposed as a possible key component (Leung et al., 2008). Therefore, in order to clarify the mechanism underlying the ability of EDX to inhibit the MPT induced by phosphate, the effects of EDX on the active ANT content were evaluated (Fig. 2.6). Titrations with CATR revealed that EDX, even at high concentrations (50 nmol/mg protein), does not significantly decrease the active ANT content (Fig. 2.6). Moreover, mitochondrial phosphorylation efficiency is only compromised at concentrations much higher than those required to inhibit

MPT (Figs. 2.1 and 2.2). According to these data, neither the ANT nor the phosphate carrier seems to be involved in the inhibition of MPT by EDX.

An important factor contributing to the regulation of MPT pore opening is oxidative stress (Halestrap et al., 1997; Kushnareva and Sokolove, 2000). Considering that both TAM and OHTAM have been shown to scavenge peroxyl radicals (Custódio et al., 1994), and that TAM inhibits the MPT induced by pro-oxidants (Cardoso et al., 2004), we have investigated the ability of EDX to prevent oxidative stress. At concentrations that inhibit phosphate induced-MPT, EDX significantly inhibits oxidative stress (Fig. 2.7), suggesting that the MPT inhibition by EDX may be related with its antioxidant properties. However, other mechanisms may be involved in this effect and cannot be excluded at this point.

A recent study has described the protective effect of TAM on the MPT induced by CATR, due to a diminution of membrane fluidity (Hernández-Esquivel et al., 2011a). TAM is a highly hydrophobic molecule that strongly partitions in biomembranes (Custódio et al., 1991), affecting the dynamic properties of lipids (Custódio et al., 1993a; Kazanci and Severcan, 2007). Similar effects were reported for OHTAM (Custódio et al., 1993b). Although the effects of EDX on biomembranes have not yet been described, it is plausible that due to its structural resemblance with OHTAM, which has an additional *N*-methyl group, it may also affect membrane dynamics. This ability to interfere with membrane fluidity could lead to a configurational change of the ANT and explain the effects of EDX on MPT. On the other hand, another possible mechanism would be related with the interaction with CypD, inhibiting the pore assembly in a similar way to what was described for CyA (Zoratti and Szabo, 1995). Therefore, the MPT inhibition by EDX may be related with different effects exerted by this drug on mitochondria and further studies are required to clarify them.

Therapeutic concentrations of EDX are in the 10-150 nM range (Borges et al., 2006). However, tissue concentrations of EDX are higher, especially in breast tumors, where they appear to be 10-60 times more, i.e., above 10 µM (Lien et al., 1991). In our experiments, a concentration of 10 nmol/mg protein was used to inhibit MPT. At 0.25-0.5 mg protein/mL, this translates to 2.5-5 µM of EDX. Therefore, at therapeutic plasma concentrations, EDX levels in the tissues are probably above the concentration required to inhibit induction of the MPT. Furthermore, in our bioenergetic studies, the concentrations of EDX were between 5-70 nmol/mg protein, which at 0.5-1 mg protein/mL, corresponds to 2.5-70 µM. Considering

that bioenergetic functions were only significantly affected by EDX at 50 nmol/mg protein (25-50 μM), it is possible that, within the range of concentrations found in breast cancerous tissue, EDX may not compromise mitochondrial bioenergetics, while inhibiting MPT. In fact, studies in healthy humans have shown that single oral doses of EDX are safe and well tolerated and allow to obtain effective systemic levels reliably (Ahmad et al., 2010b).

In conclusion, this work demonstrates that EDX, in the range of concentrations reached in tissues, slightly affects the mitochondrial bioenergetic functions, in contrast with the deleterious effects of TAM on liver mitochondria. Moreover, at concentrations that do not affect the phosphorylation capacity of mitochondria and present antioxidant activity, EDX inhibits the MPT. These data suggest that the increased therapeutic benefit obtained from the administration of EDX instead of the prodrug TAM, by eliminating the interindividual variability associated with TAM metabolism and avoiding potential serious drug interactions, may be accompanied by a significant reduction on mitochondrial toxicity, which is closely related with the hepatotoxicity observed in patients treated with TAM.

CHAPTER III

EFFECTS OF ALL-*TRANS*-RETINOIC ACID ON THE PERMEABILITY TRANSITION AND BIOENERGETIC FUNCTIONS OF RAT LIVER MITOCHONDRIA IN COMBINATION WITH ENDOXIFEN

[Ribeiro, M.P., Santos, A.E., Santos, M.S. and Custódio, J.B. (2013) Effects of all-*trans*-retinoic acid on the permeability transition and bioenergetic functions of rat liver mitochondria in combination with endoxifen. *Life Sci*. 93, 96-107]

Abstract

The clinical utilization of the combinations of all-*trans*-retinoic acid (RA) with antiestrogens, which present synergism of action in breast cancer, has been limited by RA adverse effects, including hepatotoxicity, which may be related with mitochondrial damage. This work evaluated the effects of RA alone and in combination with the antiestrogen endoxifen (EDX) on liver mitochondria. Mitochondrial permeability transition (MPT) was assessed by using Calcium Green-5N fluorescence and a tetraphenylphosphonium selective electrode. Oxidative stress was evaluated by oxygen consumption and thiobarbituric acid method. Mitochondrial bioenergetic parameters were monitored by measuring oxygen consumption and mitochondrial membrane potential ($\Delta\Psi$). Osmotic volume changes of mitochondria were followed at 540 nm. EDX prevents the MPT induced by RA, allowing mitochondria preincubated with RA to accumulate Ca^{2+} and inhibiting the depolarization of $\Delta\Psi$. RA above 10 nmol/mg protein depresses the phosphorylation capacity of mitochondria, as shown by the increase in the time required for ADP phosphorylation as well as by the decrease in state 3 respiration. At 20 nmol/mg protein, RA decreases the $\Delta\Psi$ and increases the state 4 respiration, suggesting that high concentrations of RA permeabilize the membrane to protons, possibly due to a proton leak through the Fo fraction of complex V. Moreover, the effects of RA on mitochondrial bioenergetics are not changed by EDX. RA-induced hepatotoxicity may be related with induction of MPT and alterations in bioenergetic parameters; the combination with EDX, which reduces mitochondrial dysfunction and synergistically potentiates the anticancer activity, may provide a safer therapeutic strategy.

3.1 - Introduction

All-*trans*-retinoic acid (RA), the most abundant natural retinoid, has been widely used in the treatment of visual and skin conditions, such as acne and psoriasis (Bushue and Wan, 2010; Tang and Gudas, 2011). RA exhibits antiproliferative and proapoptotic activity, and its use has been extensively evaluated in cancer prevention and therapy (Zusi et al., 2002; Xun et al., 2012). However, the severe side effects induced by RA, including skin toxicities, teratogenic effects and elevation of serum cholesterol and triglycerides, have been a major obstacle to its clinical utilization (Lee et al., 1993; Zusi et al., 2002; Warren and Griffiths, 2008). The combination of RA with other agents can possibly afford superior therapeutic benefit, while minimizing the side effects related with retinoid therapy. In fact, the effects of RA in combination with several agents, such as cisplatin, taxol and tyrosine phosphatase inhibitors, have been studied in a variety of cancer cell lines (Karmakar et al., 2007; Liu et al., 2008; Clark et al., 2013). Noteworthy, the combination of RA with antiestrogenic compounds, namely tamoxifen (TAM), has revealed a synergistic action in breast cancer cell lines (Danforth, 2004; Wang et al., 2007; Searovic et al., 2009; Koay et al., 2010) and recent work has pointed to the interplay between retinoid and estrogen signaling in breast cancer (Rousseau et al., 2003; Hua et al., 2009; Ross-Innes et al., 2010), suggesting that the combination of RA with antiestrogens is a promising strategy for cancer therapy.

Endoxifen (EDX), a key metabolite of TAM, is considered a promising anticancer agent, as it presents higher affinity and specificity to estrogen receptors (ER) than TAM (Lim et al., 2005) and its anticancer activity is not limited by CYP3A4 and CYP2D6 polymorphisms or by the coadministration of CYP2D6 inducers or inhibitors (Kelly et al., 2010; Lammers et al., 2010; Binkhorst et al., 2012). Furthermore, our recent studies demonstrate that, as observed in breast cancer cells, the combination of RA with EDX synergistically inhibits the proliferation of melanoma cells (Ribeiro et al., 2013a). Thus, studies that evaluate the contributing factors for the therapeutic efficacy and side effects of RA in combination with EDX are required.

Mitochondria, implicated as key effectors in RA-mediated differentiation process (Xun et al., 2012) and in several cases of hepatotoxicity reported in the course of dietary supplementation with retinoids (Stickel et al., 2011) or during therapy with RA in acute

promyelocytic leukemia patients (de- Medeiros et al., 1998), may be involved in both the therapeutic action and toxic effects induced by RA. In fact, it has been reported that RA modulates gene expression through its receptors in mitochondria (Everts and Bernadier, 2002) and induces mitochondrial permeability transition (MPT) pore opening (Rigobello et al., 1999; Notario et al., 2003). This phenomenon ultimately results in cell death and it is widely recognized as a mechanism of drug-induced liver injury (Nadanaciva and Will, 2009; Russmann et al., 2009) and may be closely related with the hepatotoxicity induced by RA. On the other hand, it has been reported that RA induces the disruption of the mitochondrial membrane (Stillwell and Nahmias, 1983), which may contribute to RA-induced hepatotoxicity, while EDX at the concentrations reached in tissues does not affect mitochondrial bioenergetic functions and inhibits the MPT induced by calcium and phosphate (Ribeiro et al., 2013b). However, the possible toxicity of this combination of drugs in mitochondria was not evaluated.

Therefore, considering that RA and EDX treatment is likely to synergistically amplify their therapeutic potential, the effects of the combinations of RA with EDX on mitochondrial functions were investigated and compared to those promoted by RA in combination with TAM and the other TAM active metabolite, 4-hydroxytamoxifen (OHTAM).

3.2 - Materials and methods

Chemicals

RA, EDX, TAM and OHTAM were purchased from Sigma-Aldrich Quimica SA (Sintra, Portugal). Calcium Green-5N was obtained from Life Technologies (Paisley, United Kingdom). All other chemicals were commercial products of the highest purity grade available. RA, TAM and OHTAM were dissolved in absolute ethanol and EDX was dissolved in dimethyl sulfoxide (DMSO). All other solutions were prepared in deionized ultrapure water.

Animals

Wistar rats (250-350g), of either sex, were maintained at 22 ± 2 ºC under artificial light for 12 h light/dark cycle and with access to water and food *ad libitum*. The experiments

reported here were carried out in accordance with the National Requirements for Vertebrate Animal Research (DL 129/92) and in accordance with the European Convention for the Protection of Animals used for Experimental and other Scientific Purposes (CETS no.123).

Isolation of rat liver mitochondria

Mitochondria were isolated from the liver of Wistar rats as previously described (Custódio et al., 1998). Briefly, liver was quickly removed from decapitated rats, finely minced and homogenized in an ice-cold homogenization medium containing 250 mM sucrose, 10 mM 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA), and 0.1 % bovine serum albumin (BSA). The homogenate was centrifuged for 10 min at 800×g and mitochondria were recovered from supernatant by centrifugation at 10 000×g for 10 min. The mitochondrial pellet was resuspended twice in the final washing medium adjusted to pH 7.2, in the absence of EGTA and BSA. All the previously described procedures were performed at 0-4 ºC. Mitochondrial protein was determined by the Biuret method (Gornall et al., 1949) using BSA as the protein standard.

Measurements of mitochondrial permeability transition pore (MPT)

The Ca^{2+}-dependent MPT was characterized by Ca^{2+} fluxes evaluation and mitochondrial membrane potential ($\Delta\Psi$) dissipation as previously described (Custódio et al., 1998; Moreira et al., 2007).

The mitochondrial Ca^{2+} uptake and release was monitored using the fluorescence probe Calcium Green 5-N, as described elsewhere (Moreira et al., 2007). Fluorescence was recorded continuously at 37 °C, using a Perkin Elmer LS 50B luminescence spectrometer with excitation and emission wavelengths of 506 and 531 nm, respectively. Liver mitochondria (0.5 mg protein) were suspended in the standard MPT reaction medium, containing 200 mM sucrose, 10 mM Tris-3-[N-Morpholino]propanesulfonic acid (Mops), 1 mM KH_2PO_4, 10 μM EGTA, pH 7.4, supplemented with 3 μM rotenone and 100 nM Calcium Green-5N. Then, before starting the reactions, mitochondria were incubated for 3 min in the absence (control) and in the presence of RA (10 nmol/mg protein) alone and in combination with 1 μM of cyclosporine A (CyA) or with the antiestrogens EDX, TAM and OHTAM (10 nmol/mg protein). After a stable baseline was obtained (ca. 1 min of recording),

Ca^{2+} (60-80 nmol/mg protein) was added to the reaction medium and mitochondria energized with 5 mM succinate (ca. 2 min of recording).

For the $\Delta\Psi$ determination, mitochondria (1 mg protein) were suspended in the standard MPT reaction medium, supplemented with 3 µM rotenone and 3 µM tetraphenylphosphonium (TPP^+), at 37 °C, and energized with 5 mM succinate. After reaching a steady-state distribution of TPP^+, Ca^{2+} was added (60-80 nmol/mg protein) and $\Delta\Psi$ was measured with a TPP^+ selective electrode and using a $Ag/AgCl_2$ electrode as reference (model MI 402; Microelectrodes, Inc., Bedford, NH) and estimated as previously described (Custódio et al., 1998; Moreira et al., 2007; Ribeiro et al., 2013b). Mitochondria were incubated in the absence (control) and in the presence of 5 nmol RA/mg protein alone or in combination with CyA (1 µM) or the antiestrogens EDX, TAM and OHTAM (10 nmol/mg protein) for 3 min before the addition of succinate. In other experiments, CyA, EDX, TAM or OHTAM was added after Ca^{2+} loading as indicated in the figure legends. In experiments using the thiol group protecting agents, glutathione (GSH, 1 mM), *N*-acetyl-L-cysteine (NAC, 200 µM) and cysteine (Cys, 200 µM), or the adenine nucleotide translocase (ANT) ligands, ADP (75 µM) and ATP (150 µM), these compounds were added to the reaction medium prior to the addition of RA and allowed to incubate for 3 min before starting the reactions.

Evaluation of oxidative stress

Oxidative stress was evaluated by oxygen consumption monitored polarographically at 37 °C using a Clark-type oxygen electrode (YSI Model 5331, Yellow Springs Inst) placed in a glass chamber equipped with magnetic stirring (Moreira et al., 2007). Reactions were carried out in 1 mL of reaction medium containing 175 mM KCl, 10 mM Tris, pH 7.4, supplemented with 3 µM rotenone and 1 mg protein of liver mitochondria. The reactions were started by the addition of ADP/Fe^{2+} (1 mM/0.1 mM) and the changes in oxygen tension calculated assuming a concentration of 240 nmol O_2/mL in the experimental medium at 37 °C. Mitochondria were incubated without (control) and with RA (5-30 nmol/mg protein) or in combination of RA (5 nmol/mg protein) with EDX (10 nmol/mg protein) for 3 min before the addition of ADP/Fe^{2+}. Ten min after the addition of ADP/Fe^{2+}, aliquots of 0.5 mL of mitochondrial suspensions were collected and the extent of lipid peroxidation was determined by measuring thiobarbituric acid reactive substances

(TBARS), using the thiobarbituric acid assay, modified as described elsewhere (Santos et al., 2001). The amount of TBARS was calculated using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as percentage of control.

Mitochondrial membrane potential

The $\Delta\Psi$ was monitored with a TPP^+ selective electrode as described above. The reactions were carried out in an open vessel with magnetic stirring in the standard respiratory medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 5 mM KH_2PO_4, 5 mM HEPES, pH 7.4, supplemented with 3 µM TPP^+ and glutamate/malate (5 mM/2.5 mM), at 37 °C. The assays were initiated by adding 1 mg of mitochondrial protein and after a steady-state distribution of TPP^+ had been reached (ca. 1 min of recording), mitochondria were allowed to incubate for 3 min in the absence (control) or in the presence of different concentrations of RA (5-30 nmol/mg protein) or 5-20 nmol RA/mg protein in combination with the antiestrogens EDX, TAM and OHTAM at 10 nmol/mg protein, prior the addition of ADP (150-250 nmol/mg protein) (Custódio et al., 1998; Moreira et al., 2007).

Mitochondrial respiration

Oxygen consumption was assessed polarographically with a Clark oxygen electrode (YSI model 5331, Yellow Spring Inst.) connected to a suitable recorder in a 1 mL thermostatted water-jacket closed chamber with magnetic stirring (Moreira et al., 2006). Mitochondria (1 mg) were suspended in the standard respiratory medium, at 37 °C. State 2 respiration was initiated with 10 mM glutamate/5 mM malate and ADP (150-250 nmol/mg protein) was added to induce the state 3 respiration. After phosphorylation of all the ADP, the oxygen consumption rate decreased and state 4 respiration was resumed. The state 4 in the presence of oligomycin (state 4 olig) was induced by the addition of oligomycin (1 µg/mL) plus ADP to evaluate the proton leak through the Fo fraction of complex V (Moreira et al., 2011). The mitochondrial suspension was incubated in the absence (control) or in the presence of different concentrations of RA (5-30 nmol/mg protein) or 5-20 nmol RA/mg protein in combination with the antiestrogens EDX, TAM and OHTAM at 10 nmol/mg protein, for 3 min before starting the reactions. Respiration rates were calculated assuming an oxygen concentration of 240 nmol O_2/mL. The respiratory control ratio (RCR) is calculated as the ratio between state 3 (consumption of oxygen in the presence of substrate

and ADP) and state 4 (consumption of oxygen after ADP phosphorylation). The ADP/O ratio is the number of ADP molecules added to the medium per oxygen atom consumed during phosphorylation (Ribeiro et al., 2013b).

Mitochondrial swelling

Mitochondrial osmotic volume changes were followed by monitoring the decrease in absorbance (light scattering) at 540 nm with a Perkin Elmer, Lambda 45 UV/VIS spectrometer (Cardoso et al., 2002a). The reactions were conducted at 25 ºC with 1 mg mitochondrial protein suspended in the standard MPT reaction medium. Mitochondria were incubated in the absence (control) or in the presence of RA (30 nmol/mg protein) or Triton X-100 (0.01 %), which was added to the mitochondrial suspension after 2 min of recording, as indicated in the figure legend.

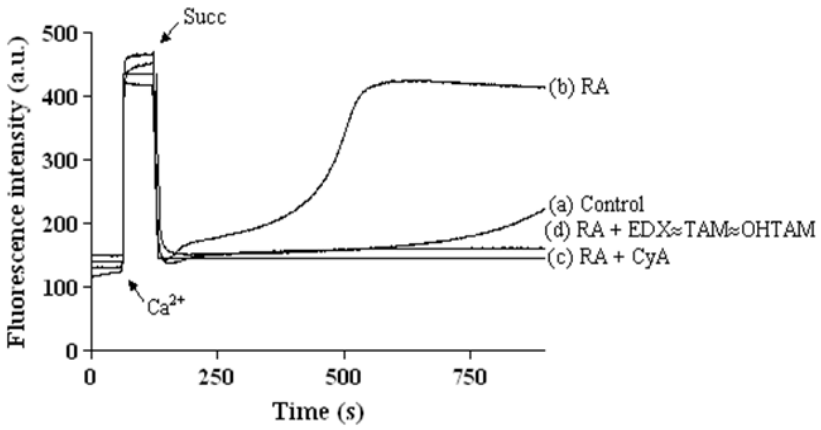
Statistical analysis

The results are presented as mean ± S.E.M. of the indicated number of experiments. Statistical significance between the different assays was determined using the one-way analysis of variance (ANOVA), followed by the Tukey post-test, for multiple comparisons. A p value <0.05 was considered statistically significant.

3.3 - Results

Effects of EDX, TAM or OHTAM on the MPT induced by RA

The effect of the antiestrogen EDX on RA-induced MPT of rat liver mitochondria was evaluated by monitoring Ca^{2+} release (Fig. 3.1) and $\Delta\Psi$ dissipation (Fig. 3.2). As shown in figure 3.1, in the control condition, after energization with succinate, mitochondria accumulated Ca^{2+} (60-80 nmol/mg protein) over the next 8-10 min (Fig. 3.1, trace a). In contrast, mitochondria previously incubated with RA released the accumulated Ca^{2+} to the reaction medium, as indicated by the increase in the fluorescence intensity (Fig. 3.1, trace b and table). This effect was significantly inhibited in the presence of CyA (Fig. 3.1, trace c and table). Similarly to what was observed for CyA, mitochondria were able to accumulate and to sustain Ca^{2+} when incubated with RA in combination with EDX (Fig. 3.1,



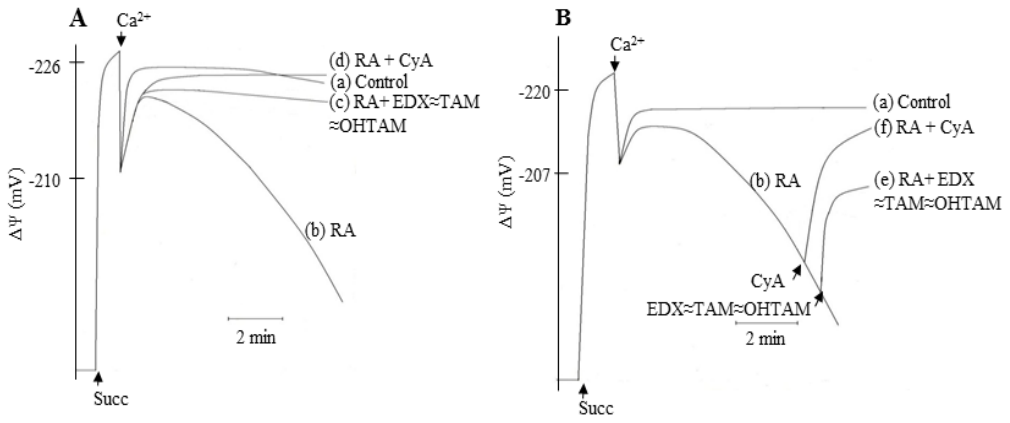
	Fluorescence intensity (a.u.)			
Time (s)	**120**	**200**	**500**	**700**
Control **(a)**	455.4 ± 19.5	114.6 ± 15.4	114.6 ± 15.8	123.5 ± 19.8
RA **(b)**	473.8 ± 17.0	125.5 ± 17.7	412.6 ± 41.0***	466.0 ± 31.3***
RA + CyA **(c)**	488.2 ± 41.8	110.9± 24.5	118.2 ± 25.2+++	118.6 ± 25.2+++
RA + EDX **(d)**	458.9 ± 30.5	91.9 ±11.4	96.2 ± 3.2+++	109.2 ± 10.5+++
RA + TAM **(d)**	460.2 ± 20.7	96.5 ±10.2	99.4 ± 2.8+++	112.9 ± 12.3+++
RA + OHTAM **(d)**	455.7 ± 33.4	89.2 ±11.8	93.2 ± 3.4+++	103.1 ± 8.9+++

Fig. 3.1 - Inhibitory effect of the antiestrogens EDX, TAM and OHTAM on RA-induced MPT as assessed by following Ca^{2+} fluxes. Ca^{2+} fluxes were monitored using the fluorescence probe Calcium Green 5-N, as described in the Materials and methods section. After obtaining a stable baseline, Ca^{2+} was added and mitochondria were energized with succinate (Succ). Before starting the reactions, mitochondria were incubated in the absence (control, trace a) and in the presence of 10 nmol/mg protein of RA alone (trace b) or in combination with 1 µM of CyA (trace c) and 10 nmol/mg protein of the antiestrogens EDX, TAM or OHTAM (trace d). The traces are typical recordings of four separate experiments with different mitochondrial preparations. The data in the table below correspond to the fluorescence intensity (a.u.) at the indicated time points and are expressed as the mean ± S.E.M. of four experiments obtained from different mitochondrial preparations. *** $p<0.001$ vs control. +++ $p<0.001$ vs RA.

trace d and table). Likewise, the incubation of mitochondria with RA in the presence of TAM or OHTAM prevented the release of the accumulated Ca^{2+} (Fig. 3.1, table).

The inhibitory effect of EDX on the induction of MPT by RA was further confirmed by studying its effects on RA-induced depolarization of ΔΨ (Fig. 3.2A). Under succinate energization, mitochondria built up a membrane potential of approximately −225 mV. Upon the addition of Ca^{2+} (60-80 nmol/mg protein), the potential drops to −210 mV, due to the transport of Ca^{2+} into mitochondria by the Ca^{2+} uniport and then rises to nearly its initial value (Fig. 3.2A, trace a, control). When mitochondria were previously incubated with RA, an irreversible depolarization of mitochondria was observed 3 min after the addition of Ca^{2+} (Fig. 3.2A, trace b and table). On the contrary, mitochondria preincubated with the combinations of RA with EDX (Fig. 3.2A, trace c and table) or with CyA (Fig. 3.2A, trace d and table) were able to sustain the ΔΨ. In a similar manner, the combinations of RA with TAM or OHTAM were able to prevent the mitochondrial depolarization of ΔΨ induced by RA (Fig. 3.2 and table), in accordance with the results regarding mitochondrial Ca^{2+} fluxes (Fig. 3.1 and table). The effects of the antiestrogens EDX, TAM or OHTAM were also studied on the reversion of MPT induced by RA. As indicated in figure 3.2B, the addition of EDX, TAM or OHTAM during the depolarization induced by RA also reversed the ΔΨ depolarization associated to MPT induction (Fig. 3.2B, trace e). Again, CyA exhibited similar effects, but being able to completely restore the ΔΨ (Fig. 3.2B, trace f).

In order to clarify the mechanisms involved in RA-induced MPT and its prevention by antiestrogens, described as intramembranous antioxidants (Custódio et al., 1994; Ribeiro et al., 2013b), we evaluated the effects of EDX and RA on the mitochondria oxidative stress induced by the pro-oxidant pair ADP/Fe^{2+} assessing the oxygen consumption (Fig. 3.3A) and lipid peroxidation by analysis of TBARS formation (Fig. 3.3B). In the absence of drugs (control) and after the addition of the pro-oxidant pair, it was possible to observe an initial phase characterized by slow oxygen consumption (lag phase). It is commonly assumed that this lag phase reflects the time required for the generation of sufficient perferryl ion complex which is thought to initiate lipid peroxidation. The lag phase was followed by a rapid oxygen consumption phase that is probably related with the oxidation of the polyunsaturated fatty acid acyl chain of membrane phospholipids by reactive oxygen species and, consequently, to the propagation of lipid peroxidation. In the range of 5-20 nmol/mg protein,



	ΔΨ (-mV)					
Time (min)	**0**	**0.5**	**1**	**3**	**5**	**6**
Control **(a)**	224.6 ± 1.6	197.5 ± 6.3	219.8 ± 1.9	220.0 ± 1.7	218.8 ± 2.0	218.8 ± 2.0
RA **(b)**	223.7 ± 1.0	194.0 ± 8.2	209.5 ± 6.2	204.0 ± 4.0**	164. 8± 21.7***	140.0 ± 5.6***
RA + EDX **(c)**	220.4 ± 1.7	199.4 ± 3.7	215.6 ± 2.5	216.4 ± 2.2	215.8 ± 2.0^{+++}	215.8 ± 2.0^{+++}
RA + TAM **(c)**	222.1 ± 1.3	198.2 ± 2.9	216.1 ± 2.1	216.9 ± 2.9	216.6 ± 2.7^{+++}	216.4 ± 2.4^{+++}
RA + OHTAM **(c)**	219.3 ± 2.0	193.6 ± 3.4	215.9 ± 2.6	216.6 ± 2.3	216.3 ± 1.9^{+++}	216.2 ± 1.9^{+++}
RA + CyA **(d)**	215.5 ± 4.7	190.3 ± 10.2	212.5 ± 5.6	213.5 ± 6.2	212.3 ± 6.3^{+++}	212.3 ± 6.3^{+++}
RA + EDX **(e)**	-	-	-	196.1 ± 2.3**	203.6 ± 2.2^{+}	203.3 ± 1.6***,$^{+++}$
RA + TAM **(e)**	-	-	-	194.6 ± 3.1**	202.0 ± 1.8^{+}	202.1 ± 1.7***,$^{+++}$
RA + OHTAM **(e)**	-	-	-	193.8 ± 2.9**	201.2 ± 2.3^{+}	202.4 ± 1.3***,$^{+++}$
RA + CyA **(f)**	-	-	-	195.3 ± 3.5**	212.4 ± 2.4^{++}	213.7 ± 1.9^{+++}

Fig. 3.2 - Inhibitory effect of the antiestrogens EDX, TAM and OHTAM on RA-induced ΔΨ depolarization associated with MPT induction. The dissipation of ΔΨ was monitored as described in the Materials and methods section. Mitochondria were energized with succinate (Succ) and after the steady-state distribution of TPP^+ had been reached, Ca^{2+} was added. (A) Mitochondria were previously incubated in the absence (control, trace a) and in the presence of 5 nmol RA/mg protein alone (trace b) or in combination with 10 nmol/mg protein of EDX, TAM or OHTAM (trace c) and 1 μM of CyA (trace d). (B) EDX, TAM, OHTAM or CyA at the same concentrations was also added after the ΔΨ depolarization induced by 5 nmol RA/mg protein, as indicated by the arrows (traces e and f). The traces are typical recordings of four independent experiments and the data in the table below correspond to the ΔΨ at the indicated time points and are expressed as the mean ± S.E.M. of four experiments obtained from different mitochondrial preparations. *** $p<0.001$, ** $p<0.01$ vs control. +++ $p<0.001$, ++ $p<0.01$, + $p<0.05$ vs RA.

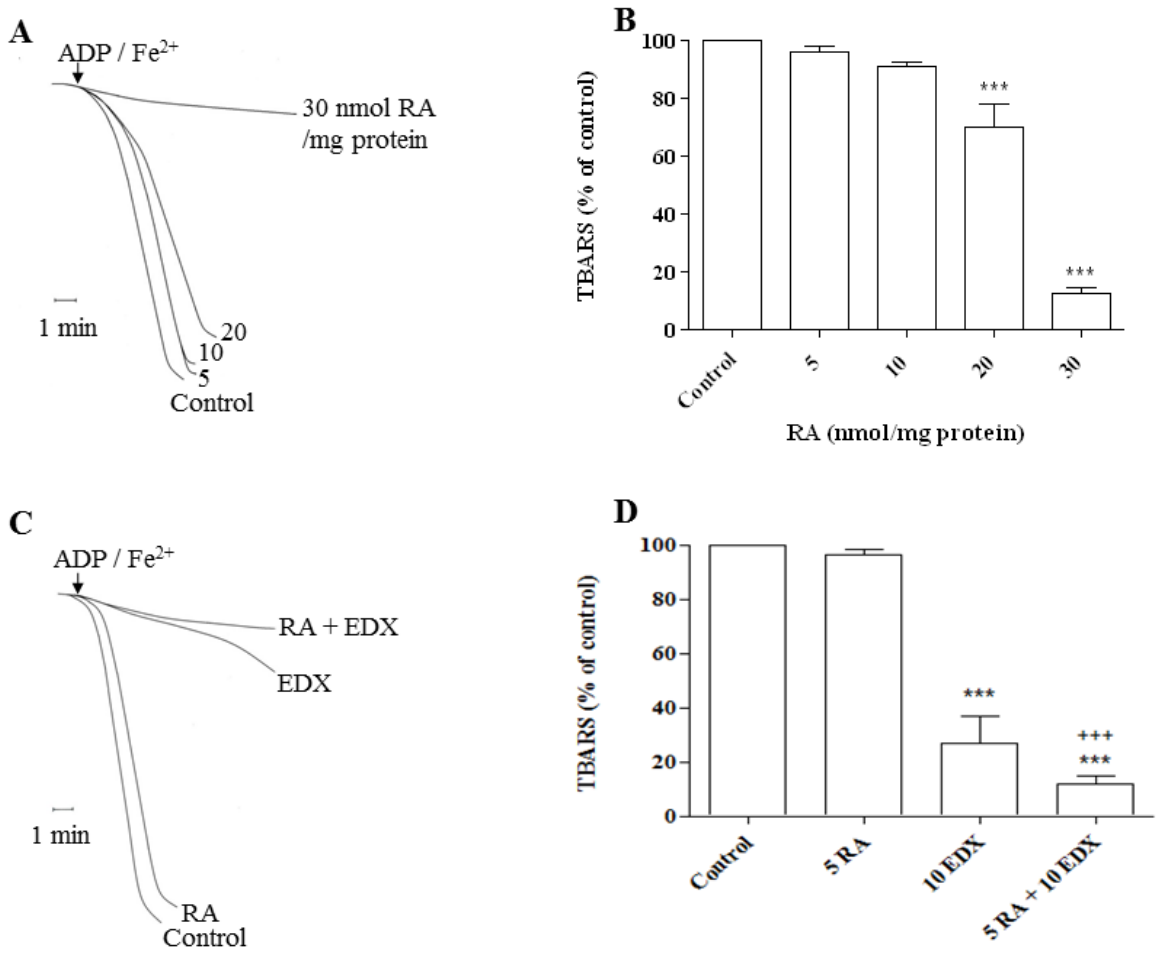


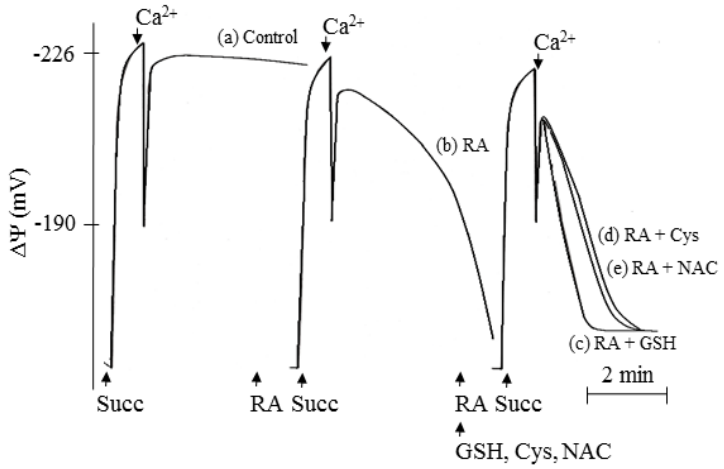
Fig. 3.3 - Protective effect of RA and EDX on mitochondria oxidative stress induced by the pro-oxidant pair ADP/Fe^{2+}. Oxidative stress was evaluated by oxygen consumption as described in the Materials and methods section. Before initiating the reaction by adding ADP/Fe^{2+}, mitochondria were preincubated in the absence (control) and in the presence of different concentrations (nmol/mg protein) of RA (A, B) and in the presence of RA (5 nmol/mg protein) and EDX (10 nmol/mg protein), alone or in combination (C, D). The traces in A and C represent the oxygen consumption rate and are typical recordings of four experiments obtained from different mitochondrial preparations. The amount of TBARS (B, D) was determined after 10 min of the addition of ADP/Fe^{2+}. TBARS, expressed as percentage of control, correspond to the mean ± S.E.M. of four independent experiments. *** p<0.001 vs control. +++ p<0.001 vs RA.

RA slightly enlarged the lag phase before the oxygen uptake burst induced by the complex ADP/Fe^{2+}, and decreased the rate of the rapid oxygen consumption phase, in a concentration-dependent manner. At 30 nmol/mg protein, RA inhibited almost completely the oxygen consumption (Fig. 3.3A). In agreement with the oxygen consumption evaluation, 5-10 nmol RA/mg protein slightly diminished TBARS formation (Fig. 3.3B). This effect of RA was more pronounced at 20 nmol/mg protein that induced a decrease in TBARS levels to about 70.1 % of control and at 30 nmol/mg protein a decrease to approximately 12.6 % of control was observed (Fig. 3.3B). Then, to evaluate whether the combination of RA with EDX could also prove beneficial against mitochondria oxidative stress, we used a combination of RA at 5 nmol/mg protein with EDX at 10 nmol/mg protein, which were the concentrations used to induce and to prevent the ΔΨ depolarization associated to MPT, respectively (Figs. 3.3C-D). As shown in figure 3.3C, EDX by itself significantly diminished lipid peroxidation as previously described (Ribeiro et al., 2013b) and when RA was combined with EDX the decrease in lipid peroxidation was significantly enhanced. Accordingly, RA and EDX individually diminished TBARS formation to about 96.2 and 26.7 % of control, respectively, and their combination decreased TBARS formation to approximately 11.7 % of control, which is significant when compared with the effects exerted by RA alone (Fig. 3.3D).

In addition, we investigated the effects of the thiol group protecting agents GSH, Cys and NAC (Fig. 3.4) and of the ANT ligands ADP and ATP (Fig. 3.5) on the RA-induced ΔΨ depolarization associated to MPT. The incubation of mitochondria with RA in the presence of 1 mM GSH (trace c), 200 μM Cys (trace d) or 200 μM NAC (trace e), before energization with succinate, did not prevent the ΔΨ depolarization due to the RA-induced MPT (Fig. 3.4). In contrast, mitochondria coincubated with RA and ADP or ATP (Fig. 3.5, traces c and d) were able to significantly sustain the ΔΨ in comparison with RA 7 min after the addition of Ca^{2+} (Fig. 3.5, table), indicating that ANT ligands prevented the MPT induced by RA.

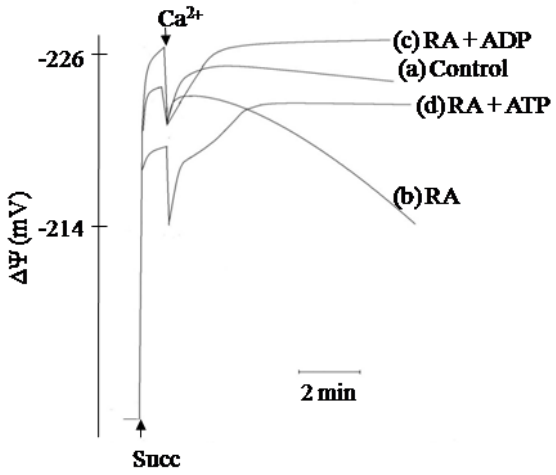
Effects of RA on bioenergetic functions of rat liver mitochondria

To assess whether RA affects mitochondrial bioenergetic functions, we evaluated the ΔΨ developed and the fluctuations associated with the phosphorylation cycle induced by ADP. A typical recording of the phosphorylation cycle induced by ADP is shown in figure



	ΔΨ (-mV)					
Time (min)	**0**	**0.5**	**1**	**3**	**5**	**7**
Control **(a)**	232.1 ± 3.3	201.8 ± 12.6	229.8 ± 3.4	230.0 ± 3.5	229.6 ± 3.5	229.6 ± 3.5
RA **(b)**	231.7 ± 3.8	204.1 ± 8.1	223.0 ± 2.8	213.4 ± 1.6	190.7 ± 5.9**	140.6 ± 6.5***
RA + GSH **(c)**	229.1 ± 3.5	202.0 ± 10.2	209.8 ± 2.4**,+	161.7 ± 13.2**,++	140.0 ± 5.8***,++	141.0 ± 6.0***
RA + Cys **(d)**	230.2 ± 3.4	201.7 ± 9.3	211.7 ± 1.9**,+	169.5 ± 11.1**,++	143.2 ± 4.7***,++	141.2 ± 5.6***
RA + NAC **(e)**	231.5 ± 3.1	203.4 ± 11.0	213.2 ± 2.6**,+	172.8 ± 12.3**,++	144.9 ± 5.0***,++	141.1 ± 5.3***

Fig. 3.4 - Thiol protecting agents do not prevent RA-induced MPT evaluated by ΔΨ depolarization. ΔΨ was measured as described in the Materials and methods section. Mitochondria were preincubated in the absence (control, trace a) or in the presence of 5 nmol/mg protein RA alone (trace b) or in combination with 1 mM GSH (trace c), 200 µM Cys (trace d) or 200 µM NAC (trace e) before energization with succinate (Succ). After a steady-state distribution of TPP^+ had been reached, Ca^{2+} was added. The traces are typical of four separate experiments and the data in the table below correspond to the ΔΨ at the indicated time points and are expressed as the mean ± S.E.M. of four experiments obtained from different mitochondrial preparations. *** $p<0.001$, ** $p<0.01$ vs control. ++ $p<0.01$, + $p<0.05$ vs RA.



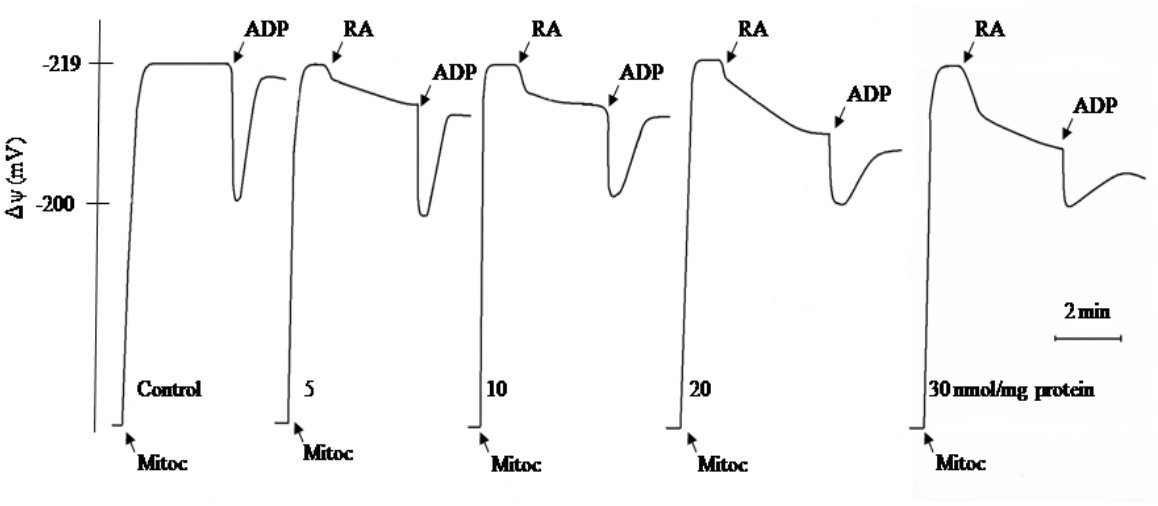
	ΔΨ (-mV)					
Time (min)	**0**	**0.5**	**1**	**3**	**5**	**7**
Control (a)	229.6 ± 3.5	205.7 ± 8.7	226.8 ± 3.9	227.5 ± 3.7	226.9 ± 3.7	226.9 ± 3.7
RA (b)	229.4 ± 3.6	206.7 ± 5.5	221.5 ± 2.7	214.9 ± 1.0*	197.9 ± 13.1*	145.0 ± 5.7***
RA + ADP (c)	221.8 ± 2.9	199.5 ± 5.8	223.3 ± 4.8	226.7 ± 4.8	226.4 ± 4.5^{+}	226.4 ± 4.5^{+++}
RA + ATP (d)	215.2 ± 6.2	204.0 ± 7.4	216.8 ± 3.5	219.5 ± 1.8	219.0 ± 1.8	219.0 ± 1.8^{+++}

Fig. 3.5 - Inhibitory effect of ANT ligands on RA-induced ΔΨ depolarization associated to MPT induction. ΔΨ was measured as described in the Materials and methods section. Mitochondria were energized with succinate (Succ) and after the steady-state distribution of TPP^+, Ca^{2+} was added. Mitochondria were preincubated in the absence (control, trace a) or in the presence of 5 nmol RA/mg protein alone (trace b) or in combination with 75 μM ADP (trace c) or 150 μM ATP (trace d). The traces are typical of four separate experiments and the data in the table below correspond to the mean ± S.E.M. of the ΔΨ evaluated in four different mitochondrial preparations. *** $p<0.001$, * $p<0.05$ vs control. +++ $p<0.001$, + $p<0.05$ vs RA.

3.6, while the table underneath shows the mean ± S.E.M. of the indicated parameters. The data demonstrated that mitochondria energized with glutamate/malate in the absence of RA (control) developed a ΔΨ of 219.8 ± 0.6 mV (negative inside) and the addition of ADP dropped the ΔΨ in 16.5 ± 1.1 mV (ADP depolarization). The time required for the phosphorylation of ADP (lag phase) was 66.8 ± 2.3 s and after ADP phosphorylation, the ΔΨ returned to 218.9 ± 0.6 mV (repolarization of ΔΨ), which is close to its initial value. While RA at 5 nmol/mg protein did not significantly affect the ΔΨ and the phosphorylation cycle induced by ADP, at 10 nmol/mg protein the phosphorylation lag phase was significantly increased and above 20 nmol/mg protein, RA also significantly decreased the ΔΨ developed, the ADP depolarization as well as the repolarization of ΔΨ (Fig. 3.6, table).

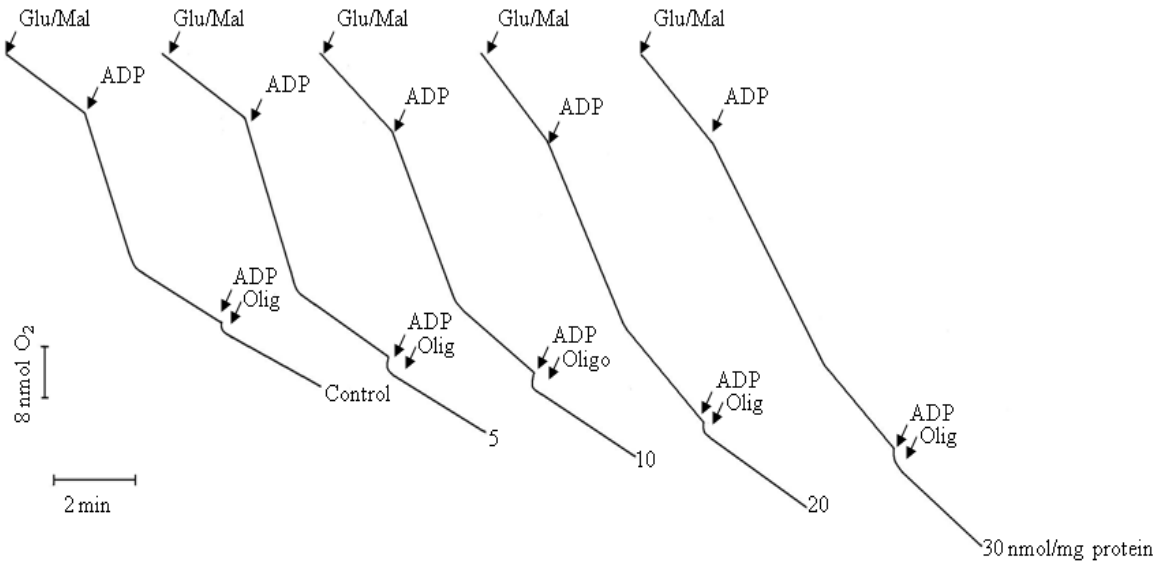
To provide further evidence regarding the effects of RA on mitochondrial bioenergetics, we also investigated the respiration parameters of mitochondria as assessed by measuring oxygen consumption (Fig. 3.7). As shown in figure 3.7 recordings, respiration was initiated by adding glutamate/malate (state 2 respiration) and after the addition of 150-250 nmol/mg protein of ADP, the rate of oxygen consumption increased (state 3 respiration) and when all the ADP was phosphorylated, the oxygen consumption rate decreased (state 4 respiration). Oligomycin was added in order to assess the proton leak through the Fo fraction of complex V (state 4 olig). At 5 nmol/mg protein, RA did not significantly affect the respiration parameters (Fig. 3.7). RA at 10 nmol/mg protein did not present significant effects on state 4 respiration, but significantly depressed the state 3 respiration, the ADP/O ratio, as well as the RCR, which is decreased as a consequence of the significant inhibition of state 3 respiration (Fig. 3.7, table). At concentrations above 20 nmol/mg protein, RA decreased the state 3, the RCR and the ADP/O and the state 4 respiration was significantly stimulated, but it was decreased in the presence of oligomycin (Fig. 3.7).

In order to clarify whether the effects of a high concentration of RA (30 nmol/mg protein) on the ΔΨ and on the respiration rates could be due to a loss of mitochondrial membrane integrity, this parameter was evaluated by following the light scattering at 540 nm (Fig. 3.8). In the absence of RA (control), the light scattering of mitochondrial suspension was about 1.5 and it was sustained over 30 min (Fig. 3.8 trace a, and table). After 25 min of recording, the light scattering at 540 nm in the presence of RA was approximately 1.4, which was not significant relative to control (Fig. 3.8, trace b and table), whereas triton



(nmol/mg protein)	ΔΨ (-mV)			Phosphorylation lag phase (s)
	Glu/Mal energization	ADP depolarization	Repolarization	
Control	219.8 ± 0.6	16.5 ± 1.1	218.9 ± 0.6	66.8 ± 2.3
5	217.7 ± 1.0	16.6 ± 1.2	216.2 ± 1.0	76.7 ± 3.1
10	216.3 ± 0.9	15.0 ± 0.9	215.0 ± 1.0	91.5 ± 2.6***
20	212.8 ± 1.0***	10.5 ± 1.0**	209.6 ± 1.0***	109.2 ± 3.8***
30	210.1 ± 1.2***	7.4 ± 1.7***	208.4 ± 1.0***	107.0 ± 7.8***

Fig. 3.6 - Effects of RA on the ΔΨ and phosphorylation cycle induced by ADP. Mitochondria were energized with glutamate/malate (Glu/Mal) and after a steady-state distribution of TPP^+ had been reached, RA was added and incubated for 3 min prior to the addition of ADP. The traces are typical recordings representative of several experiments obtained in the absence of drugs (control), and in the presence of different concentrations of RA (nmol/mg protein) as indicated by the numbers adjacent to traces. The table underneath shows the mean ± S.E.M. of the ΔΨ and phosphorylation lag phase at the indicated concentrations of RA for several independent experiments. *** $p<0.001$, ** $p<0.01$ vs control.



(nmol/mg protein)	State 3	State 4	State 4 olig.	RCR	ADP/O
Control	54.9 ± 2.6	12.2 ± 0.8	8.5 ± 0.8	4.5 ± 0.3	4.9 ± 0.2
5	46.8 ± 2.2	12.9 ± 0.7	8.4 ± 0.7	3.8 ± 0.1	4.6 ± 0.2
10	41.4 ± 1.7***	16.2 ± 1.0	11.1 ± 1.1	2.6 ± 0.1***	4.1 ± 0.1*
20	34.7 ± 1.3***	24.3 ± 2.4***	14.9 ± 1.6ººº	1.6 ± 0.1***	3.6 ± 0.2***
30	33.6 ± 3.3***	27.6 ± 2.3***	17.0 ± 1.5ººº	1.3 ± 0.2***	3.4 ± 0.0*

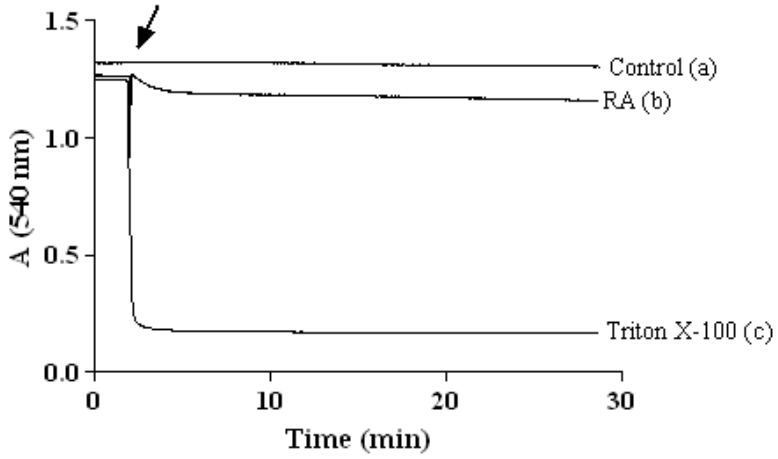
Fig. 3.7 - Effects of RA on the respiration parameters of rat liver mitochondria. Oxygen consumption was assessed with a Clark oxygen electrode as described in the Materials and methods section. Mitochondria were energized with glutamate/malate (Glu/Mal). ADP was added to induce state 3 respiration and when all the ADP was phosphorylated (state 4), state 4 oligomycin (state 4 olig) was induced by adding oligomycin plus ADP. The traces are typical recordings representative of several experiments obtained in the absence (control) and in the presence of different RA concentrations (nmol/mg protein) as indicated by the numbers adjacent to traces. The table underneath shows the mean ± S.E.M. of respiratory parameters at the indicated concentrations of RA of four independent experiments. State 3, state 4 and state 4 olig are expressed in nmol O_2/mg protein/min. *** $p<0.001$, * $p<0.05$ vs control. ººº $p<0.001$ vs state 4.

X-100 promoted an extensive swelling, significantly decreasing the light scattering to about 0.3 (Fig. 3.8, trace c and table).

Effects of EDX, TAM or OHTAM in combination with RA on the ΔΨ and O_2 consumption

In addition to the action in the MPT, the effects of EDX (10 nmol/mg protein) in combination with increasing concentrations of RA (5-20 nmol/mg protein) on the ΔΨ developed and the phosphorylation cycle induced by ADP (Fig. 3.9) and on the mitochondrial respiration parameters (Fig. 3.10) were also evaluated using glutamate/malate. The antiestrogen EDX did not significantly affect the ΔΨ developed or the phosphorylation cycle induced by ADP (Fig. 3.9). The combination of 5 nmol/mg protein of RA with the antiestrogen at 10 nmol/mg protein did not induce significant effects on any of the parameters evaluated, either in comparison with control (0) or the compounds individually (Fig. 3.9). The combination of EDX with 10 nmol/mg protein of RA significantly increased the phosphorylation lag phase and decreased the ΔΨ developed and the repolarization, while the ADP depolarization was unaffected, as compared with the control (0), but the effects were not significant relative to the compounds individually (Fig. 3.9). When EDX was combined with 20 nmol/mg protein of RA the phosphorylation lag phase was significantly increased, while the ΔΨ, the depolarization and the repolarization of ΔΨ were decreased in comparison with control and EDX (Fig. 3.9). The combinations of RA with TAM or OHTAM presented similar effects to those observed with EDX, but the effects of TAM were slightly more pronounced on depolarization and phosphorylation lag phase (Fig. 3.9).

The effects on oxygen consumption (Fig. 3.10) demonstrated that EDX either individually (10 nmol/mg protein) or in combination with 5 nmol/mg protein of RA did not affect the state 3, the state 4 and the ADP/O ratio. However, the RCR was significantly depressed by the combination relative to the control, reflecting the effects on both states 3and 4. EDX combined with 10 nmol/mg protein of RA did not affect the state 4 respiration, but significantly decreased the state 3 respiration, the RCR and the ADP/O ratio relative to the control. However, these effects were not significant in comparison with the compounds



	A (540 nm)			
Time (min)	2	2.5	3	25
Control (a)	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.2
RA (b)	1.5 ± 0.2	1.5 ± 0.2	1.4 ± 0.2	1.4 ± 0.2
Triton X-100 (c)	1.4 ± 0.2	0.9 ± 0.3	0.6 ± 0.2	0.3 ± 0.1*

Fig. 3.8 - Effect of a high concentration of RA on mitochondrial swelling. Mitochondrial volume changes were followed as described in the Materials and methods section. Mitochondria were incubated in the absence (control, trace a) or in the presence of 30 nmol RA/mg protein (trace b) and a positive control using Triton X-100 (trace c) was performed. The compounds were added where indicated by the arrow. The traces are typical of three separate experiments with different mitochondrial preparations. The data in the table correspond to the absorbance at the indicated time points and are expressed as the mean ± S.E.M. of three experiments obtained from different mitochondrial preparations. * $p<0.05$ vs control.

individually (Fig. 3.10). The combination of EDX with 20 nmol/mg protein of RA significantly depressed the state 3 respiration, the RCR and the ADP/O ratio, and stimulated the state 4 relative to the control and the antiestrogen (Fig. 3.10); the state 4 respiration was inhibited in the presence of oligomycin (Fig. 3.10). The combinations of RA with TAM or OHTAM promoted similar effects to those described for EDX on oxygen consumption. However, when RA at 5 nmol/mg protein was combined with TAM, the effects on the RCR were much more drastic than the combinations with OHTAM or EDX, occurring a decrease in the RCR that was also significant in comparison with RA individually (Fig. 3.10).

3.4 - Discussion

Our experiments demonstrate that RA is an inducer of the MPT, in agreement with already published literature (Rigobello et al., 1999; Notario et al., 2003), since its action is inhibited by CyA, a specific and potent inhibitor of the MPT (Broekemeier et al., 1989). EDX resembles CyA in the ability to prevent the MPT induced by RA, allowing mitochondria preincubated with RA to accumulate Ca^{2+} (Fig. 3.1) and inhibiting the depolarization of $\Delta\Psi$ after Ca^{2+} uptake (Fig. 3.2A). Moreover, the inhibition of RA-induced depolarization occurs regardless of whether EDX is added before or after energization with succinate and Ca^{2+} loading (Figs. 3.2A and 3.2B), supporting that EDX prevents the membrane depolarization induced by RA due to a direct influence on the proteins that form the MPT pore complex, and excludes an interference with Ca^{2+} uptake across the uniport. Similarly to EDX, the antiestrogens TAM and OHTAM also prevented and reversed the MPT promoted by RA (Figs. 3.1 and 3.2). These observations are consistent with previous reports that have shown that antiestrogenic compounds inhibit the MPT induced by several agents (Cardoso et al., 2002b; Custódio et al., 1998; Hernández-Esquivel et al., 2011a, b; Ribeiro et al., 2013b).

Since MPT induction is an important mechanism associated with cell death, the inhibition of MPT by the antiestrogens would possibly compromise the anticancer activity of RA. However, the combination of RA with antiestrogenic compounds has revealed a synergism of action in breast cancer (Danforth, 2004; Wang et al., 2007; Searovic et al., 2009; Koay et al., 2010) and melanoma cells (Ribeiro et al., 2013a), indicating that MPT

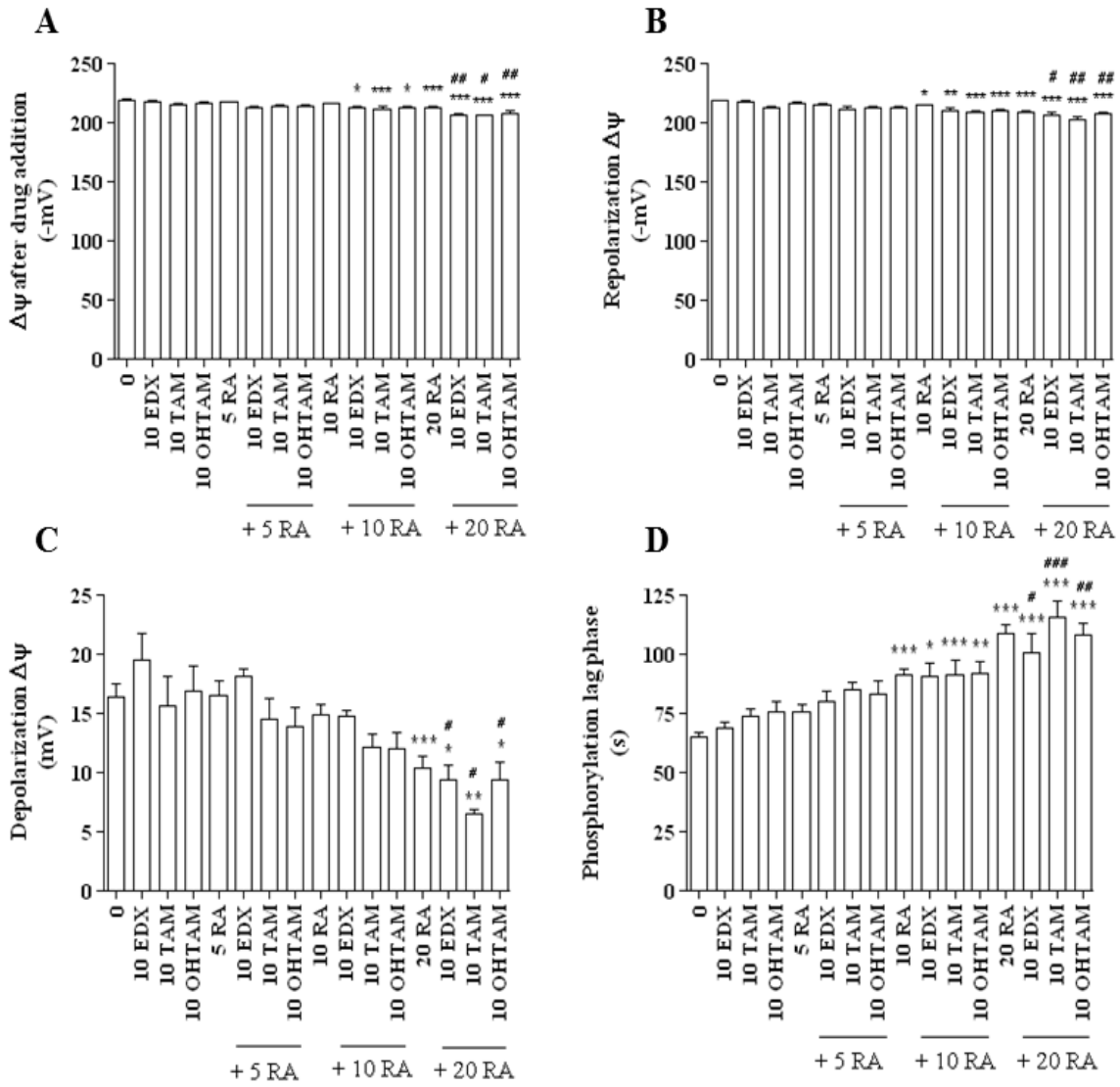


Fig. 3.9 - Effects of the antiestrogens EDX, TAM and OHTAM in combination with RA on the ΔΨ and the phosphorylation cycle induced by ADP. Mitochondria were incubated with the drugs RA (5, 10 and 20 nmol/mg protein) and antiestrogens (10 nmol/mg protein) and the experiments were performed as described in the legend of figure 3.6. The data represent the mean ± S.E.M. of five independent experiments. *** p<0.001, ** p<0.01, * p<0.05 vs control. ### p<0.001, ## p<0.01, # p<0.05 vs 10 nmol/mg protein of the respective antiestrogen.

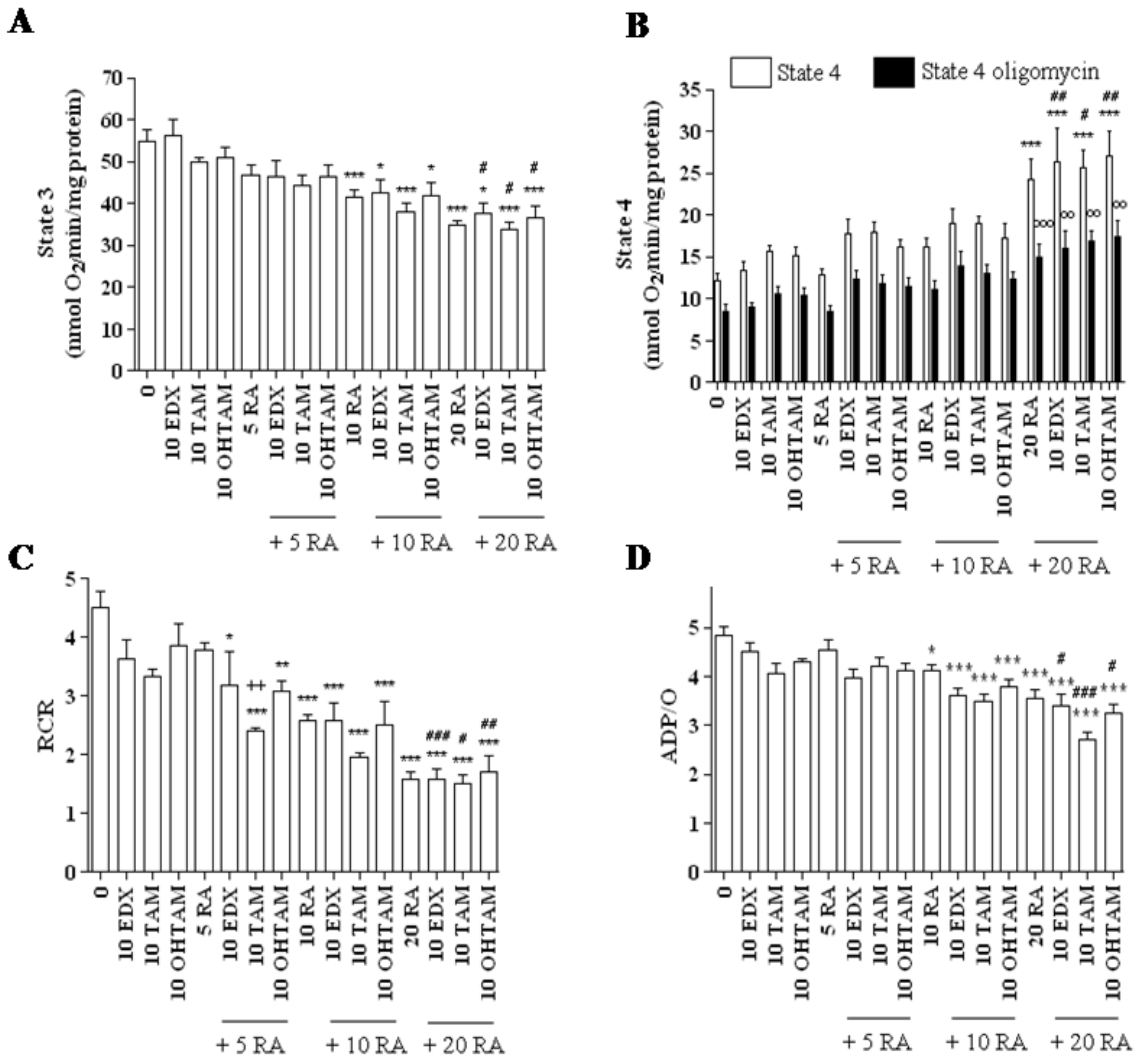


Fig. 3.10 - Effects of the antiestrogens EDX, TAM and OHTAM in combination with RA on the respiration parameters of rat liver mitochondria. Mitochondria were incubated with the drugs RA (5, 10 and 20 nmol/mg protein) and antiestrogens (10 nmol/mg protein) and the assays were carried out as described in the legend of figure 3.7. The data represent the mean ± S.E.M. of several independent experiments. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ vs control. ### $p<0.001$, ## $p<0.01$, # $p<0.05$ vs 10 nmol/mg protein of the respective antiestrogen. ^{ooo} $p<0.001$, ^{oo} $p<0.01$ vs state 4. ++ $p<0.01$ vs 5 nmol/mg protein of RA.

induction is not required for RA to exert toxicity against tumor cells. On the contrary, the protection afforded by the antiestrogens against RA-induced MPT may contribute to prevent or reduce the hepatotoxicity promoted by RA, as several inducers of MPT have been shown to be hepatotoxic (Nadanaciva and Will, 2009).

An important factor contributing to the regulation of the induction of MPT is the oxidative stress (Halestrap, 2009). Considering the recent reports on the antioxidant activity of EDX (Ribeiro et al., 2013b), we investigated the possible involvement of oxidative stress mechanisms in the prevention of RA-induced MPT by EDX. RA at a low concentration (5 nmol/mg protein) that we showed to induce the MPT did not present antioxidant activity, but the combination with EDX significantly enhanced the effects of RA (Fig. 3.3). These experiments suggest that the protection against MPT induction by RA may be related with the antioxidant properties of the antiestrogen. However, the thiol protecting agents tested GSH, Cys and NAC did not prevent the depolarization induced by RA (Fig. 3.4), indicating that some factor other than the antioxidant properties of EDX may account for the protection against the induction of the MPT by RA. On the other hand, considering a recent study which has proposed the mitochondrial phosphate carrier as a possible key component of the MPT pore complex in addition to the regulatory role played by the ANT (Leung et al., 2008), we evaluated the effects of ANT ligands on RA-induced depolarization (Fig. 3.5). Both ADP and ATP inhibit the depolarization of $\Delta\Psi$ induced by RA (Fig. 3.5), indicating a direct interaction between RA and the ANT. These results are in agreement with previous studies that have demonstrated that RA can be considered an ANT modulator (Notario et al., 2003). However, as shown in figures 3.9 and 3.10, none of the three antiestrogens tested significantly affects the mitochondrial phosphorylation efficiency at concentrations that inhibit the MPT, suggesting that neither the ANT nor the phosphate carrier seems to be related with the inhibition of MPT by EDX and the other antiestrogens TAM and OHTAM. Therefore, it is plausible that the inhibition of RA-induced MPT by these compounds may be related with the interaction with cyclophilin D, in a similar way to CyA (Zoratti and Szabo, 1995). Another possible explanation would be related with the effects of antiestrogens on biomembranes, as they are hydrophobic molecules that affect the dynamic properties of lipids (Custódio et al., 1991, 1993a, b; Kazanci and Severcan, 2007). The interference with membrane dynamics can lead to a configurational change of the ANT,

explaining the inhibition of RA-induced MPT by the antiestrogens, as previously suggested (Hernández-Esquivel et al., 2011a).

Beyond the MPT, the disruption of mitochondrial bioenergetics has also been recognized to participate in the lethal cell injury induced by xenobiotics (Wallace et al., 1997). In our experiments, RA at 10 nmol/mg protein increases the time required for ADP phosphorylation (Fig. 3.6) and diminishes the state 3 respiration, the RCR, as well as the ADP/O ratio (Fig. 3.7), indicating that at this concentration RA depresses the phosphorylation capacity of mitochondria. These results are in agreement with other studies which have reported that RA above 5 nmol/mg protein significantly inhibits the ANT activity (Notario et al., 2003). Additionally, at 20 nmol/mg protein, RA decreases the $\Delta\Psi$ developed after substrate addition and the repolarization of $\Delta\Psi$ (Fig. 3.6) and increases the respiratory rate of state 4 (Fig. 3.7), suggesting that high concentrations of RA induce a permeabilization of mitochondrial membrane to protons. Since high concentrations of RA do not decrease the light scattering of mitochondria (Fig. 3.8), and the stimulatory effect on state 4 is inhibited in the presence of oligomycin (Fig. 3.7), the effects on state 4 and on $\Delta\Psi$ are possibly related to a proton leak through the Fo fraction of complex V. Our results differ from those described by Stillwell and Nahmias (1983) that reported that RA above 0.25 nmol/mg protein induces the uncoupling of mitochondria by disrupting the mitochondrial membrane. On the other hand, our observations correlate with a recent work by Xun *et al* (2012) in a clonal human neuroblastoma cell line that has demonstrated that RA increases the rate of oxygen consumption and that this stimulation, which decreases in the presence of oligomycin, may play a role in the therapeutic efficacy of RA.

Bioenergetic studies revealed that the combinations of RA (5-10 nmol/mg protein) with EDX (10 nmol/mg protein), at the concentrations used to induce and inhibit MPT, do not present effects significantly different from those induced by the compounds individually either on the $\Delta\Psi$ and the phosphorylation cycle induced by ADP or on the oxygen consumption (Figs. 3.9 and 3.10). Similar effects were observed when RA at 5-10 nmol/mg protein was incubated with 10 nmol/mg protein of TAM or OHTAM, but the combination of TAM with 5 nmol/mg protein of RA significantly diminished the RCR relative to RA, suggesting that the combination with TAM has more drastic effects on mitochondrial bioenergetics than the combinations with its active metabolites EDX or OHTAM (Fig. 3.10). As recent studies confirmed an association between CYP2D6 polymorphisms or the

coadministration of CYP2D6-inhibiting medication with the clinical outcome in women treated with TAM (Kelly et al., 2010; Lammers et al., 2010), it is an emerging view that the active metabolite EDX may be a more suitable drug for clinical utilization in therapeutic association than its prodrug TAM. When the antiestrogens at 10 nmol/mg protein are combined with a high concentration of RA (20 nmol/mg protein), the toxic effects induced by these combinations are significantly enhanced relative to those induced by antiestrogens individually, but not in comparison with RA alone (Figs. 3.9 and 3.10). These results suggest that the eventual toxicity of the combination of antiestrogens with high concentrations of RA is related to RA. Accordingly, a phase I/II study performed in patients with advanced breast cancer which has reported that 20 mg TAM/day and RA up to 190 mg/m^2/day can be given together with acceptable toxicity, but patients treated with 230 mg/m^2/day of RA present unacceptable toxic effects (Budd et al., 1998).

In the presence of Ca^{2+}, the concentration of RA necessary to induce the MPT is 5-10 nmol/mg protein, which corresponds to 2.5 µM. The antiestrogens were able to effectively inhibit RA-induced MPT at 10 nmol/mg mitochondrial protein which translates to 2.5-5 µM. In breast cancer cells, a concentration of 1 µM of RA and TAM was sufficient to obtain a synergism of action (Wang et al., 2007). However, the RA receptors and the cellular retinoic acid binding protein that keep RA in mitochondria (Ruff and Ong, 2000; Everts and Bernadier, 2002), as well as the synergism with Ca^{2+}, explain that RA compromises intact cells at lower concentrations than those necessary to affect isolated mitochondria, suggesting that RA is able to regulate the MPT at physiological concentrations. On the other hand, the plasma concentrations of EDX are in the 10-150 nM range, but tissue concentrations are much higher, close to 10 µM (Lien et al., 1991; Borges et al., 2006), whereas the tissue concentrations of TAM are within the range of 3-60 µM (Jordan, 1990; Custódio et al., 1991; Lien et al. 1991; Ingle et al., 1999). Therefore, it is plausible that at the concentrations reached in tissues the antiestrogens can prevent the induction of MPT by RA, thus contributing to decrease the RA side effects.

In conclusion, here we report that EDX inhibits the induction of MPT by RA, without enhancing the deleterious effects on mitochondrial bioenergetics induced by RA. Altogether, these data suggest that the synergistic action of the combination of retinoids and antiestrogens observed in breast cancer and melanoma cells may parallel with a decrease in the hepatotoxic effects induced by RA, as a consequence of the inhibition of MPT. Since the

use of RA has been limited by its adverse effects, the therapeutic association with EDX may be a promising strategy to turn RA into a more suitable drug for clinical utilization.

CHAPTER IV

THE COMBINATION OF THE ANTIESTROGEN ENDOXIFEN WITH ALL-*TRANS*-RETINOIC ACID HAS ANTIPROLIFERATIVE AND ANTIMIGRATION EFFECTS ON MELANOMA CELLS WITHOUT INDUCING SIGNIFICANT TOXICITY IN NON-NEOPLASIC CELLS

[Ribeiro, M.P., Silva, F.S., Paixão, J., Santos, A.E. and Custódio, J.B. (2013) The combination of the antiestrogen endoxifen with all-*trans*-retinoic acid has anti-proliferative and anti-migration effects on melanoma cells without inducing significant toxicity in non-neoplasic cells. *Eur. J. Pharmacol*. 715, 354-362]

Abstract

Melanoma incidence is dramatically increasing and the available treatments beyond partial efficacy have severe side effects. Retinoids are promising anticancer agents, but their clinical use has been limited by their toxicity, although a combination with other agents can possibly generate a therapeutic action at lower dosage. Thus, we investigated the effects of all-*trans*-retinoic acid (RA) combined with the antiestrogen endoxifen (EDX) on melanoma cell proliferation and the effects were compared with its prodrug tamoxifen (TAM). Moreover, we evaluated the effects of these combinations on non-neoplasic cells and assessed mitochondrial bioenergetic functions, to predict their potential toxicity. Individually, RA and the antiestrogens EDX and TAM decreased melanoma cell biomass, cell viability and DNA synthesis, without increased cell death, suggesting that the compounds inhibited cell proliferation. Noteworthy, EDX decreased cell proliferation more efficiently than TAM. The combination of EDX with RA enhanced the antiproliferative effects of the compounds individually more potently than TAM, which did not enhance the effects induced by RA alone, and blocked cell cycle progression in G1. Moreover, the combination of RA with EDX significantly decreased melanoma cells migration, whereas the combination with TAM did not present significant effects. At the concentrations used the compounds did not induce cytotoxicity in non-neoplasic cells and liver mitochondrial bioenergetic function was not affected. Altogether, our results show for the first time that a combined treatment of RA with EDX may provide an antiproliferative and antimigration effect upon melanoma cells without major toxicity, offering a powerful therapeutic strategy for malignant melanoma.

4.1 - Introduction

Malignant melanoma remains among the most notoriously aggressive and treatment-resistant human cancers and its incidence has increased dramatically over the last decades (Jemal et al., 2009). In recent years, there has been a great progress in understanding the biology and molecular basis of melanoma, leading to the identification of several disease variants with different subpopulations of cells within one tumor, and the involvement of multiple signaling pathways, rather than the existence of a crucial individual alteration (Ko and Fisher, 2011). Considering this scenario, it is unlikely that a single therapeutic agent will provide a satisfactory response and new combinations of drugs with specific and complementary mechanisms of action will be required in order to increase the therapeutic benefit while allowing to reduce the adverse effects of the anticancer therapy.

Retinoids, the naturally occurring derivatives of vitamin A, play an important role in the regulation of several cellular processes, namely cell differentiation, development, growth, and apoptosis, through the regulation of gene transcription (Bushue and Wan, 2010). Retinoids have been widely used in the treatment of skin conditions, such as acne and psoriasis, and have also been shown to inhibit the proliferation and survival of cancer cells, including melanoma cells (Lotan, 1979; Drewa and Schachtshhabel, 1985). In 1995, the Food and Drug Administration (FDA) approved all-*trans*-retinoic acid (RA) for the treatment of a rare leukemia, acute promyelocytic leukemia (APL), which is caused by translocations of the retinoic acid receptor (RAR)-α gene (Tang and Gudas, 2011). However, the clinical utilization of retinoids has been limited by their adverse effects, including varying degrees of teratogenicity, mucocutaneous cytotoxicity, chondrogenesis inhibition, and hypertriglyceridemia (Zusi et al., 2002). Possibly, the combination of retinoids with other agents may allow to obtain therapeutic efficacy with lower doses through a synergism of action.

Tamoxifen (TAM) is a selective estrogen receptor (ER) modulator used routinely in the treatment of breast carcinoma. However, some ER-negative breast cancers also respond to TAM treatment, suggesting that it has alternative ways to suppress tumor cell proliferation independent of the expression of ER (Toma et al., 1999). On the other hand, *in vitro* experiments established that TAM effectively inhibits melanoma cell proliferation and migration (Kanter-Lewensohn et al., 2000; Matsuoka et al., 2009). Nevertheless, the clinical

use of TAM in the management of malignant melanoma has been controversial. Several studies have tried to establish the benefit of the inclusion of TAM in combination schemes, but the results remain contradictory (Beguerie et al., 2010). However, to our knowledge, these studies in melanoma patients have not considered the fact that the efficacy of TAM in breast cancer therapy is limited by CYP2D6 polymorphisms or by CYP2D6-inhibiting medication (Kelly et al., 2010; Lammers et al., 2010). Therefore, to avoid the therapeutic inefficacy due to the variability on TAM metabolism, we propose the use of endoxifen (EDX), a key active metabolite of TAM that has been considered responsible for its anticancer activity (Kiyotani et al., 2012), instead of the prodrug. Moreover, EDX has been shown to be less toxic for mitochondria than TAM (Cardoso et al., 2001; Ribeiro et al., 2013b).

Both antiestrogens and retinoids exhibit a cytostatic action in breast cancer cells, by blocking cell cycle progression in G1 (Wilcken et al., 1996) and the therapeutic combination of retinoids or retinamides with antiestrogens, has revealed synergism of action in breast cancer cell lines (Danforth, 2004; Searovic et al., 2009; Koay et al., 2010). Additionally, recent work has pointed to the interplay between retinoid and estrogen signaling in breast cancer (Hua et al., 2009; Ross-Innes et al., 2010). Thus, considering the activity of both retinoids and antiestrogens individually on melanoma cells and their synergistic interaction in breast cancer cells, we aimed to investigate the benefits of a combined therapy of RA and the antiestrogens EDX and TAM against melanoma cells (K1735-M2) proliferation. Moreover, the effects of these combinations were also evaluated on non-neoplasic cells and on rat liver mitochondrial bioenergetics in order to provide the first clues on their potential toxicity.

4.2 - Materials and methods

Reagents

RA, EDX and TAM were obtained from SIGMA-Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and antibiotic/antimycotic solution (10 000 units penicillin, 10 mg streptomycin, 25 µg amphotericin B per mL) were purchased from SIGMA-Aldrich (St Louis, MO, USA), Fetal Bovine Serum (FBS) and trypsin were obtained from Gibco, Invitrogen Life Technologies (Carlsbad, California, USA). All of the other

chemicals were purchased from SIGMA-Aldrich (St Louis, MO, USA) and were of the highest grade of purity commercially available. RA and TAM stock solutions were prepared in absolute ethanol. EDX was kept in dimethyl sulfoxide (DMSO).

Animals

Wistar rats (250-350g), of either sex, were maintained at 22 ± 2 ºC under artificial light for 12 h light/dark cycle and with access to water and food *ad libitum*. The experiments reported here were carried out in accordance with the National Requirements for Vertebrate Animal Research and in accordance with the European Convention for the Protection of Animals used for Experimental and other Scientific Purposes.

Cell culture

K1735-M2 mouse melanoma cells were cultured in DMEM, supplemented with 10 % heat-inactivated FBS and 1 % antibiotic/antimycotic solution, and kept at 37 ºC in a humidified incubator with 5 % CO_2. Cells were plated 6.1×10^4 cells/cm^2. The drugs RA, EDX and TAM were added to the cultures from diluted stocks 24 h after plating. Vehicle controls were performed.

Sulforhodamine B (SRB) assays

The SRB assay allows to determine the toxicity induced by the anticancer drugs in melanoma cell cultures and it is based on the labeling of the cellular protein content (Holy et al., 2006). At designated time points, the plate was fixed with absolute methanol containing ice-cold 1 % acetic acid, and stored at −20 ºC overnight. Methanol was then decanted and the plate air-dried. SRB (0.5 % in 1 % acetic acid) was added to each well, and the plate incubated at 37 °C for 1 h. Plates were rinsed with 1 % acetic acid, air-dried, and the bound dye eluted with 10 mM Tris buffer, pH 10. The absorbance was measured in a Synergy HT plate reader at 540 nm. As SRB binds to basic amino acid of cellular proteins of adherent cells, the absorbance at 540 nm provides an estimate of total protein mass (biomass), which is related to cell number. The absorbance value obtained in control cultures was considered 100 %. The number of independent experiments is indicated in figure legends.

Cell viability assessment by trypan blue dye exclusion

The effect of anticancer drugs on cell viability was investigated by staining cell suspensions with trypan blue, based on the principle that live cells with an intact cell membrane exclude the dye (Houben et al., 2009). At selected time points cells were trypsinized and the content of two wells was centrifuged and the pellet resuspended in 0.4 % trypan blue for 2-3 min and then cells were counted in a hemocytometer under a transmitted light microscope. Cells presenting a blue stained cytoplasm were considered as dead cells while the cells excluding the dye were considered as viable. The number of independent experiments is indicated in figure legends.

5-bromo-2'-deoxyuridine (BrdU) incorporation assay

K1735-M2 cell proliferation was quantitated based on the measurement of BrdU incorporation during DNA synthesis in proliferating cells, which is a nonradioactive alternative to the [^{3}H]-thymidine incorporation assay. We used the Cell Proliferation ELISA, BrdU, colorimetric kit (Roche) following the manufacturer's protocol. After 48 h of incubation with the drugs, cultured cells were placed in BrdU-labeling solution for 90 min. Afterwards, the cells were fixed and the DNA denaturated with the FixDenat solution provided with the kit, and then incubated with a monoclonal antibody conjugated with peroxidase (anti-BrdU-POD) to bind the BrdU in the newly synthesized cellular DNA. The immune complexes were detected by using the 3,3',5,5'-tetramethylbenzidine (TMB) substrate and quantified by measuring the absorbance in a Synergy HT plate reader at 370 nm. The absorbance values directly correlate to the amount of DNA synthesis and, therefore, to the number of proliferating cells. The experiments were carried out in triplicate for each independent experiment and the absorbance value obtained in control cultures was considered 100 %.

Cell cycle analysis by flow cytometry

The effects of the drugs on cell cycle were monitored by flow cytometry (Carmo et al., 2011). Cells were plated in 6-multiwell plates and incubated with RA, EDX and TAM for 48 h. At the end of the incubation period, cells were trypsinized and centrifuged at 1 500 rpm for 10 min, the culture medium was discarded and the pellet was fixed overnight at 4 ºC

with a solution of cold 70 % ethanol. The cells were then centrifuged at 1 500 rpm for 10 min, the pellet was resuspended in a solution of phosphate-buffered saline (PBS) containing RNAse and, after 45 min, propidium iodide was added and incubated for 1 h in the dark, at room temperature (the final concentrations of RNase and propidium iodide were 10 μg/mL and 20 μg/mL, respectively). The propidium iodide fluorescence was measured on a FACScan flow cytometer (BD FACSCalibur™) and the data were gated to exclude cell debris and aggregates. Data were analyzed using the ModFit LT 3.0. software. The experiments were carried out in duplicate for each independent experiment and the results are expressed as % of total cells.

Wound healing assay

The effect of anticancer drugs on melanoma cell migration ability was monitored *in vitro* using a wound healing assay, which is based on the observation that after doing a scratch on a confluent cell monolayer the cells on the edge of the newly created gap will move towards that space to close the gap and establish new cell-cell contacts (Liang et al., 2007). Thus, cells were seeded on glass coverslips and grown to confluency. The monolayer of cells was mechanically injured by scoring a line with a 21-gauge needle. Loose cells were removed by washing the well with culture medium and then the drugs were added to cells, except to control cells which were incubated with the vehicle. At 0 h, photographic images were captured by transmitted light microscopy under ×10 magnification (Axiovert, Zeiss). The multiwell plate was further incubated at 37 °C, 5 % CO_2 for 3 h, and then photographic images were captured again. To determine cell migration we selected in the images obtained at 0 h an area of the scratch lacking cells (a rectangle marked by dotted lines) and subsequently superimposed this rectangle on the scratch area of the images obtained at 3 h after scratch. Then, the total number of cells migrating into the selected area at 3 h was counted. This assay was performed within a short time interval to minimize the influence of cell proliferation in the migration assay. Experiments were performed in triplicates for each independent experiment.

Primary cultures of bovine aortic endothelial cells

The bovine aortic endothelial cells (BAEC) were isolated from thoracic aorta as described elsewhere (Paixão et al., 2011). Briefly, after treatment with collagenase (2

mg/mL), cells were cultured on gelatin-coated (0.2 %) tissue culture plastic in DMEM, with 100 U/mL penicillin and 100 µg/mL streptomycin, supplemented with 10 % heat-inactivated FBS, and kept at 37 ºC in a humidified incubator with 5 % CO_2. Endothelial cells were identified by their cobblestone morphology. Cells were used between the fifth and the eleventh passage. For the experiments, cells were seeded in 24-multiwell plates at a concentration of 5×10^4 cells/cm^2 and allowed to recover for 24 h prior to the addition of drugs. Cell viability was assessed by trypan blue dye exclusion after 72 h of incubation with the drugs. Experiments were performed in duplicates for each independent experiment.

Isolation of rat liver mitochondria

Mitochondria were isolated from the liver of Wistar rats as previously described (Custódio et al., 1998). Briefly, liver was quickly removed from decapitated rats, finely minced and homogenized in an ice-cold homogenization medium containing 250 mM sucrose, 10 mM 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA), and 0.1 % bovine serum albumin (BSA). The homogenate was centrifuged for 10 min at 800×*g* and mitochondria were recovered from supernatant by centrifugation at 10 000×*g* for 10 min. The mitochondrial pellet was resuspended twice in the final washing medium adjusted to pH 7.2, in the absence of EGTA and BSA. All the previously described procedures were performed at 0-4 ºC. Mitochondrial protein was determined by the Biuret method (Gornall et al., 1949) using BSA as the protein standard.

Evaluation of mitochondrial membrane potential and oxygen consumption

Mitochondria (1 mg) were suspended in the standard respiratory medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 5 mM KH_2PO_4, 5 mM HEPES, pH 7.4, supplemented with 3 µM tetraphenylphosphonium (TPP^+) and energized with glutamate/malate (10 mM/5 mM), at 37 °C. Mitochondrial membrane potential ($\Delta\Psi$) was assessed by measuring the movements of the lipophilic cation TPP^+ across the mitochondrial membrane with a TPP^+ selective electrode and using a $Ag/AgCl_2$ electrode as reference (model MI 402; Microelectrodes, Inc., Bedford, NH) as described elsewhere (Moreira et al., 2007). Oxygen consumption was assessed polarographically with a Clark oxygen electrode (YSI model 5331, Yellow Spring Inst.) connected to a suitable

recorder in a thermostatted water-jacket closed chamber with magnetic stirring (Ribeiro et al., 2013b). State 3 respiration (V3) was initiated upon the addition of 150-250 nmol/mg protein of ADP and after the phosphorylation of all the added ADP, the oxygen consumption rate decreased and state 4 respiration (V4) was resumed. Respiration rates (V3 and V4) were calculated assuming an oxygen concentration of 240 nmol O_2/mL and are expressed as nmol O_2/min/mg protein.

Statistical analysis

Results are presented as the mean ± S.E.M. of the indicated number of independent experiments. Statistical significance between the different assays was determined using the one-way analysis of variance (ANOVA), followed by the Tukey post-test, for multiple comparisons. A p value <0.05 was considered statistically significant. These statistical analyses were performed using the software package GraphPad Prism 4.

4.3 - Results

In order to investigate the cytotoxic effects of a combined therapy of RA and antiestrogens on a melanoma cell line we initially studied the dose-dependent effect of RA alone in a time-course experiment to establish the lowest effective RA concentration to pursue our work. Thus, melanoma cells were exposed to RA (0.5-40 µM) and the cytotoxic effects were assessed by the SRB assay, which evaluates the total cell protein content (cell biomass) that correlates with cell number (Fig. 4.1). At 24 h after drug addition, only the highest-RA concentration used in our study (40 µM) induced a significant decrease in cell biomass as compared with control condition (0), i.e., cells incubated in the absence of RA (Fig. 4.1). However, at 48 and 72 h, a significant decrease in cell biomass was already observed for all the concentrations tested, relatively to control, and these effects did not differ in intensity between this time period of exposure to RA except for the concentration of 40 µM (Fig. 4.1) Thus, in the following experiments, we used RA at 0.5 or 1 µM, which are the lowest concentrations tested inducing a toxic effect on melanoma cells.

After studying the dose-dependent effect of antiestrogens in a likewise time-course experiment (data not shown), the concentration of 5 µM was considered the most suitable

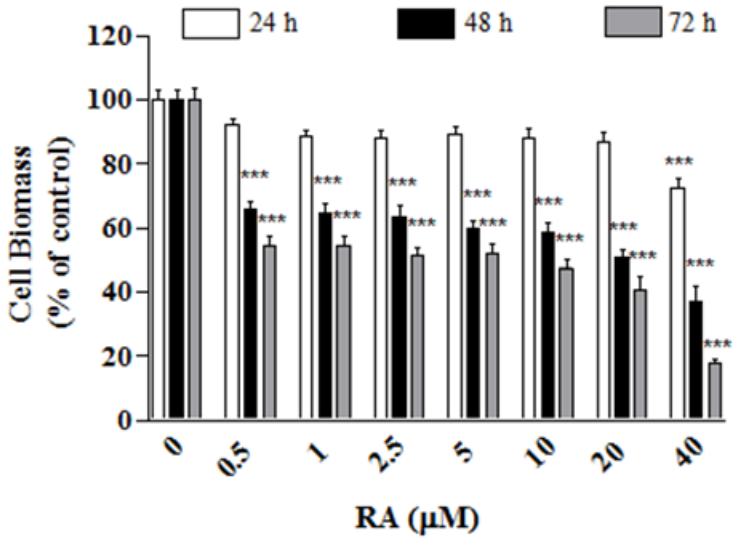


Fig. 4.1 - Time-course of RA-induced toxicity on melanoma cells. Cells were incubated in the absence (0) or in the presence of RA (0.5–40 μM), for 24, 48 and 72 h. At the selected time points, melanoma cell biomass was evaluated by the SRB assay as described in the Materials and methods section. Bars represent the mean ± S.E.M. of eight independent experiments. *** $p<0.001$ vs the respective time point in the absence of RA, One-way ANOVA followed by Tukey post-test.

for use in combination treatments, as it corresponded to the lowest concentration of EDX required to elicit a cytotoxic effect on melanoma cells. The effects of RA (0.5-1 µM) and the antiestrogens EDX and TAM (5 µM), alone or in combination, were then evaluated on melanoma cells, over 72 h (Fig. 4.2). RA at 0.5-1 µM decreased cell biomass to about 54 % of control, while the TAM active metabolite EDX at 5 µM provided a significant decrease in cell biomass to about 62 % of control (Fig. 4.2A). The combination of EDX with RA (0.5-1 µM) diminished cell biomass to about 26 % of control, which indicates that the drug combination enhances the effects induced by the compounds individually (Fig. 4.2A). In contrast, TAM at 5 µM did not significantly induce toxicity by itself, diminishing cell biomass to about 83 % (Fig. 4.2B). The combination of TAM with RA (0.5-1 µM) decreased cell biomass to about 41 %, which is significant relatively to TAM alone, but not in comparison with RA individually (Fig. 4.2B). It is noteworthy that increasing the concentration of RA up to 1 µM did not enhance the toxic effect relatively to the combinations including RA at 0.5 µM (Figs. 4.2A and 4.2B).

In order to elucidate the mechanism underlying the cytotoxic effects of the combinations of RA (0.5 µM) with antiestrogens EDX and TAM (5 µM), we assayed cell viability at selected time points by using trypan blue dye exclusion assay. As shown in figure 4.3A, RA reduced the number of viable cells within 48 and 72 h of drug incubation to about 49 % and 54 % of control condition, respectively. EDX diminished the number of viable cells to approximately 49 % and 61 %, after 48 and 72 h of drug incubation, respectively (Fig. 4.3A), while TAM did not alter the number of viable cells over 72 h (Fig. 4.3B), in agreement with the results obtained in SRB assays (Fig. 4.2). The combination of EDX with RA, at 72 h, significantly potentiated the effects of the compounds alone, decreasing the percentage of viable cells to approximately 14 % as compared with control condition (Fig. 4.3A). After 72 h of incubation, the combination of TAM with RA diminished the number of viable cells to about 30 % of control and did not further potentiate the effect of RA on the number of viable cells (Fig. 4.3B); hence, as observed in SRB assays, the effects of the combination with EDX were much more pronounced than those of the combination with TAM. On the other hand, the number of dead cells after exposure to RA and to the antiestrogens, alone or in combination, did not significantly increase during the course of 72 h (Figs. 4.3C and 4.3D). The absence of an increase in the number of dead cells within 72 h

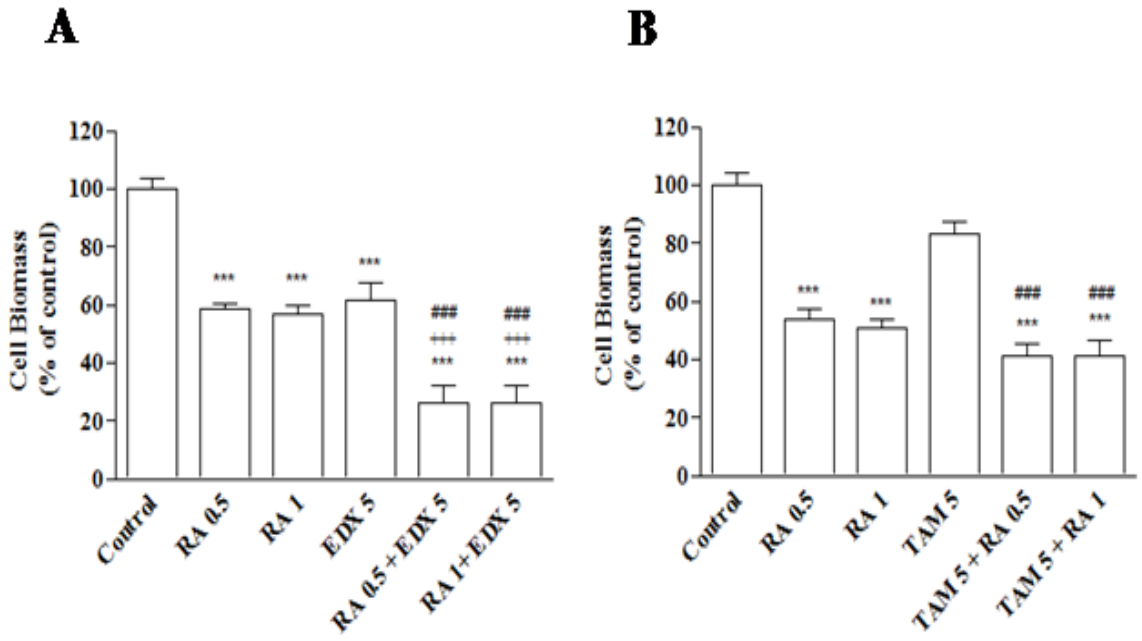


Fig. 4.2 - The combined treatment of RA with the TAM metabolite EDX enhances the toxicity on melanoma cells. Cells were incubated in the absence (control) or in the presence of RA (0.5-1 µM), 5 µM EDX (A) or 5 µM TAM (B), alone or in combination, for 72 h. The melanoma cell biomass was evaluated by the SRB assay after the incubation period. The combination of RA with the TAM metabolite EDX enhanced the decrease in cell biomass induced by the treatment with each compound individually. Bars represent the mean ± S.E.M. of eight independent experiments. *** $p<0.001$ vs control. +++ $p<0.001$ vs the respective RA concentration. ### $p<0.001$ vs the antiestrogen, One-way ANOVA followed by Tukey post-test.

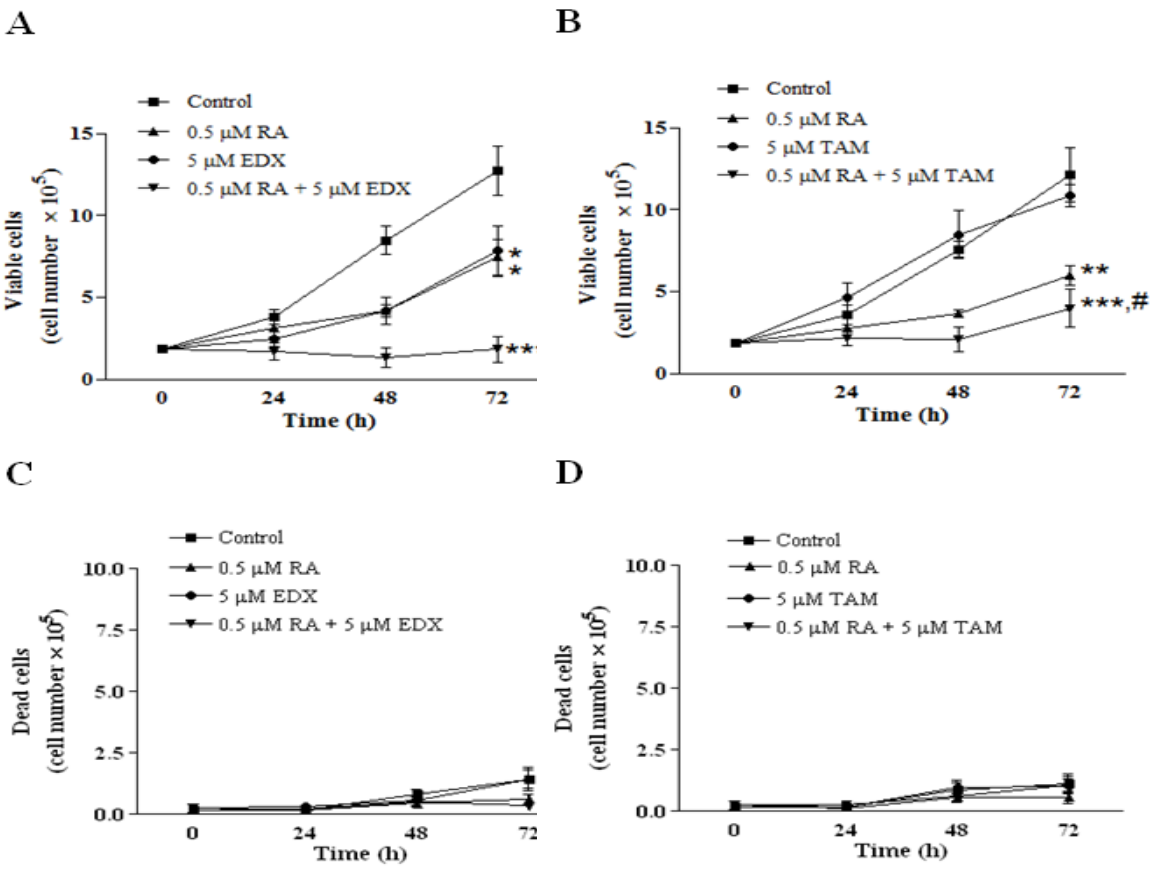


Fig. 4.3 - Cell viability of melanoma cells treated with RA and the antiestrogens EDX and TAM. Melanoma cells were incubated in the absence (control) or in the presence of RA (0.5-1 µM), 5 µM EDX (A, C) or 5 µM TAM (B, D), alone or in combination, and after 0, 24, 48 and 72 h of incubation, cell viability was assessed by the trypan blue dye exclusion assay as described in the Materials and methods. The number of trypan blue-negative (viable) cells (A, B) and trypan blue-positive (dead) cells (C, D) is presented in the graphs. The combinations of RA and antiestrogens decreased the number of viable cells without increasing the number of dead cells. Data represent the mean ± S.E.M. of six independent experiments. Statistics for 24 and 48 h are not shown. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ vs control. + $p<0.05$ vs RA. # $p<0.05$ vs the antiestrogen, One-way ANOVA followed by Tukey post-test.

of treatment with the drugs was confirmed by the lactate dehydrogenase (LDH) assay which, as the trypan blue assay, is a cytotoxicity test based on cell membrane integrity. The RA and the antiestrogens EDX and TAM, either alone or in combination, did not increase the activity of LDH in the extracellular medium of melanoma cells (data not shown). Thus, since we observed a reduction in the cell number without a simultaneous increase in cell death, our results suggest that the toxic effects of RA and antiestrogens on melanoma cells might be due to a decrease in cell proliferation.

Therefore, we investigated the effects of the drugs on melanoma cell proliferation by means of the BrdU incorporation in DNA synthesis after a 48 h treatment with RA (0.5 μM), alone or in combination with antiestrogens EDX and TAM (5 μM). As shown in figure 4.4, RA significantly decreased the incorporation of BrdU to approximately 63 % of control, i.e., in the absence of drugs. The incubation of cells with EDX decreased BrdU incorporation to about 42 %, whereas its combination with RA diminished it to approximately 12 % (Fig. 4.4). TAM diminished the BrdU incorporation to about 78 %, and the combination with RA induced a larger decrease in cell proliferation to about 12 %, as observed with EDX (Fig. 4.4). Thus, our results indicate that RA and the antiestrogens have a cytostatic effect on melanoma cells, which is intensified when the drugs are applied in a combined treatment.

To confirm whether the rate of proliferation of melanoma cells was in fact affected by the combinations of RA and antiestrogens, and that the reduction in BrdU signal was not a consequence of the decrease in cell number, the effect of the drugs on the cell cycle was analyzed by flow cytometry (Fig. 4.5). Untreated cells (control) were characterized by a long and a well defined G1 peak, a slightly prominent S peak, a least prominent G2 peak and relatively low G0/G1 fraction, which was considered as the apoptotic fraction (Fig. 4.5). The incubation of cells with 0.5 μM of RA increased the percentage of cells in the G1 phase and decreased the number of cells in S and G2 phases while 5 μM EDX or TAM did not change the population of cells in each cell cycle phase relatively to the control condition (Fig. 4.5). The combination of RA with the antiestrogen EDX significantly enhanced the effects on cell cycle induced by the compounds individually (Fig. 4.5). In contrast, the combination of RA with TAM did not potentiate the effects of RA alone (Fig. 4.5).

On the other hand, we sought to investigate whether RA and the antiestrogens could inhibit cancer cell migration that is related to metastatic potential. For that purpose, we

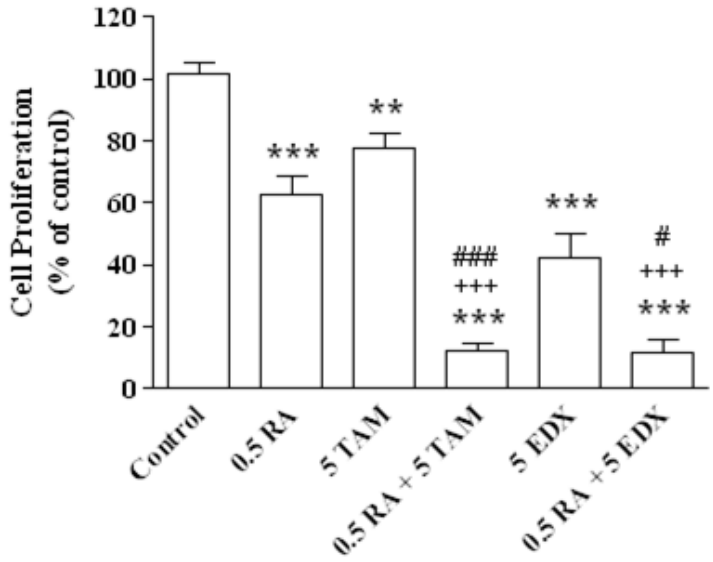


Fig. 4.4 - Melanoma cell treatment with RA and the antiestrogens EDX and TAM reduces cell proliferation. Cells were grown in the absence (control) or in the presence of RA (0.5 μM), alone or in combination with 5 μM of EDX or TAM, for 48 h. Cell proliferation was assessed by the BrdU incorporation assay as described in the Materials and methods. The combination of RA with EDX and TAM enhanced the effects on cell proliferation induced by the compounds individually. Bars represent the mean ± S.E.M. of four independent experiments performed in triplicates. *** $p<0.001$, ** $p<0.01$ vs control. +++ $p<0.001$ vs RA. ### $p<0.001$, # $p<0.05$ vs the antiestrogen, One-way ANOVA followed by Tukey post-test.

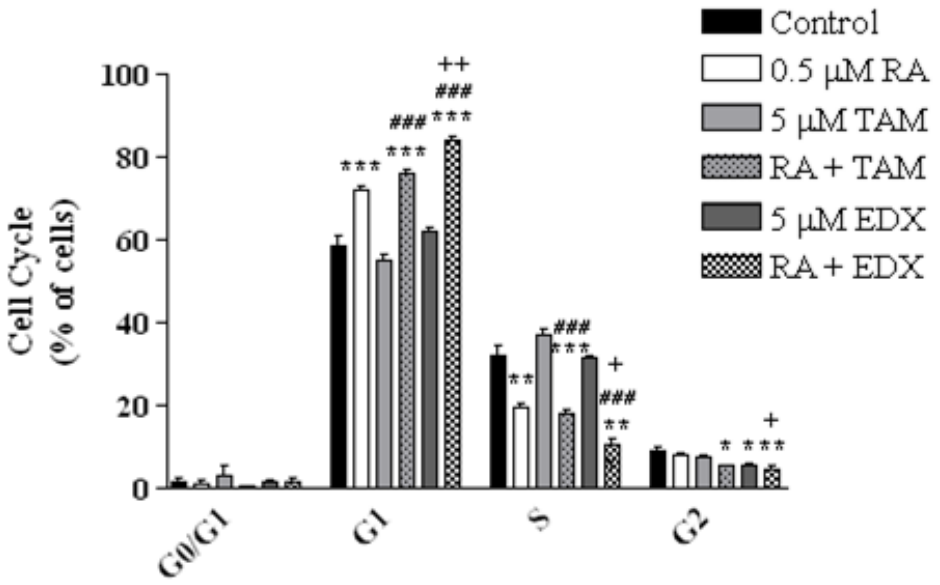


Fig. 4.5 - Melanoma cell treatment with RA and EDX blocks cell cycle progression in G1. Cells were grown in the absence (control) or in the presence of RA (0.5 µM), alone or in combination with 5 µM of EDX or TAM, for 48 h. Cell cycle distribution was evaluated by flow cytometry analysis of the content of DNA labeled with propidium iodide. The combination of RA with EDX enhanced the effects on the cell cycle induced by the compounds individually. Data are the mean ± S.E.M. of three independent experiments performed in duplicates. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ vs control. ++ $p<0.01$, + $p<0.05$ vs RA. ### $p<0.001$ vs the antiestrogen, One-way ANOVA followed by Tukey post-test.

performed the wound healing assay and analyzed the effect of the drugs on melanoma cell migration after 3 h of exposure to the combinations of RA (0.5-1 µM) with antiestrogens EDX and TAM (5 µM) (Fig. 4.6). This short time point was chosen to ensure that the effects observed in the wound healing assay were only related to the migration ability of melanoma cells and did not reflect the cell proliferation. In fact, we determined the number of cells per well at 0 and at 3 h and observed that during this time interval, the number of cells did not significantly change (cell number was, at 0 h, $5.660\times10^5 \pm 0.7743\times10^5$ and at 3 h, $5.600\times10^5 \pm 0.9431\times10^5$; $p>0.05$, Student's *t* test). As shown in figure 4.6A, neither RA at 0.5 µM nor the antiestrogens, alone or in combination, affected melanoma cells migration significantly. However, when the antiestrogens were combined with RA at 1 µM, we observed a pronounced decrease of the number of migrating cells, particularly with the combinations including EDX (Fig. 4.6B). These results suggest that a combined treatment of RA with EDX, additionally to a stronger cytostatic effect, is much more efficient in the inhibition of the metastatic potential of melanoma cells than the combination with TAM.

One possible advantage of a combined therapy of anticancer drugs is a reduction in the chemotherapy adverse effects due to a decrease in the dose of the drugs allowing a lower toxicity for non-neoplasic cells. To investigate the putative toxicity of RA (1 µM) in combination with the antiestrogens EDX and TAM (5 µM) for proliferative non-neoplasic cells, we assessed cell viability of BAEC cultures exposed to the drugs for 72 h. Cell counting with trypan blue staining revealed that RA and antiestrogens, individually or in combination, did not induce an increase in dead cells and the number of viable cells did not change significantly (Fig. 4.7).

Additionally, the potential toxicity of the combinations of RA with the antiestrogens EDX and TAM was monitored using rat liver mitochondria as a model, and several bioenergetic function parameters were determined (Fig. 4.8). In agreement with the results obtained in non-neoplasic cells, RA (5 µM) and the antiestrogens (5 µM), alone or in combination, neither affected the rates of state 3 (V3) and 4 (V4) of respiration (Fig. 4.8A) nor the phosphorylation cycle parameters (Fig. 4.8B), suggesting that liver mitochondrial bioenergetics were spared to the toxic effects of these drugs.

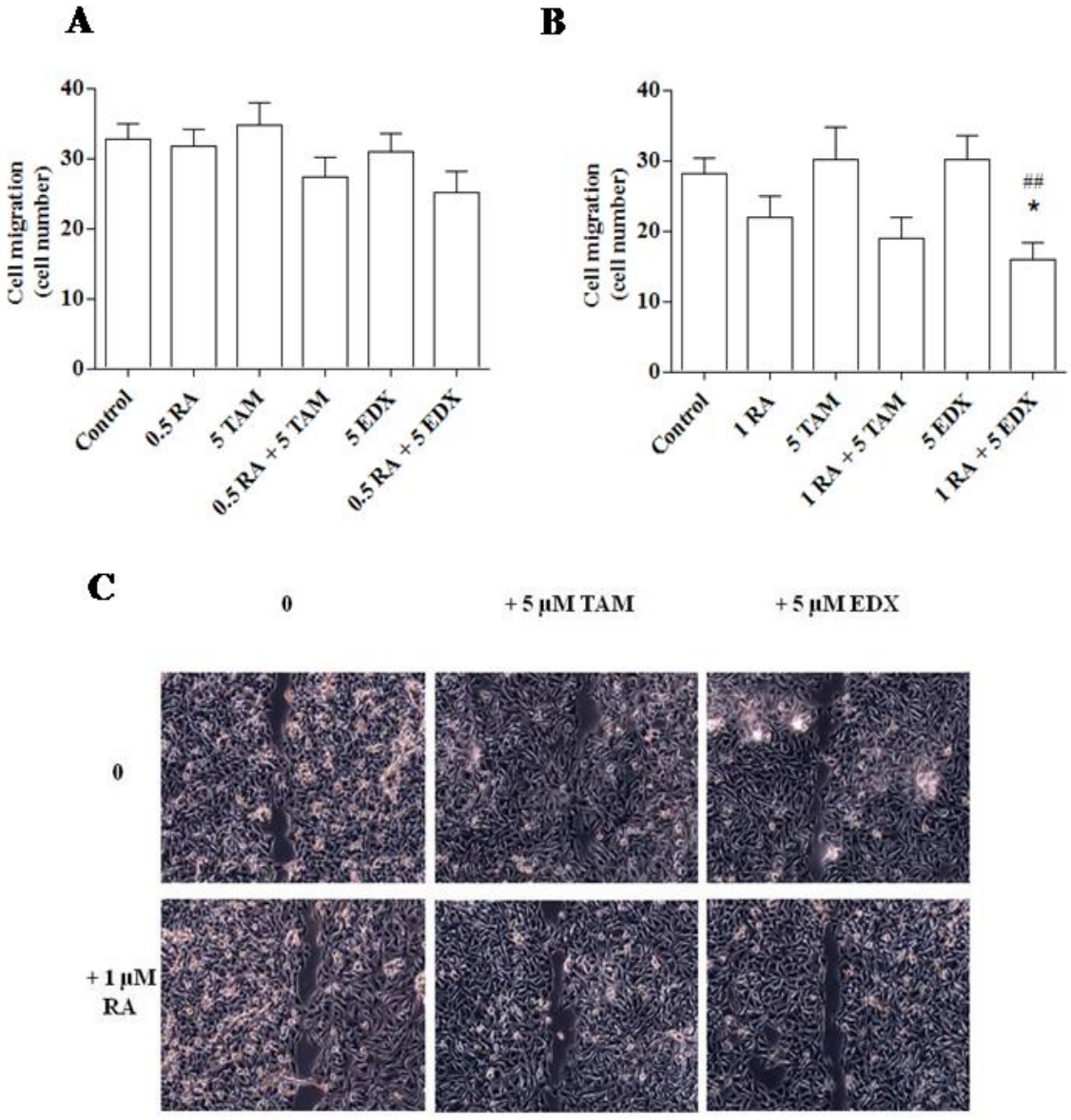


Fig. 4.6 - The combination of RA with EDX inhibits melanoma cells migration. Confluent melanoma cell cultures were scratched and incubated in the absence (control) or in the presence of RA at 0.5 µM (A) or at 1 µM (B) in combination with 5 µM of EDX or TAM. The cells were visualized by transmitted light microscopy and images of the melanoma cells were acquired at 0 h and 3 h after scratch. The cells that moved into the gap in this time interval were counted, as described in the Materials and methods. Representative images collected at t=3 h are shown in C. Data represent the mean ± S.E.M. of six independent experiments performed in triplicates. * $p<0.05$ vs control. ## $p<0.01$ vs the antiestrogen, One-way ANOVA followed by Tukey post-test.

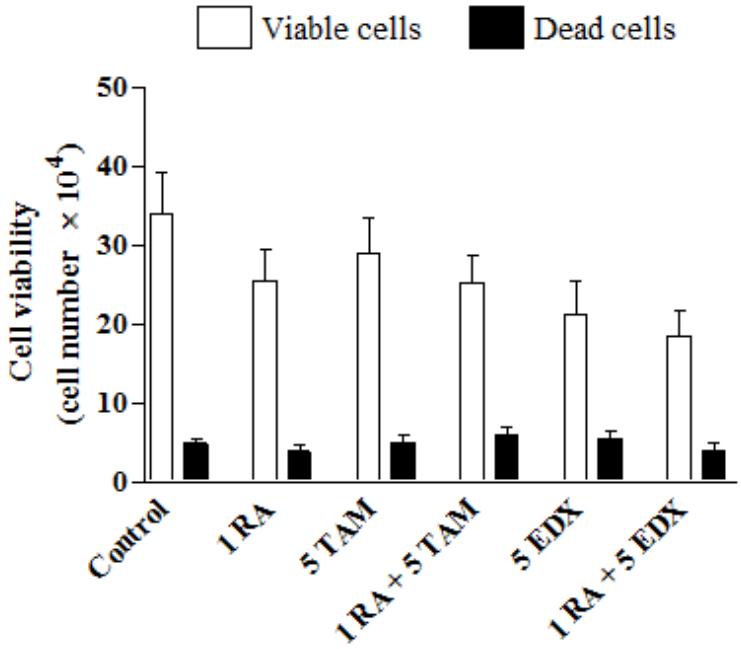
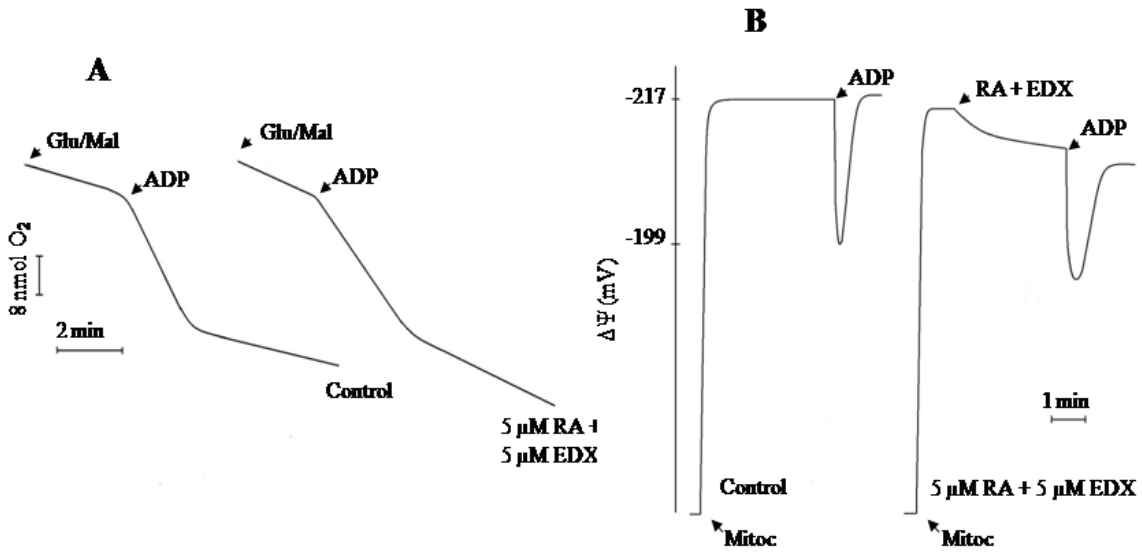


Fig. 4.7 - Cell viability of non-neoplasic endothelial cells treated with RA and the antiestrogens EDX and TAM. Cultures of BAEC were exposed for 72 h to RA (1 µM), EDX (5 µM) and TAM (5 µM), alone or in combination, and after the incubation period the endothelial cell viability was determined by the trypan blue dye exclusion assay. The number of trypan blue-negative (viable) cells and trypan blue-positive (dead) cells is presented. The RA and the antiestrogens, alone or in combination, did not induce toxicity in endothelial cells. Data represent the mean ± S.E.M. of five independent experiments performed in duplicates.



	(nmol O_2/min/mg protein)		ΔΨ (-mV)			Phosphorylation lag phase (s)
	V3	V4	Glu/Mal Energization	ADP Depolarization	Repolarization	
Control	59.4 ± 2.5	16.2 ± 1.3	220.1 ± 0.6	20.1 ± 1.7	219.8 ± 0.6	60.9 ± 1.8
5 RA	50.3 ± 2.6	17.4 ± 1.0	217.0 ± 1.2	19.3 ± 1.0	215.9 ± 1.1	75.4 ± 3.8
5 TAM	47.4 ± 5.6	15.8 ± 1.8	219.7 ± 0.5	21.0 ± 0.7	218.0 ± 0.7	69.0 ± 3.3
5 RA + 5 TAM	53.3 ± 7.2	14.3 ± 0.8	217.1 ± 1.6	18.2 ± 1.3	215.7 ± 1.9	75.0 ± 3.8
5 EDX	53.4 ± 1.8	16.3 ± 1.9	221.0 ± 1.6	21.3 ± 1.4	221.7 ± 1.4	65.3 ± 2.6
5 RA + 5 EDX	48.8 ± 2.5	15.8 ± 1.1	216.0 ± 1.2	17.4 ± 0.8	215.9 ± 1.8	73.5 ± 5.7

Fig. 4.8 - Effects of RA in combination with the antiestrogens EDX or TAM on liver mitochondrial bionenergetic function. Mitochondria (1 mg) were suspended in the standard respiratory medium and energized with 10 mM glutamate/5 mM malate. Oxygen consumption was assessed polarographically with a Clark oxygen electrode (A) and mitochondrial membrane potential (ΔΨ) was assessed using a TPP^+ selective electrode (B) as described in the Materials and methods section. The compounds RA (5 µM) and the antiestrogens (5 µM), individually or in combination, did not affect mitochondrial bioenergetic function. The traces are typical recordings representative of several experiments and the table underneath shows the mean ± S.E.M. of mitochondrial bioenergetic parameters obtained from different mitochondrial preparations.

4.4 - Discussion

Our study shows for the first time that the innovative combination of RA at concentrations of 0.5-1 µM with the TAM metabolite EDX (5 µM) enhanced the antiproliferative and antimigration effects induced by the compounds individually on melanoma cells. Importantly, RA is already approved for clinical use by the FDA and EDX has been shown to be safe and well tolerated by healthy individuals (Ahmad et al., 2010b). Thus, the fact that the drugs used in our work are already approved or in clinical trials may allow for a faster implementation of the proposed strategy.

The toxic effects of RA and antiestrogens on melanoma cells were initially assessed by the SRB assay which showed that a combined treatment of RA with the TAM active metabolite EDX was more effective in the reduction of melanoma cell biomass relatively to the compounds individually and more efficient than the combination with TAM (Fig. 4.2). Likewise, the cell viability assay with trypan blue staining revealed that the combined treatment of RA with antiestrogens induced the largest decrease in the number of viable cells, without inducing cell death (Fig. 4.3). Thus, our results suggest that the observed toxic effect of RA and antiestrogens, alone or in combination, are related to the inhibition of cell proliferation. Moreover, the evaluation of LDH activity in the supernatant of melanoma cells supports the view that the decrease in viable cells is not due to increased cell death (data not shown). The BrdU incorporation assay allowed us to confirm that RA and the antiestrogens inhibit cell proliferation with maximal efficacy when the drugs were used in combination (Fig. 4.4). The analysis of the cell cycle revealed that RA induced the cell cycle arrest in the G1 phase and the combination with EDX enhanced the number of cells in G1 phase (Fig. 4.5). On the other hand, the combination of RA (1 µM) with the TAM active metabolite EDX also decreased the migration ability of melanoma cells (Fig. 4.6), which adds to the benefits of a combined therapy with these drugs.

The RA has been widely studied for many years in a variety of chemopreventive and chemotherapeutic settings (Bushue and Wan, 2010; Tang and Gudas, 2011). The expression of RAR was reported in melanoma cell lines (Boskovic et al., 2002; Zhang et al., 2003; Fan et al., 2010) and it has been shown that the aberrant expression of retinoid receptors is a frequent event in melanoma and that the loss of retinoid receptor expression accompanies melanoma progression (Chakravarti et al., 2007). Thus, RA was found to

inhibit invasion in some tumor cell lines, including K1735-M2 melanoma cells (Helige et al., 1993), and to inhibit the proliferation of melanoma cells (Lotan, 1979; Drewa and Schachtshhabel, 1985) by inducing apoptosis and cell cycle arrest through up-regulation of p27, a cyclin-dependent kinase inhibitor that inhibits G1-to-S phase transition of the cell cycle (Zhang and Rosdahl, 2004). Nonetheless, at the concentrations used in our work, we only observed a cytostatic effect of RA (Figs. 4.3 and 4.5).

On the other hand, several studies demonstrated a strong synergistic inhibition of breast cancer cells proliferation by the combinations of retinoids with antiestrogens (Danforth, 2004; Searovic et al., 2009; Koay et al., 2010), but the effects of these combinations in other types of cancer were unknown. Our results show, for the first time, that in melanoma cells the combination of RA (0.5 μM) with antiestrogens (5 μM) enhances the antiproliferative effect of the compounds of both classes, allowing to achieve a higher cytostatic effect than when the drugs are used individually. Moreover, we show that the TAM active metabolite EDX is more effective than TAM in inhibiting melanoma cells biomass, either when antiestrogens are applied in monotherapy or when they are applied in combination with RA (Figs. 4.2 and 4.3).

The mechanisms underlying the ability of antiestrogens to decrease melanoma cell proliferation remain unclear. The presence of estrogen-binding proteins was reported in melanoma (Fisher et al., 1976; Walker et al., 1987), but further studies have failed to demonstrate their presence (Flowers et al., 1987; Cohen et al., 1990; Lecavalier et al., 1990; Kanter-Lewensohn et al., 2000). Recently, methylated ERα were detected in the tumor tissues and the sera of melanoma patients and their presence correlated with tumor progression (Mori et al., 2006). TAM has also been shown to suppress PKC/MEK/ERK and PKC/PI3K/Akt pathways and to inhibit the insulin-like growth factor-1 receptor signaling in melanoma cells (Kanter-Lewensohn et al., 2000; Matsuoka et al., 2009), suggesting that ER-independent targets may also play an important role in the biological activity of TAM in melanoma. Importantly, TAM metabolites may have additional anticancer mechanisms relatively to TAM. In fact, Eto (2010) demonstrated that retinoids and the other TAM active metabolite, 4-hydroxytamoxifen (OHTAM), but not TAM, up-regulate the expression of p27 in ER-positive and ER-negative breast cancer cells, using different pathways. Moreover, besides the 30-100-fold higher antiestrogenic potency of EDX relatively to TAM in breast cancer (Lim et al., 2005), EDX has also been shown to target ERα degradation by the

proteasome in breast cancer cells (Wu et al., 2009) and to inhibit aromatase activity, whereas similar concentrations of TAM or OHTAM do not significantly affect aromatase activity (Lu et al., 2012), suggesting that EDX may have additional mechanisms of action in breast cancer therapy in comparison with TAM. Additionally, the use of an active metabolite presents other advantages, since TAM requires CYP2D6-dependent bioconversion to its key active metabolites OHTAM and EDX to exert its anticancer activity (Kiyotani et al., 2012). In fact, it was established a correlation between a disadvantageous clinical outcome in breast cancer patients treated with TAM and CYP2D6 polymorphisms or the simultaneous administration of CYP2D6-inhibiting medication (Kelly et al., 2010; Lammers et al., 2010). To our knowledge, this is the first study using EDX in melanoma cells as an alternative to TAM. As our results demonstrate a superior cytostatic activity of EDX relatively to TAM, it is reasonable to hypothesize that, as observed in breast cancer, the steady-state levels of EDX will also have an impact on the treatment efficacy of TAM in melanoma, and the administration of the active metabolite would possibly avoid the therapeutic inefficacy due to the insufficient production of EDX *in vivo*.

A major concern with cytostatic therapies is related to the inhibition of non-neoplasic cell proliferation which leads to several side effects. Our studies revealed that at the concentrations used in our work, the combined therapy of RA and antiestrogens does not induce major toxic effects on endothelial cells (Fig. 4.7) and on rat liver mitochondrial bioenergetic function (Fig. 4.8), which suggests that the combinations of RA with antiestrogens may increase the therapeutic benefit without prohibitory side effects, particularly in the case of the combination of RA with EDX. Moreover, we showed that in isolated mitochondria EDX is less toxic than TAM, which adds to the advantages of an anticancer therapy including EDX (Cardoso et al., 2001; Ribeiro et al., 2013b). Our data are consistent with a phase I/II study performed in patients with advanced breast cancer which has reported that 20 mg TAM /day and RA up to 190 mg/m^2/day can be given together with acceptable toxicity (Budd et al., 1998). However, additional studies *in vivo* regarding the combinations of RA with the TAM metabolite EDX, which present higher cytostatic and antimigration effects, are required in order to provide more data concerning the efficacy and the possible toxic effects of these combinations.

On the other hand, the fact that the synergistic decrease of melanoma cells proliferation induced by the combinations of RA with antiestrogens was not observed in

normal cells suggests that these compounds might target specific alterations of melanoma cells. Several studies have demonstrated the existence of an interaction between retinoid and estrogen signaling in breast cancer cells (Rousseau et al., 2003; Hua et al., 2009; Ross-Innes et al., 2010). In fact, RARα is a component of the ERα transcriptional complex in breast cancer cells, where it acts as a cofactor, playing an important role in the regulation of estrogen-mediated gene expression (Ross-Innes et al., 2010). The mechanism underlying the synergistic effects of RA in combination with antiestrogens on the inhibition of proliferation of melanoma cells is a matter deserving future investigation.

In conclusion, this work reports that the combination of RA with the TAM active metabolite EDX enhances the inhibition of melanoma cell proliferation induced by the compounds individually and that this combination can also impair the migration potential of these cells. Moreover, the combinations studied are not toxic for endothelial cells or for rat liver mitochondrial bioenergetic function. Considering that the available therapies for malignant melanoma rely on very aggressive strategies that offer unsatisfactory clinical responses with high toxicity, the combinations of RA with EDX may offer a powerful strategy for the treatment of malignant melanoma, possibly with less toxic effects than the currently available treatments.

CHAPTER V

THE GLUTAMATE RECEPTOR ANTAGONIST MK-801 ACTS SYNERGISTICALLY WITH TAMOXIFEN AND ITS ACTIVE METABOLITES TO DECREASE THE PROLIFERATION OF MELANOMA CELLS

[Ribeiro, M.P., Nunes-Correia, I., Santos, A.E. and Custódio, J.B. (2013) The glutamate receptor antagonist MK-801 acts synergistically with tamoxifen and its active metabolites to decrease the proliferation of melanoma cells. Submitted for publication in *Exp. Dermatol.*]

Abstract

Recent reports suggest that *N*-methyl-D-aspartate receptor (NMDAR) blockade by MK-801 decreases tumor growth and migration. Thus, we investigated whether other ionotropic glutamate receptor (iGluR) antagonists were also able to modulate the proliferation of melanoma cells. On the other hand, the antiestrogen tamoxifen (TAM) decreases the proliferation and migration of melanoma cells, and has been included in combined therapies for malignant melanoma. As the efficacy of TAM is limited by its metabolism, we investigated the effects of the NMDAR antagonist MK-801 in combination with TAM and its active metabolites, 4-hydroxytamoxifen (OHTAM) and endoxifen (EDX). The sulforhodamine B, the trypan blue dye exclusion, the lactate dehydrogenase and the BrdU incorporation assays, as well as the cell cycle analysis by flow cytometry, were performed. The NMDAR blockers MK-801 and memantine decreased melanoma cell biomass due to diminished cell proliferation as both compounds decreased cell number and DNA synthesis without increasing cell death. In contrast, the NMDAR competitive antagonist D-2-amino-5-phosphonovaleric acid (APV) and the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and the kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline (NBQX) did not affect the proliferation of melanoma cells, suggesting that among the iGluR antagonists only the NMDAR channel blockers inhibit melanoma cell proliferation. The combination of MK-801 with the antiestrogens TAM, OHTAM and EDX synergistically enhanced the antitumoral effects induced by the compounds individually due to a decrease in cell proliferation, with the combinations including TAM metabolites promoting blockade of cell cycle progression in G1. Therefore, the data obtained indicate that these drugs in association may be useful in malignant melanoma therapy although further studies of clinical implications are warranted.

5.1 - Introduction

Emerging evidence indicates that melanoma is a very heterogenous malignancy, with several variants and with multiple signaling pathways contributing to cell proliferation constitutively activated (Herlyn, 2009). Therefore, in order to target such diversity, we need to develop combinations of drugs with specific and complementary mechanisms of action (Herlyn, 2009; Ko and Fisher, 2011).

Glutamate, the major excitatory neurotransmitter of the mammalian central nervous system, activates two classes of glutamate receptors (GluRs), the ionotropic (iGluRs) and metabotropic (mGluRs) receptors. The iGluRs form ion channels, while the mGluRs belong to the superfamily of G protein-coupled receptors (Teh and Chen, 2012). The iGluRs are divided into three groups based on structural and pharmacological similarities, and are named *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and kainate (KA) receptors, according to the type of synthetic agonist that activates them. So far, eight members of mGluRs have been identified, which are grouped in three classes based on sequence homology and downstream signaling pathways.

The fact that both neuronal embryonic progenitor cells and tumor cells have propensity to proliferate and migrate led to the investigation of the role of glutamate and its receptors on the proliferation and migration of cancer cells. It has been reported that GluR subunits are differentially expressed in a variety of tumor cell lines (Stepulak et al., 2009; Brocke et al., 2010; North et al., 2010a, b; Stepulak et al., 2011) and in samples of human tumor tissues (North et al., 2010a, b). The knockdown of selected GluR subunits has also been shown to modulate cancer cell proliferation and invasive behavior (de Groot et al., 2008; Luksch et al., 2011). Moreover, NMDA receptor (NMDAR) and AMPA receptor (AMPAR) antagonists inhibit the proliferation and migration of tumor cells and enhance the effects of cytostatic drugs, such as cyclophosphamide and thiotepa, *in vitro* and *in vivo* (Stepulak et al., 2005, 2007; North et al., 2010a, b; Rzeski et al., 2011; Stepulak et al., 2011).

Beyond the role played by mGluR signaling in melanoma cells (Marín et al., 2006; Namkoong et al., 2007; Abdel-Daim et al., 2010; Lee et al., 2011), recent reports also suggest a role for iGluRs, since functional NMDARs are expressed in this type of cells and the NMDAR antagonist MK-801 was shown to inhibit the migration and proliferation of melanoma cells and to decrease their growth *in vivo* (Song et al., 2012). In addition, the

AMPAR antagonist CFM-2, as well as the NMDAR antagonists memantine and MK-801 have been shown to alter melanocyte morphology, indicating that glutamate signaling may be relevant in melanocyte regulation (Hoogduijn et al., 2006).

On the other hand, it has been reported that tamoxifen (TAM), a selective estrogen receptor (ER) modulator widely used in the treatment and prevention of breast cancer, also decreases the growth and migration of melanoma cells (Kanter-Lewensohn et al., 2000; Matsuoka et al., 2009; Ribeiro et al., 2013a) and sensitizes melanoma cells to other chemotherapeutic agents (Flaherty et al., 1996; McClay et al., 1997). The biological activity of TAM is mediated by two active metabolites, 4-hydroxytamoxifen (OHTAM) and endoxifen (EDX), generated via cytochrome P450 (CYP) enzymes, namely CYP3A4 and CYP2D6 (Kiyotani et al., 2012). Recent studies point to an association between CYP2D6 polymorphisms and the clinical outcome in women treated with TAM (Schroth et al., 2009; Lammers et al., 2010). Furthermore, it was shown that the coadministration of CYP2D6-inhibiting medication can limit the efficacy of TAM therapy (Kelly et al., 2010). Therefore, the use of TAM active metabolites may present strong advantages relatively to the utilization of the prodrug, as it would avoid the variability related with TAM metabolism, leading to a more reliable therapeutic outcome.

Based on these findings, we investigated the effects of iGluR antagonists on the proliferation of a highly invasive melanoma cell line (K1735-M2). Additionally, since the combination of drugs with complementary mechanisms of action can provide superior therapeutic efficacy using lower concentrations, with the advantage of reducing the side effects of chemotherapy, we evaluated the effects of MK-801 in combination with antiestrogens on cell proliferation as well. We show that the NMDAR channel pore blockers, MK-801 and memantine, decrease melanoma cell proliferation due to decreased cell division. Moreover, the combined treatment of MK-801 with antiestrogens, and particularly with TAM metabolites, strongly enhances the antiproliferative effects induced by the compounds individually, supporting the view that these drugs in association may be useful in malignant melanoma therapy.

5.2 - Materials and Methods

Reagents

MK-801, Memantine TAM, OHTAM and EDX were obtained from SIGMA-Aldrich (St Louis, MO, USA). 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline (NBQX) was purchased from Tocris. D-2-amino-5-phosphonovaleric acid (APV), Dulbecco's modified Eagle's medium (DMEM) and antibiotic/antimycotic solution (10 000 units penicillin, 10 mg streptomycin, 25 µg amphotericin B per mL) were purchased from SIGMA-Aldrich (St Louis, MO, USA), Fetal Bovine Serum (FBS) and trypsin were obtained from Gibco, Invitrogen Life Technologies (Carlsbad, California, USA). All of the other chemicals were purchased from SIGMA-Aldrich (St Louis, MO, USA) and were of the highest grade of purity commercially available. GluR antagonists were kept in aqueous stocks. TAM and OHTAM stock solutions were prepared in absolute ethanol while EDX was prepared in dimethyl sulfoxide (DMSO).

Cell culture

K1735-M2 mouse melanoma cells (kindly offered by Dr. Paulo Oliveira, Center for Neurosciences and Cell Biology, Department of Zoology, University of Coimbra, Portugal) were cultured in DMEM, supplemented with 10 % heat-inactivated FBS and 1 % antibiotic/antimycotic solution, and kept in a humidified atmosphere with 5 % CO_2/95 % air, at 37 ºC.

Cells were plated with a density of 6.1×10^4 cells/cm^2 and 24 h after plating, the GluR antagonists and/or the antiestrogens were added to the cultures from diluted stocks, except in the control condition where the vehicle was added.

Sulforhodamine B (SRB) assay

The cytotoxic effects induced by the drugs on melanoma cell cultures were determined using the SRB assay, which is based on the binding of SRB to the basic amino acids of cellular proteins (Holy et al., 2006). At selected time points, the cell culture was fixed with absolute methanol containing 1 % acetic acid, and stored at −20 ºC overnight. The methanol was then decanted and the plate air-dried. The SRB solution (0.5 % in 1 % acetic acid) was added to each well, and the plate incubated at 37 °C for 1 h. The cells were rinsed

with 1 % acetic acid, air-dried, and the bound dye eluted with 10 mM Tris buffer, pH 10. The absorbance was measured in a Synergy HT plate reader at 540 nm, providing an estimate of the total protein mass (biomass) which is related to the cell number. The absorbance obtained in control cultures was considered 100 %. Experiments were performed in triplicates for each independent experiment.

Cell viability assessment by trypan blue dye exclusion

Cell viability was investigated by staining cells with trypan blue (Houben et al., 2009). At designated time points, adherent cells were trypsinized, centrifuged at 1 000 rpm for 5 min and treated with 0.4 % trypan blue for 2-3 min and then counted in a hemocytometer under a transmitted light microscope. Cells presenting a blue stained cytoplasm were considered as dead cells; cells excluding the dye were considered as viable. The number of independent experiments is indicated in figure legends.

Lactate dehydrogenase (LDH) assay

LDH is a cytosolic enzyme that is released into the extracellular medium following the loss of cell membrane integrity (Vieira et al., 2010). Thus, we investigated the ability of the compounds used in this study to induce melanoma cell death by determining the LDH activity in the cell medium. The culture medium was collected 72 h after incubation with the drugs and centrifuged at 14 000 rpm for 10 min at 4 ºC. An aliquot of supernatant (100 µL) was incubated with a substrate mixture containing 40 µM lactate in perchloric acid 3 %, and 3.6 mM nicotinamide adenine dinucleotide (NAD^+) in tris-hidrazine buffer [80 mM tris, 400 mM hydrazine, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 9.5]. LDH activity was determined by an enzymatic reaction whereby the NAD^+ is reduced to NADH by oxidation of lactate to pyruvate. Thus, the amount of NADH is directly related to LDH activity in the supernatant. Absorption of NADH was measured at 340 nm. The LDH activity is expressed as the ratio between the LDH activity in the extracellular medium and the total LDH activity obtained from the supernatant of cells lysed with Triton X-100, which was considered as 100 %. Experiments were performed in duplicates for each independent experiment.

5-bromo-2'-deoxyuridine (BrdU) incorporation assay

Melanoma cell proliferation was monitored through the evaluation of BrdU incorporation during DNA synthesis in proliferating cells. For this purpose, the Cell Proliferation ELISA, BrdU, colorimetric kit (Roche) was used according to the manufacturer's protocol. After 48 h of incubation with the drugs, cultured cells were placed in BrdU-labeling solution for 90 min. Afterwards the cells were fixed and the DNA denaturated with the FixDenat solution, provided with the kit, and then incubated with a monoclonal antibody conjugated with peroxidase (anti-BrdU-POD) to bind BrdU in the newly synthesized DNA. The immune complexes were detected using the 3,3',5,5'-tetramethylbenzidine (TMB) substrate and the absorbance was measured in a Synergy HT plate reader at 370 nm. The absorbance values correlate to the amount of DNA synthesis and, therefore, to the number of proliferating cells. The experiments were carried out in triplicate for each independent experiment and the absorbance obtained in control cultures was considered as 100 %.

Cell cycle analysis by flow cytometry

The effects of the drugs on cell cycle were monitored by flow cytometry (Carmo et al., 2011). Cells were plated in 6-multiwell plates and incubated with MK-801, TAM, OHTAM and EDX for 48 h. At the end of the incubation period, cells were trypsinized and centrifuged at 1 500 rpm for 10 min, the culture medium was discarded and the pellet was fixed overnight at 4 ºC with a solution of cold 70 % ethanol. The cells were then centrifuged at 1 500 rpm for 10 min, the pellet was resuspended in a solution of phosphate-buffered saline (PBS) containing RNAse and, after 45 min, propidium iodide was added and cells were further incubated for 1 h in the dark, at room temperature (the final concentrations of RNase and propidium iodide were 10 µg/mL and 20 µg/mL, respectively). The propidium iodide fluorescence was measured on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488 nm argon-ion laser. For aggregate/debris discrimination, in addition to propidium iodide fluorescence signal heights, areas and widths were also measured. Measurements for at least 20 000 events were collected per sample. Data were analyzed using the ModFit LT 3.0. software. The experiments were carried out in duplicate for each independent experiment and the results are expressed as % of total cells.

Statistical analysis

Results are presented as the mean ± S.E.M. of the indicated number of independent experiments. Statistical significance between the different assays was determined using the one-way analysis of variance (ANOVA), followed by the Tukey post-test, for multiple comparisons. A p value <0.05 was considered statistically significant. These statistical analyses were performed using the software package GraphPad Prism 4.

5.3 - Results

In order to establish the most effective GluR antagonists to pursue our work, we initially studied the cytotoxic effects of the NMDAR channel blockers MK-801 and memantine, and the selective NMDAR competitive antagonist APV, as well as the AMPAR antagonist NBQX, on melanoma cell biomass by using the SRB assay which correlates with cell number (Fig. 5.1). After 72 and 96 h of incubation in the presence of MK-801 and memantine, the cell biomass was decreased and significant effects were detected at 500 µM of MK-801 and at 300 µM of memantine. In contrast, the cell biomass was unaffected by 500 µM of APV or NBQX after 96 h of drug incubation (Fig. 5.1). Therefore, in the following experiments we used the NMDAR channel blockers MK-801 and memantine.

To elucidate the mechanism underlying the cytotoxic effects of the NMDAR channel blockers MK-801 and memantine, we assayed cell viability after 72 h of incubation with the drugs by using the trypan blue dye exclusion assay (Fig. 5.2). In agreement with the results obtained with SRB assay, NMDAR channel blockers induced a decrease in the number of viable cells, which was significant at 500 µM of MK-801 and 300 µM of memantine (Fig. 5.2A). Moreover, at these concentrations MK-801 and memantine did not increase the number of dead cells during the course of 72 h (Fig. 5.2B).

The absence of an increase in the number of dead cells within 72 h of treatment with the drugs was confirmed by the LDH assay which, as the trypan blue assay, is a cytotoxicity test based on cell membrane integrity. The LDH activity did not increase in the supernatant of cells grown in the presence of MK-801 or memantine (Fig. 5.3A). Therefore, we investigated whether the cytotoxic effect of the NMDAR antagonists could be due to the inhibition of melanoma cell proliferation by means of BrdU incorporation in the DNA

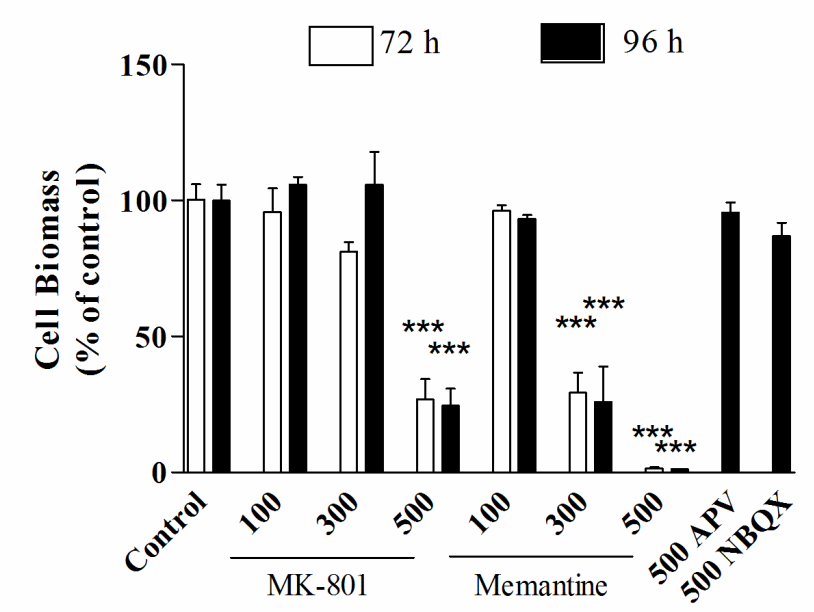


Fig. 5.1 - Effects of GluR antagonists on melanoma cell biomass. Cells were incubated in the absence (control) or in the presence of MK-801 (100-500 µM), memantine (100-500 µM), APV (500 µM) and NBQX (500 µM). At 72 and 96 h, melanoma cell biomass was evaluated by the SRB assay. For that purpose, the cells were fixed with absolute methanol containing 1 % acetic acid and incubated with SRB solution at 37 °C for 1 h. Afterwards, the plates were rinsed and the bound dye eluted with Tris buffer and the absorbance was measured at 540 nm. Bars represent the mean ± S.E.M. of four independent experiments performed in triplicates. *** $p<0.001$ vs the respective time point control, One-way ANOVA followed by Tukey post-test.

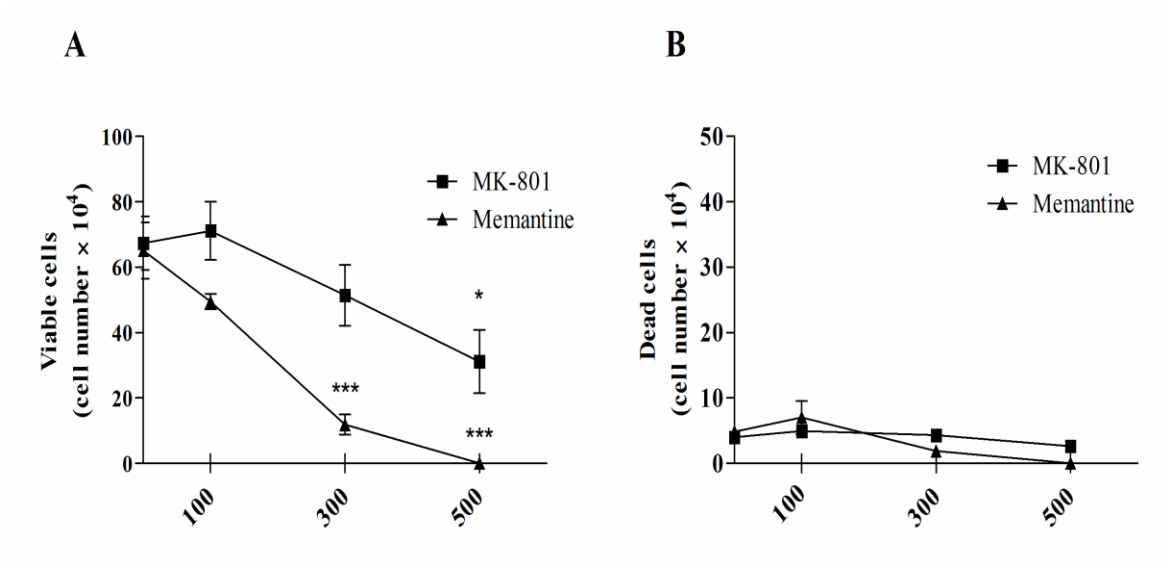


Fig. 5.2 - Cell viability of melanoma cells treated with the NMDAR channel blockers MK-801 and memantine. Cells were grown in the absence (0) or in the presence of 100-500 µM of MK-801 or memantine for 72 h and the number of viable and dead cells was determined by the trypan blue dye exclusion assay. After the incubation period, cells were trypsinized, centrifuged, treated with 0.4 % trypan blue and counted in a hemocytometer under a transmitted light microscope. The number of trypan blue-negative (viable) cells and trypan blue-positive (dead) cells is presented in the graphs A and B, respectively. Data represent the mean ± S.E.M. of four independent experiments. *** $p<0.001$, * $p<0.05$ vs control.

synthesis after a 48 h treatment with MK-801 or memantine (100-500 µM). This earlier time point was selected as the number of cells in control condition at 72 h is substantially high and could lead to absorbance values beyond the acceptable measuring range. As shown in figure 5.3B, both compounds significantly reduced BrdU incorporation at 300 µM. Taken together, our results indicate that the toxic effects of the NMDAR channel blockers MK-801 and memantine on melanoma cells might be due to a decrease in cell proliferation.

To investigate the effect of drug combinations on cell proliferation, MK-801 was the compound of choice to study in association with antiestrogenic compounds, since it has been shown to be effective and safe in several animal models of cancer (Stepulak et al., 2005; North et al., 2010a, b; Song et al., 2012), whereas exposure to memantine at the concentrations used in this study compromises mitochondrial function (McAllister et al., 2008), which can lead to drug-induced tissue injury (Labbe et al., 2008).

The dose-dependent cytotoxic effects of antiestrogens on melanoma cells were initially evaluated by the SRB assay during a time-course experiment. The TAM active metabolite concentration of 5 µM was the lowest that induced a significant decrease in cell biomass (data not shown). Therefore, 5 µM was the selected concentration to pursue the studies aiming to assess the possible co-operative effects of a combination of antiestrogens with NMDAR antagonists on melanoma cells proliferation (Fig. 5.4).

Thus, melanoma cells were subjected to treatment with MK-801 (100 µM) and antiestrogens (5 µM), alone or in combination, over 72 h (Fig. 5.4). At this concentration, the antiestrogen TAM did not significantly decrease melanoma cell biomass, whereas TAM active metabolite EDX significantly reduced cell biomass to about 82 % of control, in agreement with our previous studies (Ribeiro et al., 2013a). The other TAM active metabolite, OHTAM, significantly decreased cell biomass to approximately 66 % (Fig. 5.4). The combination of MK-801 with the antiestrogens TAM, OHTAM and EDX diminished cell biomass to approximately 46 %, 33 % and 38 % of control, respectively, which is a much stronger cytotoxic effect in comparison with that induced by the compounds individually. Noteworthy, MK-801 at a concentration that did not induce cytotoxicity, when applied individually, co-operated with the antiestrogens to potentiate their cytotoxic effects (Fig. 5.4). The effects of the combinations of MK-801 (100 µM) with antiestrogens (5 µM) on cell viability were then quantitated at selected time points through the trypan blue dye

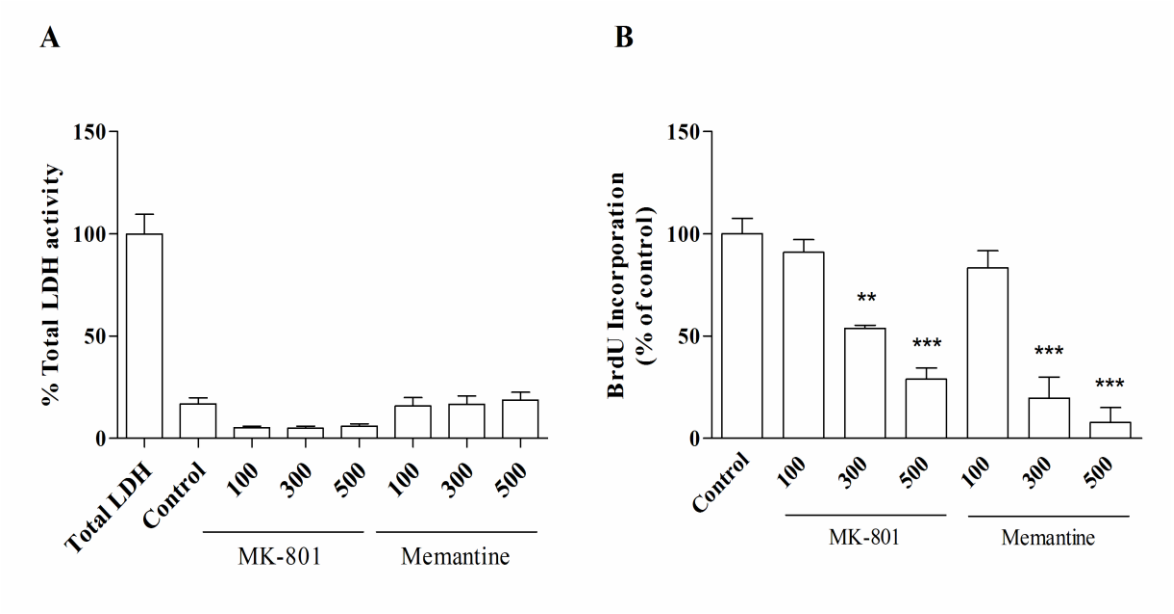


Fig. 5.3 - The NMDAR channel blockers MK-801 and memantine do not induce cell death (A) and decrease cell proliferation (B). (A) Melanoma cells were grown in the absence (control) or in the presence of 100-500 µM of MK-801 or memantine. Cell death was assessed by measuring LDH activity in the supernatant of damaged cells after 72 h in culture. Bars represent the mean ± S.E.M. of three independent experiments performed in duplicates. The statistical analysis was performed by One-way ANOVA followed by Tukey post-test. (B) Cells were grown for 48 h in the absence (control) or in the presence of 100-500 µM MK-801 or memantine and then cell proliferation was assessed by the BrdU incorporation assay as described in the Materials and methods section. Bars represent the mean ± S.E.M. of four independent experiments performed in triplicates. *** $p<0.001$, ** $p<0.01$, One-way ANOVA followed by Tukey post-test.

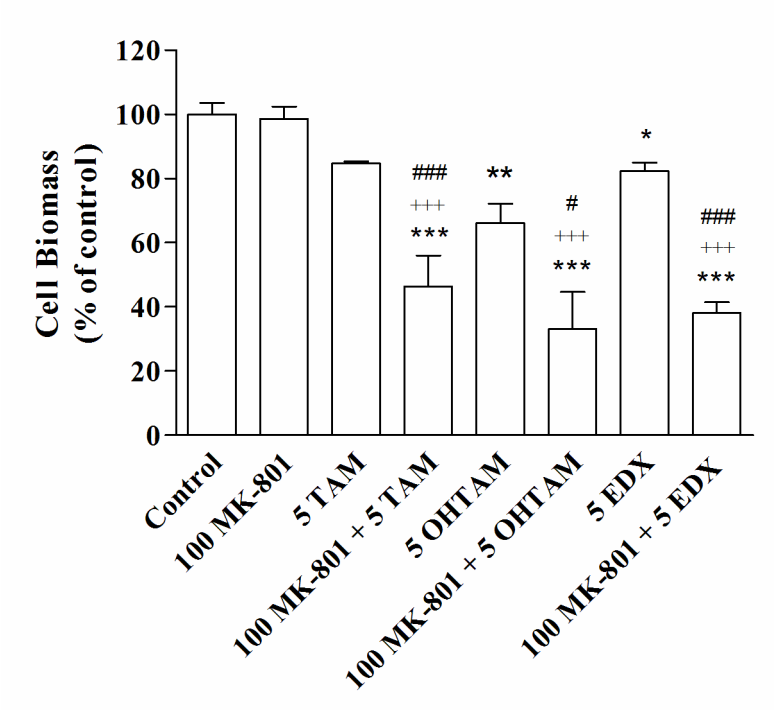


Fig. 5.4 - The combined treatment of MK-801 with antiestrogens synergistically decreases the melanoma cell biomass. Melanoma cells were grown in the absence (control) or in the presence of 100 µM of MK-801, 5 µM of the antiestrogens TAM, OHTAM and EDX, alone or in combination. The melanoma cell biomass was evaluated by the SRB assay after 72 h of incubation. Bars represent the mean ± S.E.M. of six independent experiments performed in triplicates. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ vs control. +++ $p<0.001$ vs MK-801. ### $p<0.001$, # $p<0.05$ vs antiestrogen, One-way ANOVA followed by Tukey post-test.

exclusion assay (Fig. 5.5). As shown in figure 5.5A, MK-801 and TAM did not alter the number of viable cells at 72 h, in agreement with the results obtained in SRB assays (Fig.5.4). However, a decrease in the number of viable cells was already observed at 48h when cells were treated with OHTAM (Fig. 5.5B), whereas the EDX metabolite only significantly decreased the number of viable cells at 72 h of incubation with the drug (Fig. 5.5C). The combination of any of the three antiestrogens with MK-801 induced a significantly larger decrease of viable cells already observed at 48 h of incubation when compared to the compounds applied individually (Figs. 5.5A-5.5C). On the other hand, the number of dead cells after exposure to MK-801 and to the three antiestrogens, alone or in combination, did not significantly increase during the course of 72 h (Figs. 5.5D-5.5F).

The absence of an increase in the number of dead cells within 72 h of treatment with the drugs was confirmed by the LDH assay (Fig. 5.6). Neither the compounds individually nor their combinations increased the LDH activity in the extracellular medium, in accordance with the results obtained with the trypan blue dye exclusion assay (Fig. 5.5). The results obtained thus suggest that the toxic effects induced by the combined treatment of MK-801 and antiestrogens on melanoma cells may be related to a decrease in cell proliferation. Therefore, the inhibition of cell growth induced by MK-801 in association with the antiestrogens was also investigated by means of the BrdU incorporation assay (Fig. 5.7). While 100 µM of MK-801 by itself did not affect the BrdU incorporation in melanoma cells, 5 µM of TAM, OHTAM and EDX significantly decreased the incorporation of BrdU to 80 %, 52 % and 59 % of control, respectively. Noteworthy, the combination of MK-801 at 100 µM with the three antiestrogens TAM, OHTAM and EDX significantly decreased BrdU incorporation to 35 %, 9 % and 17 % of control, respectively, which is a much stronger effect relatively to that of the compounds individually (Fig. 5.7). Thus, our results showed the combinations of MK-801 and the antiestrogens might have a cytostatic effect on melanoma cells, which is more prominent when MK-801 is combined with the TAM metabolites than with the prodrug.

To confirm our hypothesis that the rate of proliferation of melanoma cells was in fact affected by the combination of MK-801 with antiestrogens, and that the reduction in BrdU signal was not a consequence of the decrease in cell number, the effect of the drugs on the cell cycle was analyzed by flow cytometry (Fig. 5.8). Untreated cells (control) were

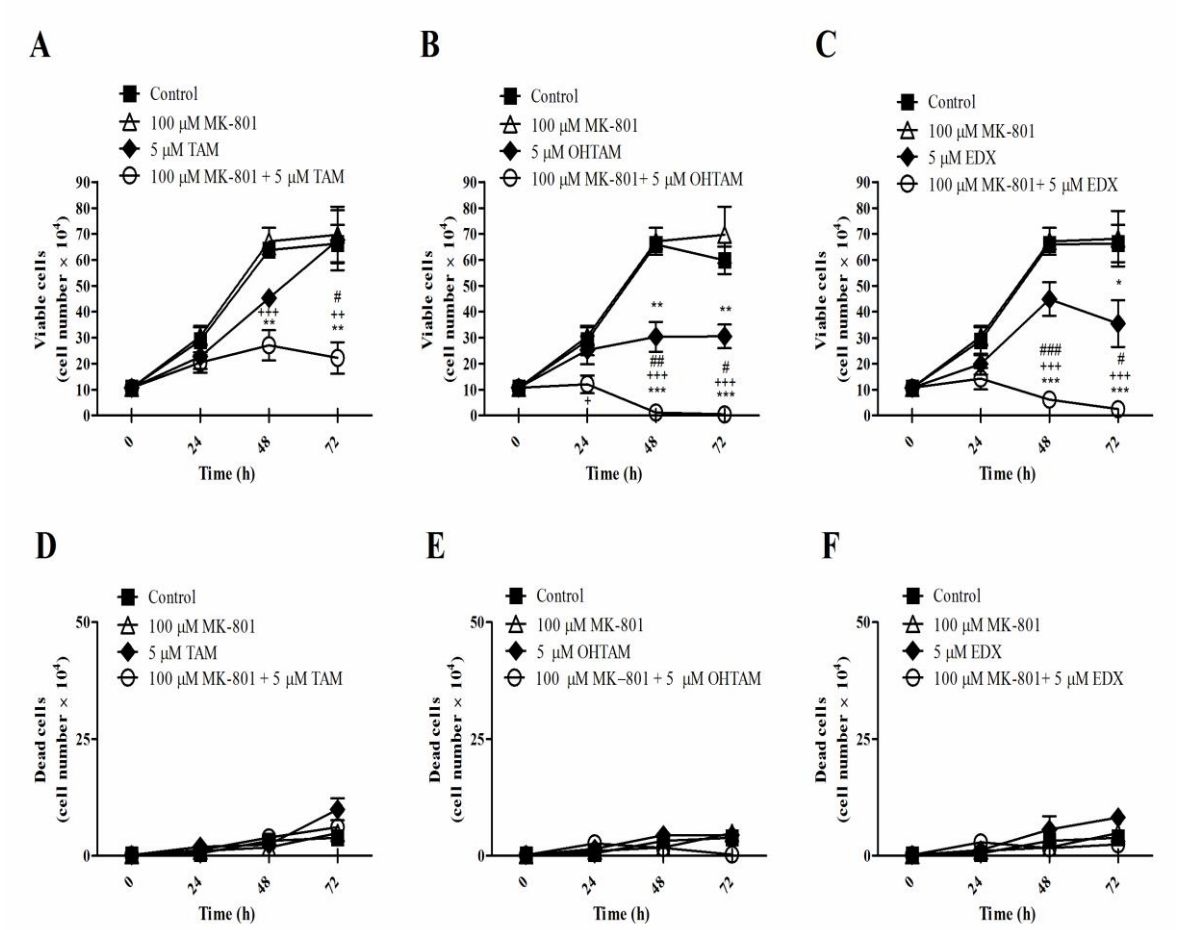


Fig. 5.5 - Cell viability of melanoma cells treated with MK-801 and the antiestrogens. Melanoma cells were grown in the absence (control) or in the presence of 100 µM of MK-801 and 5 µM of the antiestrogens TAM (A, D), OHTAM (B, E) and EDX (C, F), alone or in combination, and cell viability was assessed by the trypan blue dye exclusion assay as described in the Materials and methods section at 24 h, 48 h and 72 h. The graphs present the number of viable (A, B, C) and dead (D, E, F) cells. Data represent the mean ± S.E.M. of six independent experiments. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ vs the respective time point control. +++ $p<0.001$, ++ $p<0.01$ vs MK-801 at the respective time point. ### $p<0.001$, ## $p<0.01$, # $p<0.05$ vs the antiestrogen at the respective time point, One-way ANOVA followed by Tukey post-test.

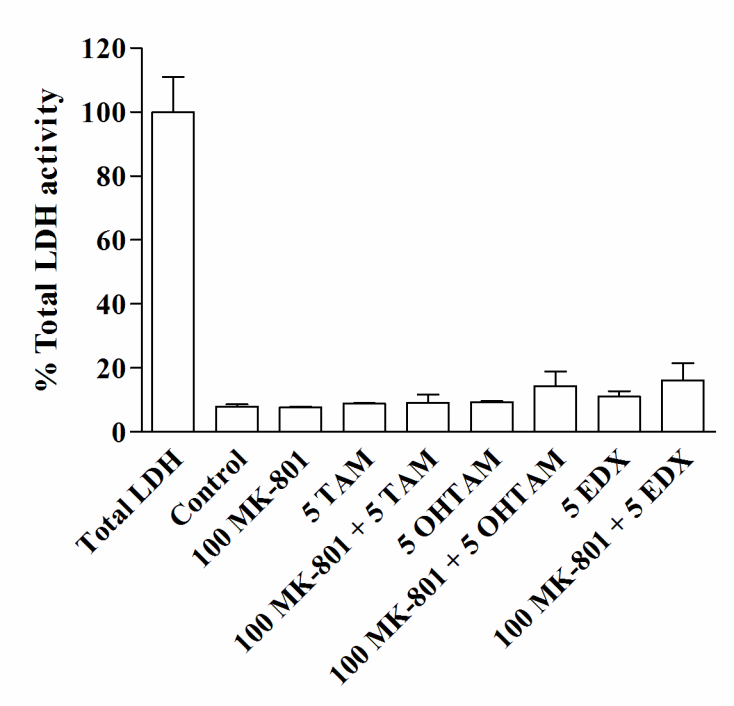


Fig. 5.6 - The combination of MK-801 with the antiestrogens does not induce melanoma cell death. Melanoma cells were grown in the absence (control) or in the presence of 100 µM of MK-801, 5 µM of the antiestrogens TAM, OHTAM and EDX, alone or in combination. Cell death was assessed by measuring LDH release from damaged cells, after 72 h in culture. Bars represent the mean ± S.E.M. of four independent experiments performed in duplicates.

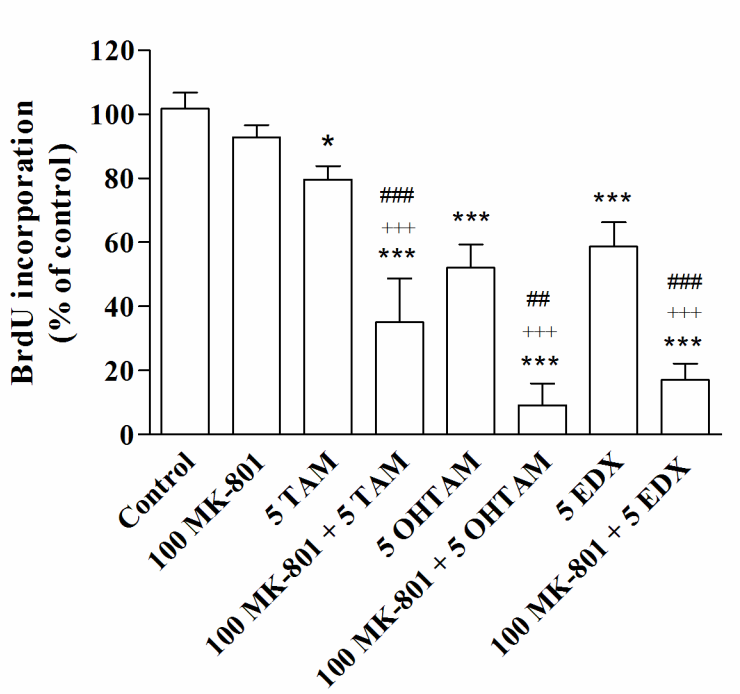


Fig. 5.7 - Melanoma cell treatment with the combination of MK-801 and the antiestrogens reduces cell proliferation. Cells were grown in the absence (control) or in the presence of 100 µM of MK-801, 5 µM of the antiestrogens TAM, OHTAM and EDX, alone or in combination, for 48 h, and then cell proliferation was assessed by the BrdU incorporation assay as described in the Materials and methods section. Bars represent the mean ± S.E.M. of four independent experiments performed in triplicates. *** $p<0.001$, * $p<0.05$ vs control. +++ $p<0.001$ vs MK-801. ### $p<0.001$, ## $p<0.01$ vs antiestrogen, One-way ANOVA followed by Tukey post-test.

characterized by a long and well defined G1 peak, a slightly prominent S phase, a least prominent G2 peak and a relatively low G0/G1 fraction, which was considered as the apoptotic fraction (Fig. 5.8). Forty-eight hours after incubation with 100 μM of MK-801 or 5 μM of antiestrogens, the population of cells in each cell cycle phase relatively to the control condition was not changed (Fig. 5.8). The combination of MK-801 with the TAM active metabolites, OHTAM or EDX, significantly increased the percentage of cells in G1 while decreasing the population of cells in the S phase, thus arresting the cell cycle in the G1 phase (Fig. 5.8).

5.4 - Discussion

Recent studies have demonstrated that melanoma cells express NMDARs and that MK-801 inhibits their migration and proliferation (Song et al., 2012). In addition, it was reported that NMDAR and AMPAR antagonists enhance the effects of cytostatic drugs on human neuroblastoma and human rhabdomyosarcoma/medulloblastoma cell lines (Rzeski et al., 2001). Thus, we investigated whether other iGluR antagonists could also affect the proliferation of melanoma cells and the possible co-operative effects of MK-801 in combination with antiestrogenic compounds, which also decrease the growth and migration of melanoma cells (Kanter-Lewensohn et al., 2000; Matsuoka et al., 2009; Ribeiro et al., 2013a). Our results show, for the first time, that the combined treatment of MK-801 with antiestrogens, and particularly with TAM active metabolites, enhances the antiproliferative action induced by the compounds individually.

The cytotoxic effects of GluR antagonists on melanoma cells were assessed by the SRB assay which showed that MK-801 and memantine reduce melanoma cell biomass (Fig. 5.1). On the contrary, the AMPAR and KAR antagonist NBQX, and the selective NMDAR competitive antagonist APV, which binds on the extracellular domain of the NMDAR, did not exhibit antiproliferative effects on melanoma cells even at high concentrations (Fig. 5.1). Although MK-801 and memantine have been traditionally considered to target the NMDAR channel, these compounds might act on other cellular targets. In fact, there is evidence that the 5-hydroxytryptamine receptor 3, the α7 and/or α4β2 nicotinic receptors and the dopamine receptors may also be involved in the biological activity of memantine (Rammes et al., 2008;

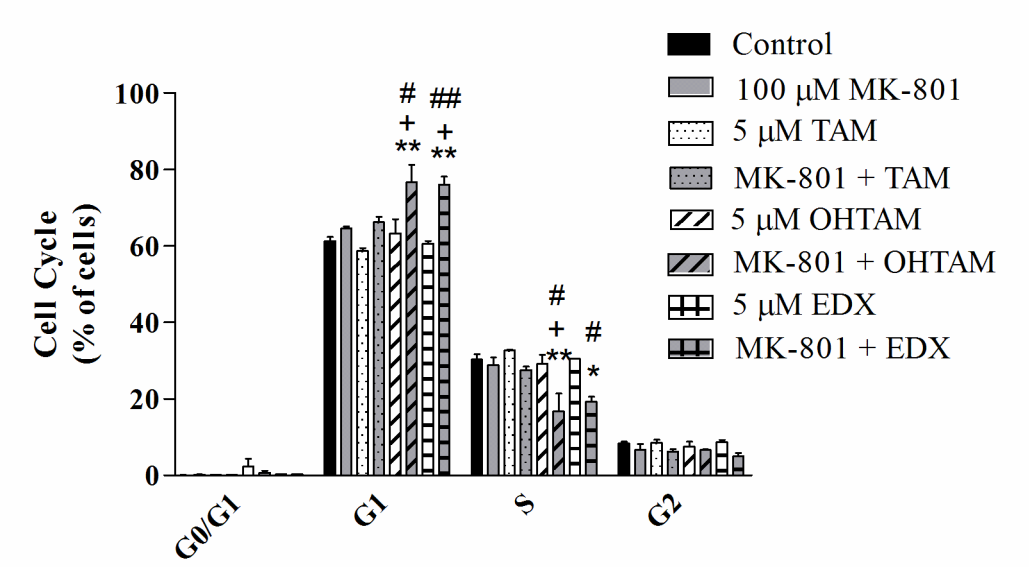


Fig. 5.8 - Melanoma cell treatment with MK-801 and TAM metabolites blocks cell cycle progression in G1. Cells were grown in the absence (control) or in the presence of MK-801 (100 µM), 5 µM of the antiestrogens TAM, OHTAM and EDX, alone or in combination, for 48 h. Cell cycle distribution was evaluated by flow cytometry analysis of the DNA content labeled with propidium iodide. Data are the mean ± S.E.M. of three independent experiments performed in duplicates. A total of 20 000 events were analyzed for each experiment. ** $p<0.01$, * $p<0.05$ vs control. + $p<0.05$ vs MK-801. ## $p<0.01$, # $p<0.05$ vs the antiestrogen, One-way ANOVA followed by Tukey post-test.

Seeman et al., 2008). In addition, acute and chronic exposure to memantine has NMDAR-independent effects on the mitochondrial function, by affecting complex I and complex IV activities (McAllister et al., 2008). Likewise, MK-801 might act on the α7 and α4β2 nicotinic receptors (Briggs et al., 1996; Buisson and Bertrand, 1998) and can also modulate thedopaminergic and serotonergic system (Rao et al., 1990; Clarke and Reuben, 1995; Iravani et al., 1999). Additionally, MK-801 was shown to inhibit protein synthesis, an effect that does not appear to be related with NMDAR inhibition (Charriaut-Marlangue et al., 1994). Thus, considering that MK-801 and memantine might interact with multiple targets, it remains unclear whether the effects on melanoma cells are mediated by NMDAR inhibition, in particular due to the lack of effect of APV. On the other hand, the absence of a cytotoxic effect of NBQX suggests that AMPAR and KAR inhibition possibly does not affect melanoma cell viability.

The cell viability assay with trypan blue staining revealed that MK-801 and memantine decrease the number of viable cells, without inducing cell death (Fig. 5.2). Moreover, the evaluation of LDH activity in the supernatant of melanoma cells (Fig. 5.3A) and the BrdU incorporation assay pointed out that MK-801 and memantine do not induce cancer cell death, but instead they inhibit cell proliferation (Fig. 5.3B). Our results correlate with a recent study that has demonstrated that MK-801 inhibits the proliferation of the human metastatic melanoma cell line WM451, and that it can also reduce melanoma cell motility and invasion (Song et al., 2012). As metastatic malignant melanoma is largely refractory to existing therapies and has a very poor prognosis, the combined cytostatic and antimigration activity of MK-801 may suggest that it is a promising drug for melanoma treatment. Moreover, MK-801 was shown to inhibit the cell growth of other tumor cell lines and to have an antitumoral effect on animal models of melanoma (Song et al., 2012), neuroblastoma and rhabdomyosarcoma (Stepulak et al., 2005), lung (Stepulak et al., 2005; North et al., 2010b) and breast cancer (North et al., 2010a).

Considering the complex machinery involved in the onset and progression of malignant melanoma, the use of combination of drugs may provide an effective strategy to increase the therapeutic benefit (Herlyn, 2009; Ko and Fisher, 2011). Therefore, the effects of MK-801 were also investigated in combination with the antiestrogens TAM, OHTAM and EDX. Our results show, for the first time, that melanoma cell treatment with the NMDAR antagonist MK-801 combined with antiestrogens strongly reduces melanoma cell

biomass in a co-operative manner when compared with the effect induced by the compounds individually (Fig. 5.4). Likewise, the assessment of cell viability with trypan blue staining revealed that the combined treatment of MK-801 with antiestrogens induces a larger decrease in the number of viable cells, without increasing the number of dead cells (Fig. 5.5), suggesting that the observed cytotoxic effect of the combinations of MK-801 with antiestrogens are due to decreased cell proliferation. Indeed, the evaluation of LDH activity in the supernatant of melanoma cells confirmed that the decrease in viable cells is not due to increased cell death (Fig. 5.6), whereas the BrdU incorporation assay pointed out that MK-801 and the antiestrogens inhibit cell proliferation with maximal efficacy when the drugs are used in combination (Fig. 5.7). Moreover, the analysis of the cell cycle revealed that the combination of MK-801 with TAM metabolites, OHTAM or EDX, induce cell cycle arrest in G1 (Fig. 5.8). These results are in line with other studies that have shown that GluR antagonists co-operate with other cytostatic drugs, such as cyclophosphamide, thiotepa (Rzeski et al., 2001) and docetaxel (Haas et al., 2007) enhancing the antiproliferative action.

According to our previous studies (Ribeiro et al., 2013a), TAM active metabolites were more effective than TAM in the inhibition of the proliferation of melanoma cells, either individually or in combination. As recent studies established that the CYP2D6 phenotype is an important predictor of treatment outcome (Lammers et al., 2010) and that the coadministration of CYP2D6-inhibiting medication diminishes the treatment effect of TAM (Kelly et al., 2010), the use of TAM metabolites instead of the prodrug may increase the therapeutic benefit. Importantly, the use of MK-801 in a combined therapy might allow achieving an antitumoral effect with a lower dose than that necessary if the compound would be used in a monotherapy regimen, thus increasing the possibility of using MK-801 in a chronic treatment, without major side effects. In fact, *in vivo* studies have revealed that doses of MK-801 that were able to slow breast (two daily doses of 0.3 mg/kg), melanoma (0.6 mg/kg every three days) and lung (up to 0.3 mg/kg) cancer progression were devoid of significant side effects (Stepulak et al., 2005; North et al., 2010a, b; Song et al., 2012). Noteworthy, the chronic exposure to MK-801 at concentrations up to 1.0 mg/kg was well tolerated by juvenile rhesus monkeys (Popke et al., 2002), suggesting that MK-801 might be suitable as a drug for cancer therapy. Nevertheless, others have found that these doses might influence rodents behavior (Gilbert, 1988; Tricklebank, 1989; Kawabe et al., 1998) and thus,

the MK-801 dose and the duration of treatment necessary to achieve a maximal effect on cancer proliferation without major side effects has yet to be determined.

In conclusion, we report that the NMDAR channel blocker MK-801 decreases melanoma cell proliferation and that its therapeutic potential is greatly enhanced when used in combination with antiestrogenic compounds, particularly with the active metabolites of TAM. Altogether, these data suggest that the NMDAR antagonist MK-801 may have therapeutic potential as an anticancer agent, particularly in therapeutic association.

CHAPTER VI

THE ACTIVATION OF THE G PROTEIN-COUPLED RECEPTOR GPR30 INHIBITS THE PROLIFERATION OF MELANOMA CELLS

Unpublished results

Abstract

The activation of the G protein-coupled receptor 30 (GPR30) (also termed G protein-coupled estrogen receptor 1) by its specific agonist G-1 inhibits prostate cancer and 17β-estradiol-stimulated breast cancer cell proliferation. In addition to 17β-estradiol and G-1, the antiestrogen tamoxifen (TAM) and one of its active metabolites 4-hydroxytamoxifen (OHTAM), are also known to activate the GPR30. TAM decreases melanoma cell proliferation and migration, but its mechanism of action in this malignancy and its-inclusion in melanoma therapeutic regimens remains controversial. Therefore, we investigated the expression of GPR30 in K1735-M2 melanoma cells and the effects of G-1 and the antiestrogens TAM, OHTAM, as well as the other key metabolite of TAM, endoxifen (EDX), on melanoma cell proliferation to clarify the role of GPR30 in the antiproliferative activity of antiestrogens in melanoma. Our data demonstrate that melanoma cells expressed GPR30 and that the selective agonist G-1 markedly reduced melanoma cell biomass and the number of viable cells without increasing cell death. Rather, G-1 decreased cell division, as shown by the decrease in DNA synthesis and by the cell cycle arrest in G2 phase. Likewise, the antiestrogens TAM, OHTAM and EDX exhibited an antiproliferative activity in melanoma cells due to decreased cell division. Moreover, G-1 and the antiestrogens showed a trend to decrease the levels of phosphorylated extracellular signal-regulated kinases (ERK) 1/2 after 1 h treatment, but only EDX induced significant effects. Thus, our results identify the GPR30 as a possible new target for the antiestrogens in melanoma and considering that the activation of the GPR30 inhibits the proliferation of melanoma cells, targeting the GPR30 may be an innovative strategy for the clinical management of melanoma.

6.1 - Introduction

Emerging evidences suggest that the G-protein-coupled receptors (GPCRs) play a central role in cancer development and progression (Lee et al., 2008). In fact, several GPCRs are overexpressed in diverse types of cancer tissues (Li et al., 2005) and the interference with GPCRs and their downstream targets thus represents an attractive strategy for cancer treatment. Similarly, some GPCRs have been shown to be involved in tumorigenesis and metastatic progression of melanoma (Lee et al., 2008).

The G protein-coupled receptor 30 (GPR30), also designated G protein-coupled estrogen receptor 1 (GPER1), was identified between 1996-1998 by four different laboratories as a new 17β-estradiol-binding protein structurally distinct from the classical estrogen receptors (ER) (Wang et al., 2010). It was demonstrated that the GPR30 mediates the proliferative effects of estrogen on some cancer cells (Vivacqua et al., 2006a; Albanito et al., 2007) and that its cellular activation involves the transactivation of epidermal growth factor receptor via a G protein-dependent pathway as well as the activation of adenylate cyclase, the mitogen activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) signaling pathways (Filardo et al., 2000, 2002; Ge et al., 2013). The identification of G-1, the first GPR30-selective agonist (Bologa et al., 2006), with no notable activity towards ERα and on 25 other important GPCRs (Prossnitz and Barton, 2011), provided an important tool for studying the GPR30-mediated signaling. It was reported that G-1 decreases endothelial and urothelial cells proliferation (Teng et al., 2008; Holm et al., 2011). Moreover, G-1 inhibits the proliferation of prostate cancer (Chan et al., 2010) and 17β-estradiol-stimulated breast cancer cells (Ariazi et al., 2010). The expression of GPR30 was also reported in ovarian, endometrial and thyroid cancer cells, but its activation has been shown to increase cell proliferation (Vivacqua et al., 2006a; Fujiwara et al., 2012; Du et al., 2012), suggesting that the effects resulting from the activation of the GPR30 may vary according to the cell type.

Besides the specific agonist G-1 and 17β-estradiol, the selective ER modulators tamoxifen (TAM) and 4-hydroxytamoxifen (OHTAM) have also been shown to behave as agonists for GPR30 (Maggiolini et al., 2004; Thomas et al., 2005; Vivacqua et al., 2006a; Pandey et al., 2009). TAM inhibits melanoma cell proliferation (Gill et al., 1984; Piantelli et al., 1995; Lama et al., 1998; Kanter-Lewensohn et al., 2000), invasion and metastasis

(Matsuoka et al., 2009) and it has been used in the clinical management of melanoma as a single agent or, more often, in combination with other chemotherapeutic agents (Cocconi et al., 1992; McClay et al., 1993). However, the mechanisms underlying the antitumor activity of TAM in melanoma are poorly understood and the clinical trials performed to clarify the benefit of TAM in therapeutic regimens for advanced and metastatic melanoma have produced contradictory results (Lens, 2003; Beguerie et al., 2010).

Based on these findings, we investigated the expression of GPR30 and the effects of GPR30 ligands on the proliferation of a highly invasive melanoma cell line (K1735-M2) and the signaling pathways involved. We show, for the first time, that K1735-M2 cells express GPR30 and that G-1 decreases melanoma cell proliferation due to decreased cell division, similarly to TAM and its active metabolites, OHTAM and endoxifen (EDX). Noteworthy, the TAM active metabolites are more potent that the prodrug in the inhibition of proliferation of melanoma cells and EDX was the most powerful compound regarding the decrease in extracellular signal-regulated kinases (ERK) 1/2 activation. Therefore, our results support the view that the targeting of GPR30 may be an innovative and powerful strategy in malignant melanoma therapy and lay the foundation for future development of GPR30-based therapies for melanoma.

6.2 - Materials and Methods

Reagents

G-1, TAM, OHTAM and EDX were obtained from SIGMA-Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and antibiotic/antimycotic solution (10 000 units penicillin, 10 mg streptomycin, 25 µg amphotericin B per mL) were purchased from SIGMA-Aldrich (St Louis, MO, USA), Fetal Bovine Serum (FBS) and trypsin were obtained from Gibco, Invitrogen Life Technologies (Carlsbad, California, USA). All of the other chemicals were purchased from SIGMA-Aldrich (St Louis, MO, USA) and were of the highest grade of purity commercially available. TAM and OHTAM stock solutions were prepared in absolute ethanol. G-1 and EDX were kept in dimethyl sulfoxide (DMSO). Primary specific rabbit polyclonal antibody to GPR30 and anti-mouse and anti-rabbit IgG secondary antibodies were obtained from Abcam (Cambridge, UK); primary specific rabbit polyclonal antibodies to phospho-Akt, total-Akt and phospho-ERK 1/2 were obtained from

Cell Signaling Techonology (MA, USA); primary specific rabbit polyclonal antibody to total-ERK 1/2 was purchased from Millipore (MA, USA); mouse monoclonal antibody to β-actin was purchased from SIGMA-Aldrich (St Louis, MO, USA).

Cell culture

K1735-M2 mouse melanoma cells (kindly offered by Dr. Paulo Oliveira, Center for Neurosciences and Cell Biology, Department of Zoology, University of Coimbra, Coimbra, Portugal) were cultured in DMEM, supplemented with 10 % heat-inactivated FBS and 1 % antibiotic/antimycotic solution, and kept in a humidified incubator with 5 % CO_2/95 % air, at 37 ºC. Cells were plated 6.1×10^4 cells/cm^2. The drugs were added to cells 24 h after plating. The volumes added did not exceed 0.5 % (v/v). Vehicle controls were performed.

Western-blot (WB) analysis

Protein expression was evaluated by WB analysis. For the preparation of total cell extracts, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and placed on lysis buffer, containing 50 mM 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 1 % Triton X-100, 0.1 % sodium dodecyl sulfate (SDS), 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaF, 2 mM Na_3VO_4, 1 mM dithiothreitol (DTT), pH 7.4, supplemented with 1 mM phenylmethanesulphonyl fluoride (PMSF) and a cocktail of protease inhibitors (P8340, Sigma). Extracts were submitted to three freeze/thaw cycles and centrifuged at 14 000 rpm for 15 min at 4 ºC. Supernatants were recovered and the protein quantified by the bicinchoninic acid (BCA) assay kit (Pierce, as part of Thermo Fisher Scientific, IL, USA). Equal amounts of protein from total cell extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using polyacrylamide gels of 10 % and then transferred into a polyvinyldiene fluoride (PVDF) membrane. The membranes were blocked with tris-buffered saline (TBS)-T (20 mM Tris-HCl, 150 mM NaCl, 0.1 % Tween) with 5 % nonfat milk, for 1 h at room temperature, and then incubated overnight at 4 ºC with the primary antibody in TBS-T with 5 % nonfat milk. After extensive washing, membranes were incubated with the secondary antibody conjugated with alkaline phosphatase for 1 h at room temperature. After additional washes, the membranes were developed using the enhanced chemifluorescence (ECF) substrate, and scanned on the Typhoon 9000 scanner

(Amersham Biosciences). Appropriate controls were used to ensure equal protein loading as indicated in the figure legends. The bands were analyzed using the ImageQuant TM software from Amersham Biosciences. For subsequent reprobing, the membranes were stripped of antibody with 0.2 M NaOH, blocked again and incubated with the appropriate antibodies.

Sulforhodamine B (SRB) assay

The cytotoxic effects induced by the drugs on melanoma cells were assessed by the SRB assay (Holy et al., 2006). At designated time points, the cultures were fixed with absolute methanol containing 1 % acetic acid, and stored at −20 ºC overnight. The methanol was decanted and the plate air-dried. The labeling solution, containing 0.5 % SRB in 1 % acetic acid, was added to each well and the plate incubated for 1 h, at 37 °C. Plates were rinsed with 1 % acetic acid, air-dried, and the bound dye eluted with 10 mM Tris buffer, pH 10. The absorbance was measured in a Synergy HT plate reader at 540 nm. As SRB binds to basic amino acid of proteins of adherent cells, the absorbance at 540 nm provides an estimate of total protein mass (biomass), which is related to cell number. The absorbance obtained in control cultures was considered 100 %. The number of independent experiments is indicated in figure legends.

Cell viability assessment by trypan blue dye exclusion

The effect of drugs on cell viability was investigated by staining cell suspensions with trypan blue, based on the principle that viable cells with an intact membrane exclude the dye, while dead cells present a blue stained cytoplasm (Houben et al., 2009). After 72 h of incubation with the drugs, cells were trypsinized, centrifuged and the pellet ressuspended in 0.4 % trypan blue for 2-3 min and then cells were counted in a hemocytometer under a transmitted light microscope. The experiments were carried out in duplicate for each independent experiment.

Lactate dehydrogenase (LDH) assay

The LDH assay allows to determine the cytotoxic effects of drugs based on the release of this cytosolic enzyme into the extracellular medium following the loss of cellular membrane integrity (Vieira et al., 2010). The culture medium was collected after 72 h of incubation with the drugs and centrifuged at 14 000 rpm for 10 min at 4 ºC. An aliquot of

supernatant (100 µL) was collected and incubated with substrate mixture, containing 40 µM lactate in perchloric acid 3 %, and 3.6 mM nicotinamide adenine dinucleotide (NAD^+), in tris-hidrazine buffer (80 mM tris, 400 mM hydrazine, 5 mM EDTA, pH 9.5). The enzymatic reaction that occurs leads to the reduction of NAD^+ to NADH by oxidation of lactate to pyruvate. Thus, the amount of NADH is directly related to LDH activity in the supernatant and can be evaluated by measuring the absorbance of NADH at 340 nm. The LDH activity is expressed as the ratio between the LDH activity in the extracellular medium and the total LDH activity obtained from the supernatant of cells lysed with Triton X-100, which was considered 100 %. Experiments were performed in duplicates for each independent experiment.

5-bromo-2'-deoxyuridine (BrdU) incorporation assay

K1735-M2 cell proliferation was evaluated by measuring BrdU incorporation during DNA synthesis in proliferating cells, using the Cell Proliferation ELISA, BrdU, colorimetric kit (Roche). After 48 h of incubation with the drugs, cultured cells were placed in BrdU-labeling solution for 90 min. Afterwards the cells were fixed and the DNA denaturated with the FixDenat solution provided with the kit and then incubated with a monoclonal antibody conjugated with peroxidase (anti-BrdU-POD), which binds to the BrdU in the newly synthesized DNA. The complexes formed were detected by using the 3,3',5,5'-tetramethylbenzidine (TMB) substrate and quantified by measuring the absorbance in a Synergy HT plate reader at 370 nm. The absorbance values directly correlate to the amount of DNA synthesis and, therefore, to the number of proliferating cells. The experiments were carried out in triplicate for each independent experiment and the absorbance obtained in control cultures was considered 100 %.

Cell cycle analysis by flow cytometry

The effects of G-1 and the antiestrogens on cell cycle were monitored by flow cytometry (Carmo et al., 2011). Cells were plated in 6-multiwell plates and, after 48 h of incubation with the drugs, cells were trypsinized and centrifuged at 1 500 rpm for 10 min. Afterwards, the culture medium was discarded and the pellet was fixed overnight at 4 ºC with a solution of cold 70 % ethanol. The cells were then centrifuged at 1 500 rpm for 10 min, the pellet was resuspended in a solution of PBS containing RNAse and, after 45 min,

propidium iodide was added and cells were further incubated for 1 h in the dark, at room temperature (the final concentrations of RNase and propidium iodide were 10 µg/mL and 20 µg/mL, respectively). The propidium iodide fluorescence was measured on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488 nm argon-ion laser. For aggregate/debris discrimination, in addition to propidium iodide fluorescence signal heights, areas and widths were also measured. Measurements for at least 20 000 events were collected per sample. Data were analyzed using the ModFit LT 3.0. software. The experiments were carried out in duplicate for each independent experiment and the results are expressed as % of total cells.

Statistical analysis

Results are presented as the mean ± S.E.M. of the indicated number of independent experiments. Statistical significance between the different assays was determined using the one-way analysis of variance (ANOVA), followed by the Tukey post-test, for multiple comparisons. A p value <0.05 was considered statistically significant. These statistical analyses were performed using the software package GraphPad Prism 5.

6.3 - Results

In order to investigate the presence of GPR30 in melanoma cells, we have determined the GPR30 protein expression by means of WB analysis. As shown in figure 6.1, K1735-M2 cell line express GPR30. For positive control, MCF-7 cells, which are known to express the receptor (Filardo et al., 2000; Ariazi et al., 2010), were used (Fig. 6.1). β-actin antibody was used to ensure equal amount of protein loading.

To examine the effects of GPR30 activation in melanoma, K1735-M2 cells were treated for 72 h with G-1, a specific agonist for GPR30, and cell biomass was determined by using the SRB assay during a time-course experiment (Fig. 6.2A). After 24 h of incubation, the cell biomass was not significantly changed by G-1 up to 1 µM, but above 2.5 µM a significant decreased was observed (Fig. 6.2A). When cells were grown in the presence of G-1 for 48 and 72 h, the cell biomass was significantly decreased in the presence of 1 µM; for G-1 concentrations of 2.5 µM to 20 µM the effects were more pronounced but

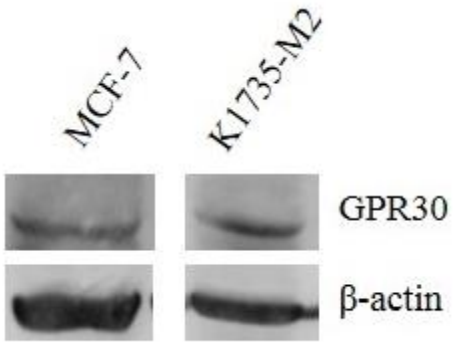


Fig. 6.1 - GPR30 is expressed in melanoma cells. 80 µg of total protein were used for immunoblot analysis with an anti-GPR30 antibody. The membrane was reprobed with a β-actin antibody to confirm an equal amount of protein loading in each gel lane. MCF-7 cell lysate was used as positive control. The experiments were performed in 3 independent preparations.

concentration-independent (Fig. 6.2A). These results indicate that the activation of GPR30 may mediate an inhibitory effect on melanoma cells proliferation. Thus, the effects of antiestrogenic compounds, TAM and OHTAM, which bind to GPR30 (Wang et al., 2010), were also monitored on melanoma cells (Figs. 6.2B and 6.2C). After 24, 48 and 72 h of incubation, TAM below 7.5 μM did not affect cell biomass, but at concentrations ≥ 7.5 μM a significant decrease was observed in comparison with the control condition (Fig. 6.2B). The treatment of melanoma cells with OHTAM up to 5 μM for 24 h did not change cell biomass, and at 7.5 and 10 μM significant cytotoxicity was observed; after 48 and 72 h of incubation, OHTAM at concentrations ≥ 5 μM significantly decreased cell biomass (Fig. 6.2C). Additionally, considering that EDX plays a key role in the antitumor activity of TAM in breast cancer, its possible cytotoxic effects on melanoma cells were also investigated (Fig. 6.2D). The cell biomass was unaffected by treatment with EDX up to 5 μM within 48 h, however, after 72 h of incubation a significant decrease in the cell biomass was observed; EDX above 7.5 μM induced a significant reduction in the cell biomass at each time point (Fig. 6.2D). Since 5 μM was the lowest concentration of TAM active metabolites that induced a significant decrease in melanoma cell biomass, that was the selected concentration to pursue our work and for further comparisons with the selective agonist G-1.

To elucidate the mechanism underlying the cytotoxic effects of G-1 and the antiestrogens TAM, OHTAM and EDX, we assayed cell viability after 72 h of incubation with the drugs at 5 μM by using the trypan blue dye exclusion assay (Fig. 6.3). G-1 at 5 μM significantly decreased the number of viable cells (Fig. 6.3). The number of viable cells was not significantly affected by TAM, whereas a significant decrease was observed after incubation with either OHTAM or EDX (Fig. 6. 3), in agreement with the results obtained with the SRB assay (Figs. 6.2B-6.2D). Moreover, at this concentration, the compounds tested did not increase the number of dead cells during the course of 72 h (Fig. 6.3).

The absence of an increase in cell death within 72 h of treatment with the drugs was confirmed by the LDH assay (Fig. 6.4) which, as the trypan blue assay, is a cytotoxicity test based on cell membrane integrity. Neither G-1 nor the antiestrogens TAM, OHTAM and EDX at 5 μM increased the activity of LDH in the extracellular medium of melanoma cells (Fig. 6.4). Thus, our results indicate that the cytotoxic effects of G-1 and of the antiestrogens on melanoma cells are not related with increased cell death.

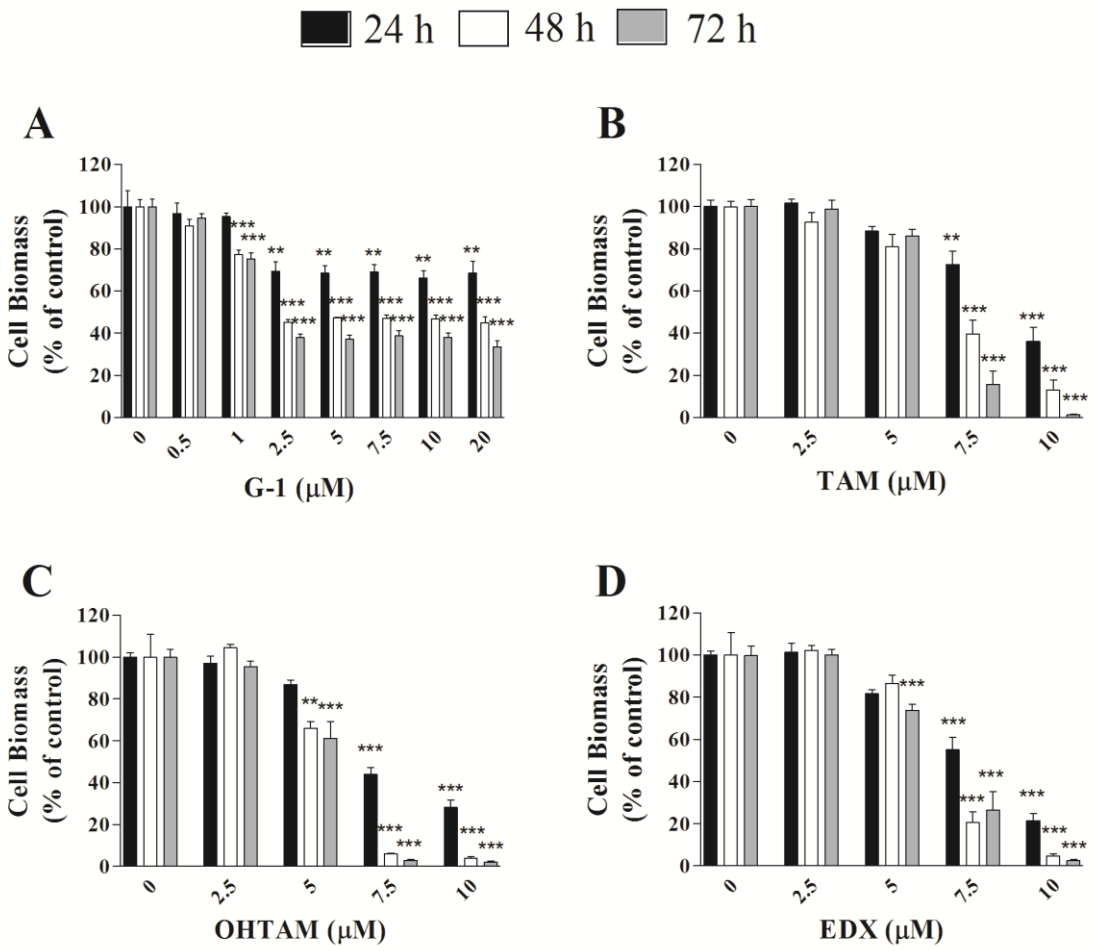


Fig. 6.2 – G-1 and the antiestrogens decrease melanoma cell biomass. Cells were incubated in the absence (0) or in the presence of G-1 (A), and the antiestrogens TAM (B), OHTAM (C) and EDX (D). At the selected time points, melanoma cell biomass was evaluated by the SRB assay as described in the Materials and methods section. Bars represent the mean ± S.E.M. of three independent experiments performed in triplicates. *** $p<0.001$, ** $p<0.01$ vs the respective time point control, One-way ANOVA followed by Tukey post-test.

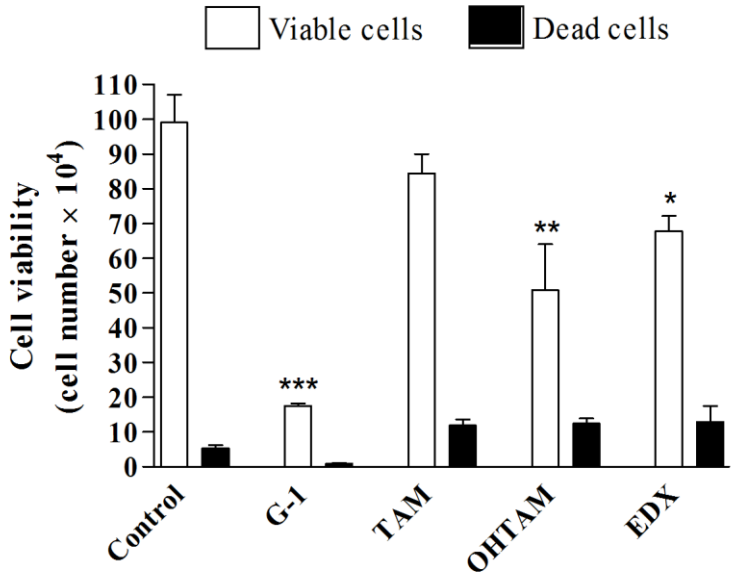


Fig. 6.3 - Cell viability of melanoma cells treated with G-1 and the antiestrogens. Melanoma cells were grown for 72 h in the absence (control) or in the presence of 5 μM of G-1, TAM, OHTAM or EDX and after the incubation period the melanoma cell viability was determined by the trypan blue dye exclusion assay. The number of trypan blue-negative (viable) cells and trypan blue-positive (dead) cells is presented. Data represent the mean ± S.E.M. of three independent experiments performed in duplicates. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ vs control, One-way ANOVA followed by Tukey post-test.

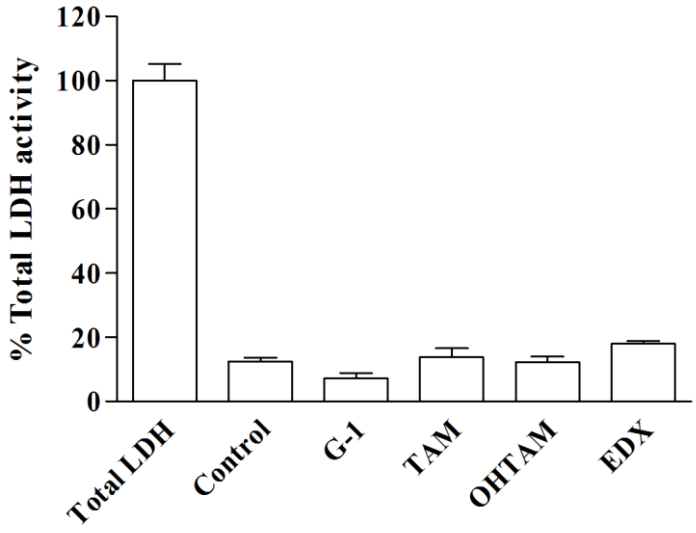


Fig. 6.4 – The GPR30 selective agonist G-1 and the antiestrogens do not induce melanoma cell death. Melanoma cells were grown in the absence (control) or in the presence of 5 µM of G-1, TAM, OHTAM or EDX. Cell death was assessed by measuring LDH release from damaged cells after 72 h in culture. Bars represent the mean ± S.E.M. of three independent experiments performed in duplicates.

The effects of the drugs on melanoma cell proliferation were then investigated by means of the BrdU incorporation in DNA synthesis after a 48 h treatment with 5 µM of G-1 or with 5 µM of the antiestrogenic compounds TAM, OHTAM and EDX (Fig. 6.5). This earlier time point was selected as the number of cells in control condition at 72 h is substantially high and could lead to absorbance values beyond the acceptable measuring range. As shown in figure 6.5, G-1 at 5 µM significantly diminished BrdU incorporation to about 42 % relative to control, whereas OHTAM and EDX diminished it to approximately 52 % and 59 %, respectively (Fig. 6.5). On the contrary, TAM at 5 µM decreased BrdU incorporation to about 80 %, which was not significant in comparison with control (Fig. 6.5). Thus, our results indicate that the cytotoxic effects induced by this concentration of G-1 and TAM metabolites on melanoma cells might be due to a decrease in cell proliferation.

Therefore, we analyzed the effect of the drugs on the cell cycle by flow cytometry (Fig. 6.6). The cell cycle of untreated cells (control) was characterized by a long and sharp G1 peak, a slightly prominent S phase, a least prominent G2 peak and a low apoptotic fraction (Fig. 6.6). The cells incubated with 5 µM of G-1 had a markedly diminished percentage of cells in the S phase, whereas a significant increase in G2 was observed (Fig. 6.6). In contrast, at this time point the antiestrogens at 5 µM did not significantly affect the cell cycle (Fig. 6.6).

Given that the MAPK and the PI3K pathways are major pathways implicated in melanoma cell proliferation and survival (Ko and Fisher, 2011), which can be activated downstream of GPR30 (Maggiolini and Picard, 2010), the effects of 5 µM of G-1, TAM and EDX on the phosphorylation of ERK 1/2 and Akt after 1 h of drug addition were evaluated (Fig. 6.7). The compounds decreased the phosphorylation of ERK, but only the effects of EDX were significant at this early time point (Figs. 6.7A and 6.7B). In contrast, no significant effects on the phosphorylation of Akt were detected at this time point (Figs. 6.7A and 6.7C).

6.4 - Discussion

The expression of GPR30 was demonstrated in human tumor tissues or cancer cell lines, including human seminoma (Chevalier et al., 2012), lung (Jala et al., 2012),

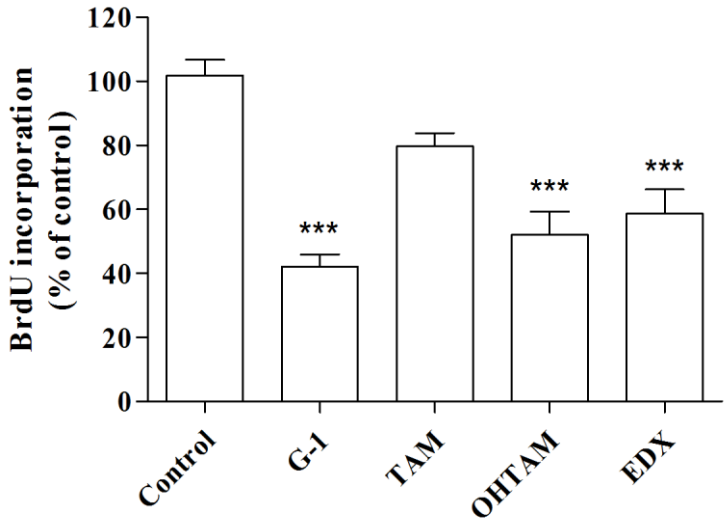


Fig. 6.5 – G-1 and the antiestrogens decrease melanoma cell proliferation. Melanoma cells were grown in the absence (control) or in the presence of 5 μM of G-1, TAM, OHTAM or EDX. After 48 h, cell proliferation was assessed by the BrdU incorporation assay as described in the Materials and methods section. Bars represent the mean ± S.E.M. of four independent experiments performed in triplicates. *** $p<0.001$ vs control, One-way ANOVA followed by Tukey post-test.

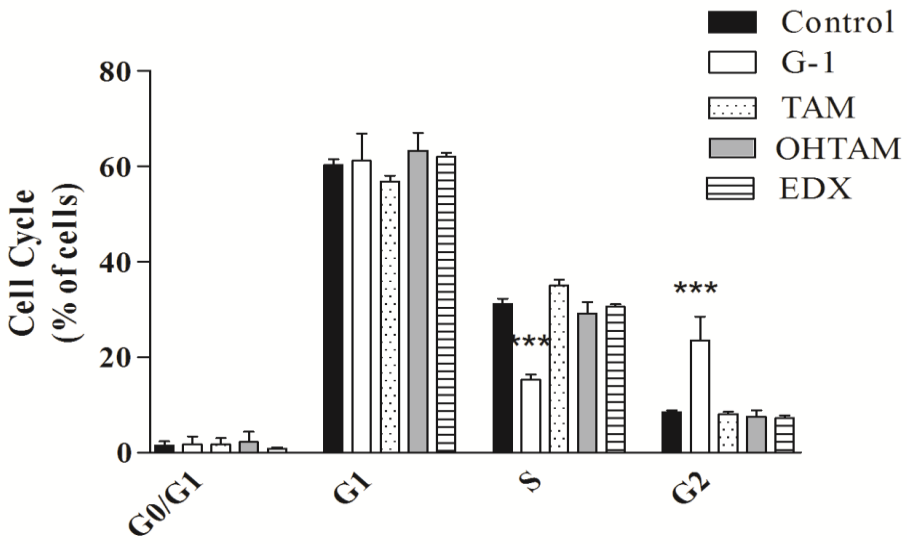


Fig. 6.6 - G-1 blocks cell cycle progression in G2. Melanoma cells were grown in the absence (control) or in the presence of 5 µM of G-1, TAM, OHTAM and EDX. Cell cycle distribution was evaluated by flow cytometry analysis of the DNA content labeled with propidium iodide after 48 h. A total of 20 000 events were analyzed for each experiment. Data represent the mean ± S.E.M. of three independent experiments performed in duplicates. *** $p<0.001$ vs control, One-way ANOVA followed by Tukey post-test.

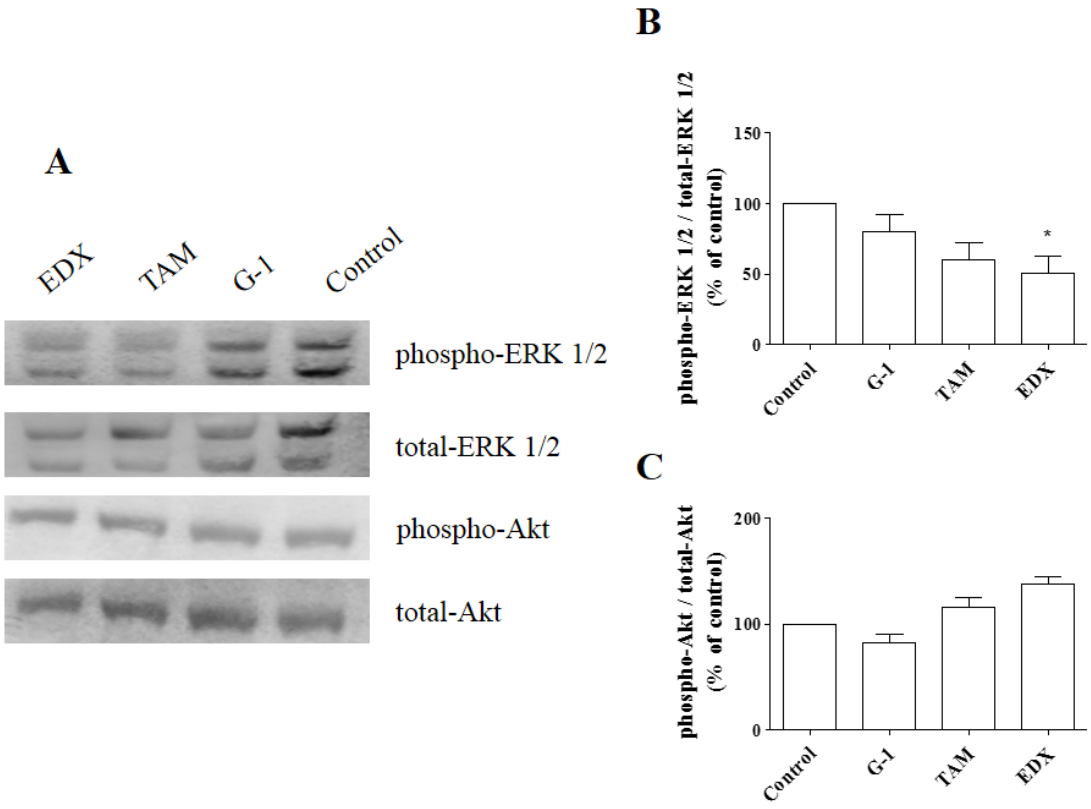


Fig. 6.7 - Effects of G-1 and the antiestrogens on ERK 1/2 and Akt phosphorylation. Melanoma cells were incubated for 1 h in the absence (control) or in the presence of G-1, TAM and EDX at 5 µM and total extracts were prepared. Sixty µg of total protein was used for immunoblot analysis with phospho-ERK 1/2 and phospho-Akt antibodies. The membrane was reprobed with total-ERK 1/2 and total-Akt antibodies. Representative blots (A) and relative densitometric values of ERK 1/2 phosphorylation (B) and Akt phosphorylation (C) are shown and were normalized to total-ERK 1/2 and total-Akt, respectively, and expressed as percentage of control. Values are the mean ± S.E.M. of nine experiments. * $p<0.05$ vs control, One-way ANOVA followed by Tukey post-test.

endometrial (Du et al., 2012), ovarian (Fujiwara et al., 2012) and breast cancer (Filardo et al., 2000, 2006). The GPR30 is also present in normal human skin fibroblast cells (Tsui et al., 2011), but whether melanoma cells express GPR30 or whether it has a role in this malignancy is not yet clarified.

Our studies show, for the first time, that melanoma cells express GPR30 (Fig. 6.1) and that its activation by G-1, a selective agonist of GPR30 (Bologa et al., 2006), induces profound cytotoxic effects on melanoma cells, at concentrations as low as 1 μM, as shown by the SRB assay (Fig. 6.2A). Increasing the concentration of G-1 from 2.5 up to 20 μM did not enhance the cytotoxic effect, which is in agreement with the saturation of the receptor. The cell viability assay with trypan blue staining revealed that G-1 decreases the number of viable cells, without increasing the number of dead cells (Fig. 6.3). Accordingly, the evaluation of LDH activity in the supernatant of cells (Fig. 6.4) point out that G-1 does not induce melanoma cell death, but instead inhibits cell proliferation, as suggested by the BrdU incorporation assay (Fig. 6.5). The cell cycle analysis by flow cytometry supports these findings, as G-1 induces cell cycle arrest in G2 (Fig. 6.6). These results are in agreement with data from the literature showing that G-1 decreases endothelial cell proliferation by accumulating cells in the S and G2 phases of the cell cycle (Holm et al., 2011), and diminishes DNA synthesis in urothelial cells (Teng et al., 2008). G-1 also inhibits prostate cancer cells proliferation and induces cell-cycle arrest in G2 (Chan et al., 2010), whereas it promotes the blockade in G1 phase in 17β-estradiol-stimulated breast cancer cells (Ariazi et al., 2010).

Therefore, our results suggest that the GPR30 is a new target in melanoma therapy and that the evaluation of GPR30 expression in human melanoma tissues will allow the identification of subgroups of patients who may benefit from a GPR30-based therapy. Noteworthy, the treatment with G-1 was found to have minimal or no effects on normal prostatic epithelial cells and on benign prostate epithelial cells (Chan et al., 2010) and in an animal model of multiple sclerosis, G-1 exerted beneficial effects without estradiol-associated side effects (Wang et al., 2009), suggesting that GPR30-based therapies may be well tolerated and have little harmful effects on patients normal tissues.

Importantly, like the selective agonist G-1, TAM and OHTAM, which bind to GPR30 (Wang et al., 2010), can also decrease melanoma cell biomass (Figs. 6.2B and 6.2C). In addition, our results show that EDX resembles OHTAM in the ability to decrease

melanoma cell biomass (Fig. 6.2D), suggesting that EDX might bind to GPR30 as well. Similarly to what was observed with G-1, the cytotoxic effects induced by TAM metabolites at 5 μM are not related with cell death, as demonstrated by the absence of an increase in the number of dead cells and in the LDH activity in the supernatant of cells (Figs. 6.3 and 6.4). Rather, and in agreement with our previous studies (Ribeiro et al., 2013a, c), TAM metabolites decrease BrdU incorporation (Fig. 6.5), indicating that TAM metabolites decrease cell division, similarly to G-1. These observations suggest that the activation of the GPR30 is a new mechanism that may contribute to the anticancer activity of antiestrogenic compounds in melanoma.

In contrast, the activation of GPR30 in endometrial and thyroid cancer has been shown to stimulate cell proliferation (He el al., 2009; Vivacqua et al., 2006a) suggesting that the effects mediated by GPR30 may vary according to the cell type. These results parallel with the observation that TAM behaves as an antiestrogen in breast cancer cells, but has mild estrogenic effects in other tissues, such as the uterus and the bone, and the cardiovascular system (McDonnell, 1999). Therefore, the factors that modulate the GPR30-mediated signaling in different tissues are worthy of further investigation.

Noteworthy, TAM active metabolites are more potent than the prodrug, inducing significant effects on cell proliferation at lower concentrations (Figs. 6.2, 6.3 and 6.5). These findings correlate with the reported superior anticancer activity of TAM metabolites in breast cancer (Lim et al., 2005; Wu et al., 2009) and suggest that the individual variability in TAM metabolism and the coadministration of CYP2D6-inhibiting medication may influence the efficacy of TAM in melanoma, as already demonstrated in breast cancer (Higgins and Stearns, 2010; Kelly et al., 2010; Lammers et al., 2010; Thompson et al., 2011). In fact, the many studies conducted until now with the goal of establishing the benefits of the inclusion of TAM in melanoma therapeutic regimens provided contradictory results (McClay et al., 1989, 1992; Cocconi et al., 1992; Rusthoven et al., 1996). Previous studies performed in our laboratory have supported the view that EDX is an effective alternative to TAM, providing superior anticancer activity than the prodrug when used in combination with other agents (Ribeiro et al., 2013a, c). The impact of the variability in TAM metabolism in the melanoma treatment efficacy is unknown at the moment, but can contribute to explain the discrepant outcomes observed in melanoma patients taking TAM and should be taken into consideration in future trials. However, we can not exclude that a possible variability in

GPR30 expression in melanoma cells might contribute as well to the discrepant outcomes regarding TAM therapy, should this drug act through the activation of this receptor.

To provide further insights on the antiproliferative mechanisms of the drugs under investigation, the effects of G-1, TAM and EDX on the phosphorylation of ERK 1/2 and Akt, which are key signaling pathways in melanoma, were also evaluated. ERK is constitutively activated in up to 90 % of melanomas (Russo et al., 2009), whereas phosphorylated Akt is detected in 49 % and 77 % of the biopsies of primary melanoma and melanoma metastases (Dai et al., 2005). The compounds decreased ERK 1/2 phosphorylation, but after 1 h treatment, only EDX was able to provide a significant reduction of ERK 1/2 activation (Fig. 6.7B). Strikingly, G-1, which is the most potent compound regarding the inhibition of melanoma cell proliferation (Figs. 6.2, 6.3, 6.5 and 6.6) is the least effective in the inhibition of ERK 1/2 activation (Fig. 6.7B). As it was reported that TAM inhibits melanoma cell migration and invasion by suppressing the phosphorylation of ERK 1/2 and Akt through the inhibition of protein kinase C (PKC) phosphorylation (Matsuoka et al., 2009), these differences may be related with effects promoted by the antiestrogens on targets other than the GPR30. In fact, the mechanisms underlying the antitumor activity of TAM in melanoma are not entirely clarified, but both genomic and nongenomic mechanisms were proposed. Earlier investigations detected the presence of ERs (Fisher et al., 1976; Walker et al., 1987), but further studies using ER-specific monoclonal antibodies have failed to support this hypothesis (Cohen et al., 1990; Lecavalier et al., 1990; Kanter-Lewensohn et al., 2000). More recently, methylated ERα were detected in the tumor tissues and sera of melanoma patients resulting in ERα gene silencing (Mori et al., 2006). On the other hand, the inactivation of the insulin-like growth factor-1 receptor (IGF-1R) was also reported as a possible mechanism of TAM-induced cytotoxicity in melanoma cells (Kanter-Lewensohn et al., 2000). The multiple actions promoted by the antiestrogens in melanoma cells may contribute to explain the absence of a significant effect on cell cycle at the time point selected, as these drugs induce effects on multiple targets rather than a single one, as occurs with the selective agonist G-1 (Fig. 6.6). No significant effects were detected on Akt activation (Figs. 6.7A and 6.7B), but further studies at different time points are needed.

A great concern regarding melanoma therapy is the development of resistance, given the great plasticity of melanoma cells that can rewire their signaling circuitries,

allowing the adaptation of tumor cells to pharmacological challenges (Herlyn, 2009; Villanueva et al., 2010). The development of resistance can be surpassed by the combination with other agents (Villanueva et al., 2010) and our previous studies demonstrate that EDX acts synergistically with glutamate receptor antagonist MK-801 (Ribeiro et al., 2013c) or all-*trans*-retinoic acid (Ribeiro et al., 2013a) to decrease melanoma cells proliferation. Moreover, the combination of EDX with all-*trans*-retinoic acid, which provides antiproliferative and antimigration effects on melanoma cells, was devoid of significant toxicity in non-neoplasic cells and in mitochondrial functions (Ribeiro et al., 2013a, d).

In conclusion, our results suggest that the pharmacological activation of GPR30 may be an appropriate therapy for melanoma treatment and opens up the opportunity for the development of new therapies targeting the GPR30. Indeed, it might have the advantage of exerting beneficial effects without overt side effects (Wang et al., 2009). Furthermore, our results suggest a new possible mechanism of action of antiestrogenic compounds in melanoma cells and can contribute to select patients who may benefit from the inclusion of antiestrogens in their therapeutic regimens against melanoma. As the prognosis for locally advanced and metastatic melanoma is poor and currently available therapies are unable to clearly alter the history of the disease (Ko and Fisher, 2011), the activation of GPR30 represents an innovative strategy for the clinical management of malignant melanoma.

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

7.1 - General discussion

Recently, a systematic review synthesized the safety data from clinical trials to elucidate the adverse events associated with several drugs used in advanced melanoma therapy, including interferon-α, dacarbazine, interleukin-2 and the newly approved ipilimumab and vemurafenib (Ma and Armstrong, 2013). It demonstrated that currently available therapies for the management of malignant melanoma are associated with the induction of severe side effects (Ma and Armstrong, 2013). Therefore, new therapeutic strategies with high efficacy and low toxic effects are required.

As described in chapter II, our results show that EDX, at concentrations much higher than those reached in tissues (Lien et al., 1991; Borges et al., 2006), depresses the phosphorylation efficiency of mitochondria (Figs. 2.1 and 2.2) and promotes membrane permeabilization to protons, whereas TAM induces a much more potent proton leak (Fig. 2.3), indicating that EDX, like OHTAM (Cardoso et al., 2002a), mildly affects membrane integrity, in comparison with TAM. The ability of TAM to unspecifically disrupt the structure of model and native membranes has been implicated in its side effects, including the hemolytic anemia (Cruz Silva et al., 2000). The fact that EDX only compromises membrane integrity at very high concentrations suggests that EDX would present less unspecific effects than the treatment with its prodrug. Such results are in accordance with the demonstration of the absence of significant alterations in hematologic and biochemical parameters, including the cholesterol and triglyceride levels, in individuals taking a daily oral dose of 4 mg of EDX, which provides a similar EDX steady-state concentration to that found in patients with normal CYP2D6 function who are administered 20 mg of TAM/day (Ahmad et al., 2010b). Altogether, our results suggest that EDX is less toxic than TAM to mitochondrial functions and therefore the use of EDX instead of TAM will minimize the adverse effects related with mitochondrial damage, membrane disruption and changes in lipid metabolism induced by TAM.

Furthermore, EDX prevents the MPT induced by Ca^{2+} and phosphate (Figs. 2.4 and 2.5). This protection was observed either when the drug was added before (Figs. 2.4A and 2.5A) or after calcium loading during membrane depolarization (Fig. 2.4B and 2.5B), suggesting a direct interference with the proteins that form the MPT complex and excluding a possible effect on Ca^{2+} uptake across the uniport or other mitochondrial effects. Likewise,

both TAM and OHTAM inhibit the MPT induced by several agents (Custódio et al., 1998; Cardoso et al., 2002b; Hernández-Esquivel et al., 2011a, b). The mechanisms underlying the inhibition of MPT by antiestrogens remain unclear. EDX at concentrations that inhibit the MPT does not compromise the active ANT content (Fig. 2.6) or phosphorylation efficiency (Figs. 2.1 and 2.2), suggesting that neither the ANT nor the phosphate carrier is related with the inhibition of MPT by EDX. The antiestrogen EDX significantly inhibits oxidative stress (Fig. 2.7), which may indicate that the MPT inhibition by EDX is related with its antioxidant properties, since the oxidation state of cysteine residues in the vicinity of the pore is a critical factor regulating MPT induction (Petronilli et al., 1994). Another possible explanation is the interference with membrane fluidity, as described for TAM and OHTAM, which leads to a configurational change of the ANT (Custódio et al., 1991, 1993a, b; Hernández-Esquivel et al., 2011a; Kazanci and Severcan, 2007). The interaction of EDX with CypD, inhibiting the pore assembly similarly to CyA (Zoratti and Szabo, 1995), is also conceivable.

On the other hand, mitochondria, which play a central role in the cellular life or death, are thought to be involved in the pharmacological action of several drugs, including RA. It was reported that RA inhibits the proliferation of both primary and metastatic melanoma cells by reducing mitochondrial viability, which is followed by alterations of the cell cycle distribution and, at higher concentrations, apoptotic cell death (Zhang et al., 2003). A recent study conducted by Xun *et al* (2012) also proposed that the effects of RA on mitochondria may be beneficial in cancer therapy. It was shown that during RA-induced differentiation of neuroblastoma cells there is a dramatic increase in the oxygen consumption rate without changes in the number of mitochondria or in the composition of the electron transport chain (Xun et al., 2012). Through a mechanism yet to be determined, RA treatment enhances the metabolic activity of neuroblastoma cells and the energetic demands in the differentiated state; as the maintenance of differentiated cells takes precedence over tumor cell growth, the availability of nutrients for tumor cells is limited and the tumor growth is supressed (Xun et al., 2012). In addition, the induction of MPT has garnered significant attention as a possible strategy to trigger cancer cell death and retinoids are known inducers of MPT (Rigobello et al., 1999; Fulda et al., 2010).

In spite of being a powerful anticancer agent, the utilization of RA in clinical practice is almost restricted to APL, as RA induces severe side effects (Zusi et al., 2002). Cases of hepatotoxicity were reported during dietary supplementation (Stickel et al., 2011)

or treatment of APL patients (de-Medeiros et al., 1998) with retinoids; hypertriglyceridemia is also common and it was shown to be mediated by RARs (Standeven et al., 1996). As mentioned previously, alterations in lipid metabolism and liver damage are closely related with mitochondrial dysfunction (Begriche et al., 2006; Labbe et al., 2008; Rolo et al., 2012). In fact, besides being an inducer of MPT (Rigobello et al., 1999), RA compromises mitochondrial bioenergetics as well, since it induces uncoupling of mitochondria (Stillwell et al., 1982; Stillwell and Nahmias, 1983). *In vivo* studies supported the view that mitochondria are a target of retinoid toxicity. In rats, vitamin A supplementation induced slight enlargement of mitochondria and a moderate decrease in cytochrome *c* oxidase activity (Leo et al., 1982). In the study conducted by de Oliveira *et al* (2009), short-term vitamin A supplementation of adult rats also induced hepatic oxidative insult and mitochondrial dysfunction. The combination of RA with other agents can possibly allow to achieve therapeutic efficacy with lower concentrations, this way minimizing the adverse effects of retinoid treatment. Indeed, the combinations of RA with TAM demonstrated a synergism of action in breast cancer cells (Wang et al., 2007; Searovic et al., 2009; Koay et al., 2010) and an interplay between retinoid and estrogen signaling was reported (Hua et al., 2009; Ross-Innes et al., 2010), suggesting that these combinations may be useful in breast cancer treatment. However, the combination of RA with TAM, which presents overlapping toxicity pattern, may raise some concerns.

Therefore, understanding the effects of RA on mitochondrial function can contribute to clarify either the pharmacological or the toxicological mechanisms of action of this agent. Furthermore, the evaluation of the effects of RA in combination with TAM on mitochondria can provide relevant mechanistic information regarding the potential toxicity of these combinations. As our results demonstrate that EDX promotes less deleterious effects on mitochondria than TAM (Ribeiro et al., 2013b), the effects of RA were evaluated in combination with EDX and OHTAM and compared to those promoted by TAM.

The data shown in chapter III demonstrate that RA induces the MPT pore opening (Figs. 3.1 and 3.2), in accordance with previous reports (Rigobello et al., 1999; Notario et al., 2003). As observed with the MPT induced by Ca^{2+} and phosphate, EDX inhibits the MPT induced by RA by directly interfering with the proteins that form the MPT pore complex (Figs. 3.1 and 3.2). RA is considered an ANT modulator (Notario et al., 2003) and the ANT ligands ADP and ATP prevent the RA-induced MPT (Fig. 3.5). However, EDX at the

concentrations that inhibit the MPT does not affect the phosphorylation efficiency (Figs. 2.1 and 2.2) and, therefore, neither the ANT nor the phosphate carrier seems to be related with the inhibition of RA-induced MPT by EDX. On the other hand, EDX enhances the antioxidant activity of RA (Figs. 3.3C and 3.3D), but the thiol protecting agents do not prevent the MPT induced by RA (Fig. 3.4), suggesting that other mechanisms may be involved. Therefore, the protection afforded by EDX against the MPT may rely on multiple effects exerted by this drug on mitochondria, including the effects on membrane fluidity or the interaction with CypD, as proposed for the MPT induced by Ca^{2+} and phosphate.

MPT induction is often implicated in cell death and it is thought to play a role in the therapeutic efficacy of anticancer drugs (Fulda et al., 2010). However, the combination with antiestrogenic compounds, which prevent the onset of MPT, does not limit the anticancer activity of RA, according to our results in melanoma cells (Figs. 4.2-4.5) and to studies performed by others in breast cancer cells (Danforth, 2004; Wang et al., 2007; Searovic et al., 2009; Koay et al., 2010). In addition, although EGTA and CyA were able to inhibit the MPT induced by 13-*cis* RA, both agents did not prevent the release of cytochrome *c* from the IMS caused by the retinoid (Rigobello et al., 1999). Moreover, in MCF-7 cell line, CyA did not prevent the formation of fragmented nuclei characteristic from the apoptotic process or the changes in mitochondrial parameters induced by retinoids (Poot et al., 2002). Mitochondria isolated from vitamin A-treated rat liver were also more sensitive to calcium regarding the redox status, but the insult elicited by calcium was independent of the MPT, since it was not prevented by CyA (de Oliveira et al., 2009). These evidences support that MPT induction may not be critical for RA to exert its antitumoral activity. On the contrary, the protection afforded by the antiestrogens against RA-induced MPT may contribute to prevent or reduce the adverse effects promoted by RA, including the hepatotoxicity, as several inducers of MPT are hepatotoxic (Labbe et al., 2008; Nadanaciva and Will, 2009).

RA decreases the phosphorylation efficiency in a concentration-dependent manner and at high concentrations induces the permeabilization of mitochondrial membrane to protons, which is possibly related with a proton leak through the Fo fraction of complex V (Figs. 3.6-3.8). As these effects promoted by RA are dose-dependent (Figs. 3.6 and 3.7), using a low dose of RA may avoid compromising mitochondrial bioenergetics and this way the toxicity of RA. The combinations of low concentrations of RA (5 nmol/mg protein) with

antiestrogens (10 nmol/mg protein) do not present significant effects on mitochondrial functions, except for the RCR, which is decreased in comparison with control, reflecting the effects on both state 3 and state 4 (Figs. 3.9 and 3.10). The combination of TAM with 5 nmol/mg protein of RA significantly diminished the RCR relatively to RA, suggesting that the combination with TAM has more drastic effects on mitochondrial bioenergetics than the combinations with its active metabolites EDX or OHTAM (Fig. 3.10C). In contrast, the combination of high concentrations of RA (above 10 nmol/mg protein) with antiestrogens significantly affects mitochondrial bioenergetic functions (Figs. 3.9 and 3.10). Our results correlate with the study performed by Budd *et al* (1998) in patients with advanced breast cancer. RA presented dose-dependent toxicity and 190 mg/m^2/day was established as the maximum tolerated dose; the combination with 20 mg of TAM/day did not enhance the toxicity of RA (Budd et al., 1998). On the contrary, the antitumor activity of this combination in breast cancer was not convincing, since in 7 patients with measurable disease, only 2 presented partial responses; in 18 patients with evaluable nonmeasurable disease, only 7 achieved disease stabilization for more than 6 months, but remarkably among these 7 patients, 5 presented TAM-resistant disease (Budd et al., 1998). Considering our current knowledge, it is possible to speculate whether the results would have been the same if the the individual metabolism or the intake of CYP2D6-inhibiting medication were taken into consideration. However, this study of Budd *et al* (1998) reinforces the notion that RA and antiestrogens can be given together with acceptable toxicity, if the concentrations of RA are low.

Based on the remarkable progress of research into the mechanisms of cancer pathogenesis, several mechanism-based therapies have been developed in the last years. Such therapies have been considered beneficial, as they can inhibit a specific molecular target, with less off-target effects, which in principle translates into less unspecific toxicity (Hanahan and Weinberg, 2011). However, these targeted therapies lead almost inevitably to relapses. There are two main reasons that explain the failure of such approach: the existence of redundant pathways, which allow some cancer cells to survive and adapt through mutations, epigenetic reprogramming or remodeling of the tumor microenvironment; on the other hand, tumor cells can shift their dependence from one hallmark capability to another (for instance, the inhibition of angiogenesis can be surpassed by increasing invasiveness and metastasis) (Hanahan and Weinberg, 2011). Therefore, mechanism-guided combinations

that can target several hallmark capabilites of cancer can provide more effective and durable therapeutic strategies (Hanahan and Weinberg, 2011).

Considering that retinoids and TAM decrease the proliferation of melanoma cells individually, and the absence of significant toxic effects on mitochondrial functions when RA is used at low concentrations in combination with antiestrogens (Ribeiro et al., 2013d), the effects of RA in combination with TAM on melanoma cells were investigated. Moreover, the effects of RA combined with EDX were also studied, which is to our knowledge the first time that EDX is tested in melanoma cells, and compared to those promoted by the combinations with TAM.

As shown in chapter IV, RA at concentrations as low as 0.5 μM has an antiproliferative effect on K1735-M2 melanoma cells (Fig. 4.1), which is not related with increased cell death, but is due to decreased cell division (Figs. 4.3-4.5). Similar findings were reported by Helige *et al* (1993) which showed that RA at 1 μM decreases K1735-M2 melanoma cell number. Melanoma cell death after 72 h treatment with RA at 0.5 μM was not detected (Fig. 4.3), in agreement with previous studies in leukemia and in melanoma cells, which demonstrate that the induction of apoptosis by RA requires long exposure time (2 days or more) and high concentrations of drug (10 μM) (Hsu and Yung, 2000; Baroni et al., 2003; Zhang et al., 2003). The antiproliferative effect of RA in K1735-M2 melanoma cells is related with cell cycle arrest in the G1 phase (Fig. 4.5), as previously shown by others in B16 melanoma cells (Niles, 2003), or in human myeloid cell lines (Dimberg and Oberg, 2003). It was reported that the induction of apoptosis and cell cycle arrest in melanoma cells by RA involves the down-regulation of the MAPK proteins and the up-regulation of p27 (Zhang and Rosdahl, 2004), as well as an increase in p16 expression in a time- and dose-dependent manner (Zhang and Rosdahl, 2005).

The combination of RA with EDX significantly enhanced the antiproliferative activity of both compounds individually and was more effective than the combination with TAM, which was unable to potentiate the action of RA (Figs. 4.2, 4.3 and 4.5). The combination of RA with EDX did not increase cell death (Fig. 4.3), but decreased cell division, inducing cell cycle arrest in G1 (Fig. 4.5). The mechanism underlying this synergism of action in melanoma is not yet clarified. Like RA, which is known to down-regulate the MAPK pathway (Zhang and Rosdahl, 2004), EDX also down-regulates this pathway in melanoma cells as shown for the first time by our results (Fig. 6.7). In addition,

it is known that TAM inhibits PKC activity in ER− cancer cells (Gundimeda et al., 1996; Gelmann, 1997; Luo et al., 1997), which further contributes to MAPK pathway suppression (Matsuoka et al., 2009). On the other hand, it was reported that the interaction between RA and PKCα decreases PKC activity at concentrations higher than those used in our experiments (Radominska-Pandya et al., 2000). Interestingly, the antitumor activity of RA in B16 melanoma cells involves an increase in PKCα expression, but as the inhibition of the enzyme activity does not inhibit RA action, it seems that the antitumor activity of RA does not require the phosphorylation activity of PKC (Niles, 2003). The effects of RA related with PKC involve other protein-protein interactions (Niles, 2003) and, therefore, the inhibition of PKC phosphorylation by the antiestrogens does not compromise the anticancer activity of RA, but possibly adds to it.

One of the most important factors in the development of malignant melanoma are G1/S checkpoint abnormalities (Sauroja et al., 2000). The G1/S transition is regulated by the retinoblastoma protein (pRb) and p53 pathways, but while alterations in the pRb pathway are relatively common in melanomas, due to lower levels of p16 or pRb, mutations in the gene encoding the cyclin-dependent kinase (cdk) 4 or overexpression of cyclin D1, those in the p53 pathway are infrequent (Li et al., 2006). In addition, studies in melanoma cells demonstrate the increase of p27 expression in the cytoplasm and a simultaneous reduction of nuclear expression, which is the only form of p27 that functions as a cdk inhibitor (Denicourt et al., 2007). Thus, targeting the pRb pathway seems crucial in the suppression of melanoma development (Li et al., 2006). As most tumors show aberrant expression of more than one protein (Li et al., 2006), the use of combined strategies is more likely to target such a diversity and produce significant responses.

An interplay between retinoid and estrogen signaling has already been demonstrated in a breast cancer ER+ cell line, and it was shown that retinoid and antiestrogen-mediated signaling converge at the cell cycle regulation level (Wilcken et al., 1996). Both the antiestrogen and RA block cell cycle progression in G1 phase; whereas antiestrogen treatment caused a rapid inhibition of cyclin Dl gene expression, RA reduced cdk4 activity without reducing cyclin or cdk levels (Wilcken et al., 1996). However, following exposure to either RA or antiestrogen, there was a reduction in the amount of hyperphosphorylated pRb, indicating that the mechanisms of action of antiestrogens and retinoids are different but converge at pRb (Wilcken et al., 1996). Importantly, it was

demonstrated that OHTAM (but not TAM) and various retinoids up-regulate the expression of p27 in both ER+ and ER− human breast cancer cell lines, through different pathways (Eto, 2010). This piece of information may be relevant as it also supports the existence of similarities between the modes of action of retinoids and antiestrogens. Considering that the MAPK pathway activation reduces the inhibitory activity of p27 (Donovan et al., 2001), melanoma cells treated with the combination with EDX possibly present higher p27 activity than those treated with TAM, in agreement with a more pronounced inhibition of the MAPK pathway promoted by EDX (Fig. 6.7), explaining the finding that the combinations with TAM do not enhance the effects induced by RA on cell cyle.

On the other hand, it remains unknown the role played by classic ER in the synergism of action between retinoids and EDX in melanoma cells. It was shown that an interaction between ERα and RARα is necessary for effective transcriptional activity in breast cancer cells (Ross-Innes et al., 2010). Whether such interplay is also important in melanoma is not known. In addition, ER can also mediate nongenomic signaling which can interact with GPR30- and RA-mediated signaling. These are questions that should be addressed in future investigations.

In melanoma, as in most peripheral cancers, metastatic disease is more important than local tumor growth as a determinant of mortality. Therefore, drugs that combine a cytostatic action and the ability to limit tumor metastasis can bring great therapeutic advantage. RA decreases melanoma cell migration through changes in cellular adhesion (Helige et al., 1993; Situ et al., 1993) and in the production of degradative enzymes that lead to host tissue degradation (Helige et al., 1993; Hendrix et al., 1990; Jacob et al., 1998), alterations in the actin cytoskeleton (Helige et al., 1993) and decreased motility (Helige et al., 1993). Several of these alterations were observed in K1735-M2 melanoma cells under treatment with RA (Helige et al., 1993). In our studies, RA up to 1 µM does not provide significant effects (Fig. 4.6), but as pointed by Helige *et al* (1993), the directional migration study was the one revealing the most modest activity of RA in K1735-M2 cells, whereas the adhesion capacity and the organization of the actin cytoskeleton were considerably affected (Helige et al., 1993).

A recent investigation conducted by Matsuoka *et al* (2009) has provided new insights on the role of antiestrogens in the inhibition of melanoma cells migration and invasion. MMPs are key players in the invasive process and it was shown that melanoma

cells express MMP-1, MMP-2 and MMP-9 (Hofmann et al., 2000). TAM inhibits MMP expression through the suppression of the PKC/MEK/ERK and PKC/PI3K/Akt pathways in B16BL6 melanoma cells above 2.5 and 5 μM, respectively (Matuoka et al., 2009). Furthermore, mice injected with melanoma cells previously treated with TAM present less visible lung nodules, whereas the treatment with TAM after the injection of B16BL6 cells decreased metastasis, indicating that TAM inhibits either developing or clinically evident metastasis, although the latter requires higher doses (Matuoka et al., 2009). As TAM is able to inhibit developing metastasis *in vivo* at 1 mg/kg, the authors suggest that the metabolic activation that occurs *in vivo* may contribute to amplify the antimetastatic potential of TAM (Matsuoka et al., 2009). Although in our experimental setting neither TAM nor EDX decreases the migration of K1735-M2 cells, the combination with RA at 1 μM was able to significantly enhance the effects of EDX and decrease the number of migrating cells relative to control, suggesting that EDX may be a more powerful antimetastatic agent than TAM when combined with other agents (Fig. 4.6). As RA also regulates the expression of MMP-1 (Jacob et al., 1998) and down-regulates the MAPK pathway in melanoma cells (Zhang and Rosdahl, 2004), it is possible that shared pathways can account for the antimigration activity of RA combined with EDX. Several studies established a good correlation between the invasive properties of tumor cells *in vitro* and their metastatic potential *in vivo* and, therefore, it is reasonable to expect that the combined treatment of RA with EDX, which inhibits melanoma cells migration *in vitro*, may provide similar effects *in vivo*.

Most systemic chemotherapeutic regimens for cancer, either in the adjuvant setting or in the treatment of metastatic disease, use agents targeting the cell cycle, which also affect the proliferation of normal cells, resulting in marrow suppression (neutropenia), hair loss and gut toxicity (Rodriguez-Acebes et al., 2010). Therefore, to provide the first clues on the potential adverse effects of RA in combination with antiestrogens, the viability of normal cells after 72 h treatment was monitored. Our results demonstrate that neither the compounds nor their combinations affect the number of viable endothelial cells (Fig. 4.7). Importantly, the number of dead endothelial cells is also not increased by these combinations (Fig. 4.7). The effects of RA on BAEC were also investigated by Gaetano *et al* (2001) after 7 days of exposure using the same technique to assess cell viability. According to this study, RA at 1 μM does not affect endothelial cell proliferation (Gaetano et al., 2001), in agreement with our findings. Moreover, EDX and RA at the concentrations used to decrease the proliferation

and migration of melanoma cells do not affect mitochondrial functions (Fig. 4.8). Given that the toxicity induced by treatment with RA remains the major obstacle to its clinical utilization as an anticancer agent, the use of low concentrations of RA in combination with EDX may be a suitable strategy to improve the outcomes with reduced toxicity.

Accumulating evidence suggests that glutamate plays an important role in the regulation of tumor cells proliferation and migration. Accordingly, NMDAR and AMPAR antagonists limit the proliferation of several non-neuronal cancers (Rzeski et al., 2001; Abdul and Hoosein, 2005; North et al., 2010a, b), as well as their motility (Rzeski et al., 2001). More recently, it was reported that melanoma cells WM451 express functional NMDAR and that the treatment with MK-801 alters melanoma cell morphology, motility and proliferation (Song et al., 2012), but this was the only study until now reporting the effects of an iGluR antagonist on melanoma.

The results described in chapter V demonstrate that NMDAR channel blockers memantine and MK-801 decrease the proliferation of K1735-M2 melanoma cells (Figs. 5.1-5.3), in agreement with the study conducted by Song *et al* (2012) that showed that MK-801 decreases WM451 melanoma cells proliferation *in vitro* and decreases tumor volume and growth in mouse models inoculated with melanoma cells. Significant effects were achieved at 500 and 300 µM of MK-801 and memantine, respectively (Figs. 5.1 and 5.2), which is in the range of iGluR antagonists concentrations used by others to decrease cancer cell proliferation (Rzeski et al., 2001; Stepulak et al., 2005; North et al., 2010a, b). Interestingly, memantine is more effective than MK-801 in the decrease of melanoma cells proliferation (Figs. 5.1 and 5.2), in spite of having a lower binding affinity towards NMDAR than the latter. Similar findings were reported by other groups (Rzeski et al., 2001; Stepulak et al., 2005; North et al., 2010a, b) and may suggest the existence of mechanisms of action other than the blockade of NMDAR.

As the concentrations of NMDAR antagonists necessary to block tumor cell proliferation are relatively high, some concerns about the toxic effects of treatment with NMDAR antagonists may be raised. Indeed, these concentrations might influence rodents behavior (Gilbert, 1988; Tricklebank, 1989; Kawabe et al., 1998). On the other hand, MK-801 and memantine do not induce cytotoxicity in melanocytes at 100 µM, but memantine promotes a larger increase in apoptosis (Hoogduijn et al., 2006). Moreover, treatment of human skin fibroblasts and bone marrow stromal cells with MK-801 up to 250 µM for 96 h

did not affect non-malignant cells viability (Rzeski et al., 2001). The absence of significant effects on non-neoplasic cells may predict that the treatment with NMDAR channel blocker MK-801 is devoid of significant toxicity. These observations were corroborated by studies using *in vivo* models of melanoma (Song et al., 2012), breast (North et al., 2010a) and lung (Stepulak et al., 2005; North et al., 2010b) cancers, which required doses of MK-801 much lower than expected based on the corresponding *in vitro* studies. However, for the treatment of peripheral cancers, it was proposed that the use of NMDAR antagonists that do not cross the blood-brain barrier could minimize the possible adverse effects.

Memantine at the concentrations used in our study affects mitochondrial function (McAllister et al., 2008) and induces a larger increase in apoptosis of melanocytes than MK-801 (Hoogduijn et al., 2006), and therefore MK-801 was the drug of choice to study in combination with antiestrogens. The antiestrogens are able to act synergistically with MK-801 to decrease melanoma cell proliferation (Figs. 5.4, 5.5 and 5.7), and the combinations with EDX or OHTAM were more effective than the combinations with TAM, inducing cell cycle arrest in G1 phase (Fig. 5.8). The effects of MK-801, at concentrations that do not induce significant effects individually, are greatly potentiated by the antiestrogens, indicating that through the combination with other agents, the doses of MK-801 used can be significantly reduced, this way minimizing the possible adverse effects. Likewise, MK-801 enhanced the antiproliferative effect of cyclophosphamide and thiotepa in rhabdomyosarcoma/medulloblastoma, neuroblastoma, lung carcinoma and astrocytoma cells (Rzeski et al., 2001), supporting that the NMDAR antagonists possess anticancer potential that can add to the existing therapies of cancer. The mechanisms underlying the interaction between NMDAR antagonists and antiestrogens are not yet clarified. However, the activation of NMDAR in neurons results in the phosphorylation of ERK 1/2 (Kemp and McKernan, 2002; Hardingham and Bading, 2003). Accordingly, MK-801 at 250 µM decreases ERK 1/2 phosphorylation in laryngeal cancer cells (Stepulak et al., 2011) and in lung cancer cells, which results in decreased CREB phosphorylation (Stepulak et al., 2005). In addition, after 24 h of exposure to 250 µM of MK-801 cyclin D1 was down-regulated, whereas p53 and p21 were up-regulated, providing a link to the cell cycle machinery and explaining the lung cancer cell cycle arrest in G1 (Stepulak et al., 2005). Although MK-801 at 100 µM does not affect the cell cycle of melanoma cells individually, the combination with EDX and OHTAM enhanced the effects induced by the compounds individually,

promoting cell cycle arrest in G1 phase. As it seems that the MAPK pathway plays a pivotal role in NMDAR signaling in different types of cancer cells and EDX decreases ERK 1/2 phosphorylation in K1735-M2 cells (Fig. 6.7), it is possible that the synergism of action observed in melanoma cells involves this common pathway and a convergence of cell cycle regulation mechanisms.

Talampanel is a recently developed noncompetitive antagonist of the AMPAR that has provided encouraging results for the use of GluRs antagonists in cancer therapy. In patients diagnosed with recurrent glioblastoma, monotherapy with talampanel has not demonstrated useful activity, but it was well tolerated (Iwamoto et. al., 2010). However, in a second larger phase II study performed in newly diagnosed glioblastoma patients, the combination of talampanel with radiation therapy plus temozolomide, demonstrated improved survival and talampanel did not increase the toxicity of temozolomide (Grossman et. al., 2009). These data support the view that GluR antagonists may be useful agents in therapeutic association, without adding significant toxicity to the treatment.

Our results demonstrate that EDX has a more powerful cytostatic action in melanoma cells than TAM and its benefits are greatly enhanced when used in therapeutic association. Moreover, EDX is less toxic to mitochondrial functions than TAM, allowing the reduction of the possible side effects related with mitochondrial damage, and avoids the variability in TAM metabolism, as well as drug interactions. Thus, EDX is an advantageous therapeutic alternative to TAM in the management of malignant melanoma, which is a new indication for this drug that is already being tested in humans. However, the mechanisms of action of antiestrogens in melanoma remain poorly defined. The understanding of the mechanisms involved and the explanation for the superior cytostatic activity of EDX over TAM in melanoma can contribute to clarify the contradictory results obtained so far, as it is possible that certain patients, according to the specific traits of their lesions, can benefit from the treatment with antiestrogens, while others do not. The identification of the GPR30 as a new ER, which can be activated by TAM and OHTAM, led us to investigate the expression and the role played by GPR30 in melanoma cells.

The results described in chapter VI show, for the first time, that melanoma cells express GPR30 (Fig. 6.1) and that its activation by low concentrations of G-1, a selective agonist of GPR30 (Bologa et al., 2006), induces profound cytostatic effects on melanoma cells (Figs. 6.2, 6.3, 6.5 and 6.6). Similar findings were reported in prostate cancer cells

(Chan et al., 2010). Thus, our results suggest that targeting the GPR30 is a new strategy in melanoma therapy and the evaluation of GPR30 expression in samples obtained from melanoma biopsies, and the comparison with the expression in normal skin, are required to support these findings.

The ability of G-1 to decrease melanoma cell proliferation, by decreasing cell division without enhancing cell death, resembles the effects promoted by the antiestrogens (Figs. 6.2-6.5). Since both TAM and OHTAM bind to GPR30 (Wang et al., 2010) it is possible that the antiproliferative effect on melanoma cells induced by these antiestrogens, and also by EDX, is at least in part mediated by this receptor. However, some differences should be noted. Whereas the three antiestrogens decrease the cell biomass in a concentration-dependent manner (Fig. 6.2B-6.2D), increasing the concentration of G-1 from 2.5 up to 20 μM does not enhance the decrease in cell biomass, which is in agreement with the saturation of the receptor (Fig. 6.2A). Moreover, after a 48 h treatment, G-1 induced cell cycle arrest in G2 phase, but none of the antiestrogens was able to induce significant effects at this time point (Fig. 6.6). Nevertheless, TAM metabolites were able to enhance the effects on cell cyle promoted by RA (Fig. 4.5) or MK-801 (Fig. 5.8). The differences observed are likely to reflect the multiple mechanisms by which antiestrogens exert their anticancer activity (de Médina et al., 2004), in contrast to G-1, which is a selective agonist, without significant activity on classic ER or on other GPCRs (Bologa et al., 2006). The effects promoted by the antiestrogens on targets other than the GPR30 may account for the fact that, although G-1 and TAM decrease ERK 1/2 phosphorylation, at the time point selected, only EDX provides a significant reduction (Fig. 6.7). However, this finding is highly relevant, as it provides the first mechanistic information that explains the superior efficacy of EDX in comparison with TAM in the reduction of melanoma cell proliferation, either individually or in combination with RA or MK-801, which also target the MAPK pathway, as discussed above. Since melanoma cells respond to chronic inhibition of specific targets by re-wiring their signaling circuitries, allowing the tumor cells to adapt to pharmacological challenges, combination strategies targeting key oncogenic pathways should be developed for successful therapy (Villanueva et al., 2010). Therefore, the signaling pathways modulated by antiestrogens, retinoids or MK-801 should be addressed in future experiments. Studies using RNA interference are also underway in order to confirm the involvement of GPR30 in the antiproliferative action of antiestrogens in melanoma. If this is the case, the study of a

possible association between the expression of GPR30, the steady-state levels of EDX and the clinical outcome of melanoma patients treated with TAM would be the next step.

The fact that antiestrogens can act through different mechanisms would have been considered pernicious some time ago. However, it is emerging the view that in a very heterogeneous and plastic malignancy, such as melanoma, targeting a single alteration will not be sufficient to kill all tumor cells, as learned from vemurafenib. In contrast, drugs that target several alterations in cancer cells can be beneficial, if devoid of significant toxicity in normal tissues. The possible off-target effects can be minimized through the use of low concentrations of drugs in combination with other agents, according to the genetic signature of lesions of each patient (Herlyn, 2009). Therefore, the future of EDX in melanoma therapy will inevitably involve its association with other chemotherapeutic agents.

7.2 - Conclusions

Our results demonstrate that EDX, at the concentrations reached in tissues, slightly affects mitochondrial bioenergetic functions in comparison with the damaging effects promoted by TAM. Therefore, the administration of EDX instead of TAM may decrease the adverse effects related with mitochondrial dysfunction, such as the liver injury commonly observed in patients taking TAM. Similarly to TAM, EDX inhibits the MPT pore opening induced by calcium and phosphate or by RA, which can contribute to decrease the toxicity of the retinoid. As the utilization of RA has been limited by its toxicity, the combination with EDX can contribute to turn RA into a more suitable agent for clinical practice.

On the other hand, EDX presents stronger cytostatic activity in melanoma cells than TAM, which may be due to a more pronounced inhibition of the MAPK pathway. In addition, the therapeutic benefit obtained from the administration of EDX is not limited by metabolic intervariability or by the intake of drugs that can limit the extent of TAM metabolism. The antiproliferative activity of EDX in melanoma is greatly enhanced when used in combination of with RA or MK-801, blocking cell cycle progression in G1. Noteworthy, the combinations of RA with EDX, in addition to the antiproliferative effect, also decrease the migration of melanoma cells at concentrations that do not affect the proliferation of normal cells. These findings, together with the inhibition of RA-induced

MPT by EDX, suggest that the combination of RA with EDX is an effective strategy for melanoma, without prohibitory side effects.

Our results also show, for the first time, that K1735-M2 melanoma cells express GPR30 and that the activation of the receptor decreases the proliferation of melanoma cells. Therefore, GPR30 is a new target in melanoma therapy that is possibly involved in the antiproliferative activity of antiestrogens, paving the way for future investigations.

Altogether, the results obtained support that EDX is a less toxic alternative to TAM in cancer therapy and a promising drug for the clinical management of malignant melanoma. The mechanisms underlying the anticancer activity of EDX in melanoma have just begun to be elucidated. However, given the complexity of melanoma and the multiple mechanisms involved in the pharmacological action of antiestrogenic compounds, it will be a long way to run, but hopefully it will worth the effort.

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