Dissertação em Bioquímica

ANTITUMOUR ACTIVITY EVALUATION OF SYNTHETIC STEROIDAL OXIMES AND EPOXIDES

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Abstract

Cancer is a worldwide disease, causing numerous deaths every year. The metabolism of cancer cells is altered compared to the normal ones, leading to an abnormal cellular growth, which enables them to metastasize to other organs. Currently, several strategies are used to help fight cancer among them the use of antitumour chemotherapy, however, this type of treatment bears several side effects. Steroidal compounds were proven to be efficient against several types of cancer. Epoxides and oximes are two structural features frequently associated with anticancer activity. In this manner, the main goal of this dissertation was to combine these features with the steroidal backbone by synthesizing steroidal epoxides and oximes and evaluating them in several cancer cell lines, ultimately to contribute to finding new anticancer agents with fewer side effects.

The compounds 5α-androst-3-en-17-one oxime (**3,4 – OLOX**), 3α,4α-epoxy-5α-androstan-17-one oxime (**3,4 – EPOX**), androst-4-en-17-one oxime (**4,5 – OLOX**) and 4α,5α-epoxyandrostan-17-one oxime (**4,5 – EPOX**) were synthesized and their cytotoxicity evaluated in four human cancer cell lines, namely, colorectal adenocarcinoma (WiDr), non-small cell lung cancer (H1299), prostate cancer (PC3) and hepato carcinoma (HepG2) with concentrations ranging from 1-75 μM. We used the MTT assay to assess cell proliferation, flow cytometry to evaluate viability and types of cell death and fluorescence to measure ROS (reactive oxygen species) production, after treatment of cancer cells with the synthesized compounds. Two non-tumour cell lines were also used, namely MRC5, a normal lung cell line and CCD841 CoN, a normal colon cell line. Hemocompatibility was also assessed by haemoglobin release measurement.

The most effective compound in inhibiting tumour cell proliferation in all cell lines was **3,4 – OLOX.** Furthermore, this compound demonstrated the lower IC_{50} values for WiDr and PC3 cells (9.1 μM and 13.8 μM, respectively). **4,5 – OLOX** also showed promising results in the same cell lines with an IC_{50} of 14.5 μM in PC3 and 16.1 μM in WiDr cell lines. Moreover, **3,4 – OLOX** and **4,5 – OLOX** revealed to be selective towards WiDr cells as demonstrated by the proliferation assays performed in the non-tumour CCD841 CoN cells. The compounds with an olefin group in the steroidal A-ring showed remarkable antiproliferative activity. On the other hand, compounds with an epoxide function instead of an olefin presented poorly antiproliferative activity. Furthermore, the oxime presented in C-17 position seemed to increase cytotoxicity. Further studies also revealed that **3,4 – OLOX** and **4,5 – OLOX** induced a decrease in cell viability accompanied by an increase in cell death, mainly by apoptosis/necroptosis for **3,4 – OLOX** in both cell lines and for **4,5 – OLOX** in WiDr cells, and by necrosis for **4,5 – OLOX** in PC3 cells. Moreover, the increase

production of ROS levels (intracellular peroxides and superoxide anion) in both PC3 and WiDr cells, treated with **3,4 – OLOX** and **4,5 – OLOX** compared to non-treated cells, indicated that the compounds might exert their cytotoxicity by ROS production. Additionally, both compounds did not induce haemoglobin release at a concentration of 10 and 75 μM in the hemocompatibility assay.

Preliminary results suggest that **3,4 – OLOX** and **4,5 – OLOX** might have an antitumoural effect, mediated by apoptosis/necroptosis and R.O.S. production, which encourages further studies. Compounds were proved to be safe for I.V. administration.

Keywords: Steroids, epoxides, oximes, synthesis, antitumour activity

Resumo

O cancro é uma doença à escala mundial que causa inúmeras mortes todos os anos. Em relação às células normais, o metabolismo das células tumorais encontra-se alterado, o que conduz a um crescimento celular anormal que lhes permite metastizar para outros órgãos. Atualmente, inúmeras estratégias são usadas no tratamento do cancro entre elas, a quimioterapia. No entanto, este tipo de tratamento comporta inúmeros efeitos secundários. Os esteróides têm sido amplamente estudados e tem-se provado a sua eficiência em diferentes tipos de cancro. Epóxidos e oximas são duas características estruturais frequentemente associadas à atividade antitumoral. Desta forma, o objetivo principal desta dissertação foi o de combinar estas mesmas características com o esqueleto dos esteróides através da síntese de epóxidos e oximas esteroidais, com posterior avaliação da sua atividade biológica em diversas linhas celulares tumorais para, em última análise, contribuir para encontrar novos fármacos antitumorais com menos efeitos adversos.

Os compostos 5α-androsta-3-eno-17-ona oxima (**3,4 – OLOX**), 3α,4α-epoxi-5α-androstano-17-ona oxima (**3,4 – EPOX**), androsta-4-eno-17-ona oxima (**4,5 – OLOX**) e 4α,5α-epoxiandrostano-17-ona oxima (**4,5 – EPOX**) foram sintetizados e foi avaliada a sua citotoxicidade em quatro linhas celulares tumorais, adenocarcinoma colorretal (WiDr), cancro do pulmão de não-pequenas células (H1299), cancro da próstata (PC3) e carcinoma hepatocelular (HepG2) com concentrações que variaram entre 1 e 75 μM. Foi usado o teste MTT para avaliar a proliferação celular após o tratamento, citometria de fluxo para avaliar a viabilidade e morte celular e fluorescência para medir a produção intracelular de ROS (espécies reativas de oxigénio) após o tratamento das células com os compostos. Foram utilizadas, também, duas linhas celulares normais, uma de pulmão, a linha MRC5, e uma de colon, a linha CCD841 CoN. A hemocompatibilidade foi também avaliada através da medição da hemoglobina libertada.

O composto que inibiu mais eficazmente a proliferação das células tumorais, facto observado em todas as linhas celulares, foi o **3,4 – OLOX**. Para além disso, nas células WiDr e PC3 demonstrou os melhores valores de IC_{50} (9.1 μM e 13.8 μM, respetivamente). O **4,5 – OLOX** também demonstrou resultados promissores nas mesmas linhas celulares com valores de IC_{50} de 14.5 μM nas PC3 e 16.1 μM nas WiDr. Estudos adicionais de proliferação celular utilizando a linha celular CCD841 CoN, revelaram a seletividade do **3,4 – OLOX** e do **4,5 – OLOX** face às células tumorais WiDr. Os compostos que apresentam uma dupla ligação (olefina) no anel A demonstraram uma forte atividade antiproliferativa. Por sua vez, os compostos que apresentam um grupo epóxido em vez de uma dupla

ligação, demonstraram fraca atividade antiproliferativa. Além disso, a oxima presente na posição C-17 do esqueleto do esteróide parece influenciar positivamente a atividade citotóxica dos compostos. O **3,4 – OLOX** e o **4,5 – OLOX** induziram uma diminuição na viabilidade celular acompanhada de um aumento de morte celular, principalmente por apoptose/necroptose para o **3,4 – OLOX** nas duas linhas celulares e para o **4,5 – OLOX** na linha celular WiDr, e por apoptose para o **4,5 – OLOX** nas células PC3. Além disso, o aumento da produção de ROS (peróxidos e do anião superóxido intracelulares), tanto nas células PC3 como nas WiDr, tratadas com o **3,4 – OLOX** e com o **4,5 – OLOX**, comparando com as células não tratadas, pode indicar que estes compostos exercem a sua citotoxicidade através da produção de ROS. O ensaio de hemocompatibilidade realizado revelou que ambos os compostos não induzem libertação de hemoglobina nas duas concentrações testadas, 10 µM e 75 µM.

Resultados preliminares sugerem que o **3,4 – OLOX** e o **4,5 – OLOX** podem ter um efeito antitumoral mediado por apoptose/necroptose e pela produção de ROS, o que encoraja estudos futuros. Os compostos provaram também ser seguros para serem administrados intravenosamente.

Palavras-chave: Esteróides, epóxidos, oximas, síntese, atividade antitumoral

List of contents

ACRONYMS, ABBREVIATIONS AND FORMULAS

List of acronyms, abbreviations and formulas

A549	*Human lung cancer cell line*
AIDS	*Acquired immune deficiency syndrome*
AIs	*Aromatase inhibitors*
ALK	*Anaplastic lymphoma kinase*
APC	*Adenomatous polyposis coli gene*
AR	*Androgen receptor*
ATP	*Adenosine triphosphate*
AV	*Annexin V*
BAX	*BCL-2 associated X protein*
BCL-2	*B-cell lymphoma-2*
BCLC	*Barcelona Clinic Liver Cancer*
BGC-823	*Human stomach cancer cell line*
BRAF	*V-raf murine sarcoma viral oncogene homolog B1*
BRCA1	*Breast cancer type 1 gene*
BRCA2	*Breast cancer type 2 gene*
CaSki	*Human cervical cancer cell line*
$CaCl_2$	*Calcium chloride*
CCD841 CoN	*Human non-tumour colon cell line*
$CH_3COONa.3H_2O$	*Sodium acetate trihydrate*
CIMP	*CpG island methylator phenotype*
CIN	*Chromosomal Instability*
CMSs	*Consensus molecular subtypes*
CRC	*Colorectal carcinoma*

CRPC	*Castration-resistant prostate cancer*
CYP17	*Steroid 17α-monooxygenase*
$DCFH_2$-DA	*2',7'- Dichlorodihydrofluorescein diacetate*
DHE	*Dihydroethidium*
DMEM	*Dulbecco's Modified Eagle Medium*
DMSO	*Dimethyl sulfoxide*
DNA	*Deoxyribonucleic acid*
DNAse	*Deoxyribonuclease*
DU-145	*Human prostate cancer cell line*
EGFR	*Epidermal growth factor receptor*
3,4 – EP	*3α,4α-Epoxy-5α-androstan-17-one*
3,4 – EPOX	*3α,4α-Epoxy-5α-androstan-17-one oxime*
4,5 – EP	*4α,5α-Epoxyandrostan-17-one*
4,5 – EPOX	*4α,5α-Epoxyandrostan-17-one oxime*
FAP	*Familial adenomatous polyposis*
FBS	*Fetal bovine serum*
FDA	*Food and Drug Administration*
FITC	*Fluorescein isothiocyanate fluorochrome*
5-FU	*5-Fluorouracil*
GI_{50}	*Half growth inhibition concentration*
GSH	*Reduced glutathione*
GPx	*Glutathione peroxidase*
H1299	*Human lung cancer cell line*
HBV	*Hepatitis B virus*
HCC	*Hepatocellular carcinoma*

HCT-8	*Human colon cancer cell line*
H_2O_2	*Hydrogen peroxide*
HCO_2H	*Formic acid*
HCV	*Hepatitis C virus*
HeLa	*Human cervical cancer cell line*
HepG2	*Human liver cancer cell line*
HER-2	*Human Epidermal growth factor Receptor 2*
HGPIN	*High-grade intraepithelial neoplasia*
HOXβ13	*Homeobox protein Hox-B13*
17β-HSDI	*Hydroxy steroid dehydrogenase inhibitors*
HT-29	*Human colorectal cancer cells*
I.V	*Intravenous administration*
IC_{50}	*Half maximal inhibitory concentration*
JC-1	*5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide*
KB	*Nasopharyngeal cancer cell line*
KCl	*Potassium chloride*
KH_2PO_4	*Monopotassium phosphate*
KRAS	*Kristen rat sarcoma viral oncogene homolog*
LC_{50}	*Half lethal concentration*
LGPIN	*Low-grade form of prostatic intraepithelial neoplasia*
LNCap	*Human prostate cancer cell line*
LogP	*Partition coefficient*
M/A	*Monomers/Aggregates ratio*
MAPK	*Mitogen-activated protein kinase*

MCF-7	*ER^+ human breast cancer cell line*
MCF-7aro	*ER^+ human breast cancer cell line overexpressing aromatase*
MDA-MB-231	*Human triple negative breast cancer cell line*
MEK-1	*Dual threonine and tyrosine recognition kinase*
Mp	*Melting point*
MRC5	*Human non-tumour lung cell line*
MSI	*Microsatellite instability*
MTT	*3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*
NaCl	*Sodium chloride*
$Na_2HPO_4.2H_2O$	*Sodium phosphate dibasic dihydrate*
NH_2OH	*Hydroxylamine*
NRAS	*Neuroblastoma RAS viral oncogene homolog*
NSCLC	*Non-small cell lung carcinoma*
O_2^-	*Superoxide anion*
$OH^\bullet$	*Hydroxyl radical*
3,4 – OL	*5α-Androst-3-en-17-one*
3,4 – OLOX	*5α-Androst-3-en-17-one oxime*
4,5 – OL	*Androst-4-en-17-one*
4,5 – OLOX	*Androst-4-en-17-one oxime*
PBS	*Phosphate buffer saline*
PC3	*Human prostate cancer cell line*
PCa	*Prostate cancer*
PI	*Propidium iodide*
PI3K	*Phosphoinositide 3-kinase*
PIK3CA	*Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha*

PSA	*Prostate-specific antigen*
PTEN	*Phosphatase and tensin homolog*
RNA	*Ribonucleic acid*
RNAse	*Ribonuclease*
RNS	*Reactive nitrogen species*
ROS	*Reactive oxygen species*
RPMI	*Roswell Park Memorial Institute Medium*
SCLC	*Small cell lung carcinoma*
Sip-T	*Sipuleucel-T*
SOD	*Superoxide dismutase*
STSI	*Sulfatase inhibitors*
TGF-β	*Transforming growth factor beta*
TGI	*Total growth inhibition concentration*
TLC	*Thin layer chromatography*
TP53	*Tumour suppressor gene 53*
TRUS	*Transrectal ultrasound imaging*
WiDr	*Human colorectal cancer cell line*

INTRODUCTION

Cancer

Cancer is a worldwide disease that causes high death rates, not only because of the ageing and growth of the world population but also due to an increase of cancer-causing behaviours. In fact, cancer is the main cause of death in economically developed countries and the second leading cause of death in developing countries (Bray *et al.*, 2018).

The development of cancer involves the *hallmarks of cancer*, which include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg, 2000). Overall, normal cells have mechanisms to control the production and release of growth-promoting signals that are responsible for telling the cells if they should undergo division or not. Cancer cells can overcome these mechanisms by deregulating these signals allowing the uptake and metabolism of nutrients that are able to promote cell survival and are the fuel for cell growth resulting in the formation of a tumour (Hanahan and Weinberg, 2011; Courtnay *et al.*, 2015). More recent studies added four new hallmarks as a complement: avoid immune destruction, tumour-promoting inflammation, genome instability and mutation and deregulating cellular energetics (Hanahan and Weinberg, 2011). Altogether, they account for the ten *hallmarks of cancer* (Figure 1).

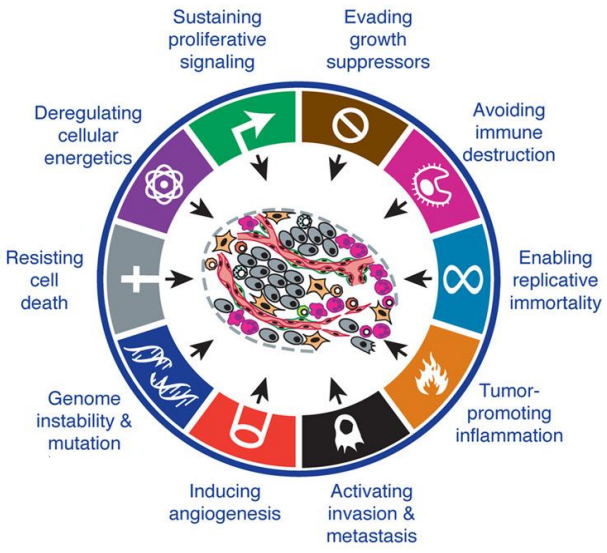


Figure 1 – Hallmarks of cancer. Printed from (Hanahan and Weinberg, 2011).

Epidemiology

The World Health Organization estimated the occurrence of 18.1 million new cases and 9.6 million cancer deaths worldwide in 2018 (Bray *et al.*, 2018). Moreover, epidemiologic studies estimated that, for both sexes, lung cancer was the most commonly diagnosed cancer (11.6% of the total cases) and the one with the highest mortality rate (18.4% of the total cancer deaths), closely followed by female breast cancer (11.6%), colorectal cancer (10.2%), and prostate cancer (7.1%) for incidence and colorectal cancer (9.2%), stomach cancer (8.2%), and liver cancer (8.2%) for mortality (Bray *et al.*, 2018) (Figure 2). In Portugal, prostate cancer was the most incident type of cancer and lung cancer was the one with a higher mortality rate, in males. In females, for incidence is breast cancer and for mortality is colorectal cancer (Figure 3 and Figure 4).

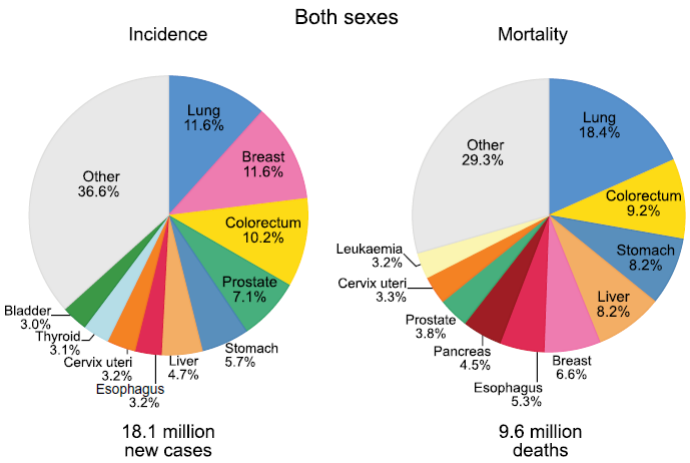


Figure 2 – Estimated incidence and mortality of the 10 Most Common Cancers in 2018 for both sexes. Printed from (Bray *et al.*, 2018).

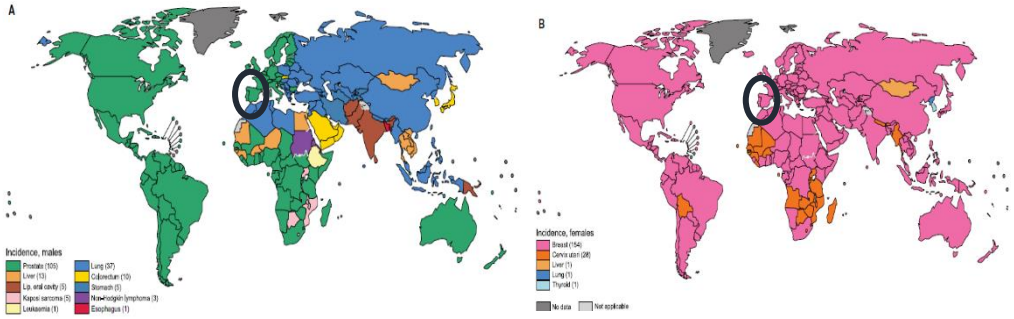


Figure 3 – Estimated most common type of cancer incidence in 2018 in each country among (A) males and (B) females with especial attention for Portugal. Adapted from (Bray *et al.*, 2018).

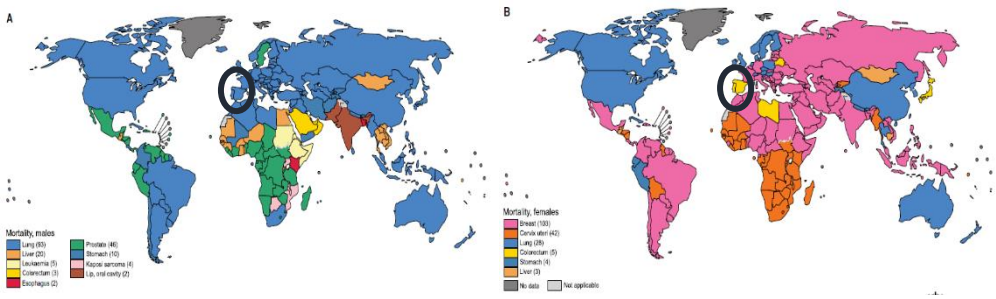


Figure 4 – Estimated most common type of cancer mortality in 2018 in each country among (A) males and (B) females with especial attention for Portugal. Adapted from (Bray *et al.*, 2018).

Colorectal cancer

Colorectal cancer (CRC) is a heterogeneous disease that occurs in the colon and the rectum, parts of the gastrointestinal system. CRC was the third most common cancer and the second in terms of mortality worldwide in 2018 (Figure 2). In Portugal, colorectal cancer is the cancer with the highest incidence in both sexes (World Health Organization, 2019) and with the highest mortality in women (Bray *et al.*, 2018).

Unlike most malignancies, CRC is highly preventable and even potentially curable, if high-risk adenomas and early stage tumours are removed. Survival of CRC patients during the first 5 years is approximately 65% and highly dependent on the tumour stage when it is diagnosed. Survival rate five years after the diagnosis is 90% for patients with localized disease, 70% for patients with regional disease and 13% for the ones with distant stages. As colorectal cancer is typically an asymptomatic cancer until it progresses to advanced stages, implementation of screening programs is essential to detect this type of cancer in an early stage (Gonzalez-Pons and Cruz-Correa, 2015).

The risk of developing this type of cancer is associated with age, family history, occurrence of inflammatory bowel diseases (*i.e*., ulcerative colitis and Chron disease), lifestyle, genetic and environmental factors (*i.e.*, diet, alcohol and tobacco abuse) (Brenner, Kloor and Pox, 2014; Aran *et al.*, 2016) (Figure 5). It is well known that CRC is mainly caused by environmental and/or acquired stimuli (Vieira, Tramonte and Lopes, 2015). Genetic factors only account for 5% of the different types of cancer, whereas environmental factors represent 95% (Encarnação *et al.*, 2015).

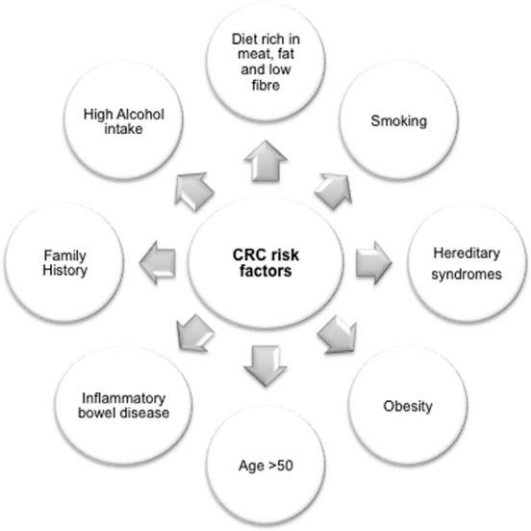


Figure 5 - Most frequent risk factors associated with colorectal cancer. Printed from (Aran *et al.*, 2016).

Understanding the molecular mechanisms underlying the development of colorectal cancer is clinically important because they are related to the prognosis and treatment response of the patient. It has been widely known that most colorectal adenocarcinomas derive from precursor lesions such as adenomas, serrated polyps or hereditary syndromes such as Lynch syndrome and familial adenomatous polyposis (FAP) (Fleming *et al.*, 2012; Pires, 2016). Generally, it can be stated that most colorectal cancers are sporadic, with no family history or genetic predisposition (Fearon, 2016).

The first model of colorectal tumourigenesis (the adenoma-carcinoma sequence represented in Figure 6) outlined four steps of the development of cancer (Fearon and Vogelstein, 1990). The first one, involves the adenoma development through inactivation of *APC* (adenomatous polyposis coli) gene, followed by mutations in *KRAS* (Kristen rat sarcoma viral oncogene homolog), genetic alterations in chromosome 18 and inactivation of P53. This model enlightened the alterations in the main oncogenes such as *KRAS*, *NRAS* (neuroblastoma RAS viral oncogene homolog), *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) and *PIK3CA* (phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha) and in the major tumour suppressor genes such as *APC*, *TP53* and *PTEN* (phosphatase and tensin homolog) (Fearon and Vogelstein, 1990; Pires, 2016). More recently, other signalling pathways have been implicated in the development of CRC, such as WNT/β-catenin, TGF-β (transforming growth factor beta), EGFR (epidermal growth factor receptor), MAPK and PI3K (Armaghany *et al.*, 2012; Dienstmann, Salazar and Tabernero, 2014; Pires, 2016).

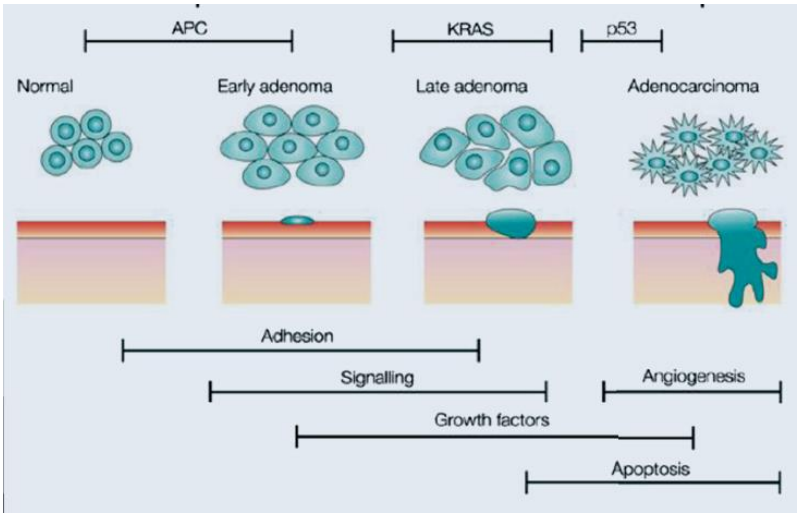


Figure 6 – The adenoma-carcinoma sequence. Adapted from (Armaghany *et al.*, 2012).

Nowadays, we can distinguish four molecular mechanisms responsible for genetic and epigenetic instability in CRC such as chromosomal instability (CIN), microsatellite instability (MSI), CpG island methylator phenotype (CIMP) and global DNA's hypomethylation (Armaghany *et al.*, 2012; Gonzalez-Pons and Cruz-Correa, 2015; Pires, 2016; Yiu and Yiu, 2016). Advances at the level of genetic expression allowed the classification of CRC in four consensus molecular subtypes (CMSs) with different molecular features namely, CMS1, CMS2, CMS3 and CMS4 (Yiu and Yiu, 2016).

Due to the slow growth rate of CRC, screening is essential in order to reduce mortality rates, since it increases the diagnosis in early stages of the tumour and ultimately increases the probability of survival (Siegel and Jemal, 2016).

The decision of the therapeutic approaches for each patient is taken considering the best options according to the staging of CRC and the risks and benefits associated with each approach. Approximately 80% of the newly diagnosed cases require surgery since it is considered the best healing approach. Surgical treatment can be preceded by a neoadjuvant treatment and/or complemented by an adjuvant treatment (Young, Hobbs and Kerr, 2011; Pires, 2016). In terms of chemotherapeutic treatment, the protocol depends not only on the CRC's staging but also on other factors, such as age and other diseases suffered by the patient (Brenner, Kloor and Pox, 2014). Conventional chemotherapeutic options include mostly fluoropyrimidines, oxaliplatin and irinotecan. First-line of treatment consists of intravenous administration of a fluoropyrimidine such as 5-fluorouracil (5-FU) or other oral fluoropyrimidines in several combinations and therapeutic approaches (Labianca *et al.*, 2010; Pires, 2016).

Liver cancer

Primary liver cancer is an extremely heterogenous malignant disease among the tumours that have been so far identified. Hepatocellular carcinoma (HCC) is the most prevalent malignant neoplasm of the liver, accounting for about 70-85% of the total primary liver cancer (Bosetti, Turati and La Vecchia, 2014). Other forms less predominant include adult cholangiocarcinoma originated from the intrahepatic biliary ducts, angiosarcoma from the intrahepatic blood vessels and childhood hepatoblastoma (Schottenfeld and Fraumeni, 2006; Bosetti, Turati and La Vecchia, 2014).

HCC emerges most commonly in the setting of chronic liver inflammation and fibrosis as a consequence of viral infections, metabolic injuries, toxic insults, or autoimmune

reactions (Li and Wang, 2016). Chronic Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infections are the major recognized risk factors for HCC worldwide, additionally to other causes such as aflatoxins, alcohol abuse, metabolic syndrome, obesity and diabetes. These are intimately related to the environmental and genetic susceptibilities to HCC, leading to alterations on signal transduction pathways. Hepatocarcinogenesis is then a complex multistep process in which different signalling cascades can be altered leading, ultimately, to the heterogeneity of liver cancer disease (Figure 7) (Forner, Reig and Bruix, 2012; Bosetti, Turati and La Vecchia, 2014; Li and Wang, 2016). Several genetic events have been related with the development of HCC, such as inactivation of the tumour suppressor gene, *TP53* (present in 25-40% of cancers, depending on tumour stage), mutations in β-catenin (about 25% predominantly in HCV-related HCC), overexpression of numerous ErbB receptor family members and overexpression of MET receptor. Furthermore, many cancer-relevant genes seem to be targeted on the epigenetic level (methylation) (Farazi and DePinho, 2006; Forner, Reig and Bruix, 2012). Genomic instability is also very common in HCC, in which several mechanisms might contribute, such as telomere erosion, chromosome segregation defects and alterations in the DNA-damage response pathways (Farazi and DePinho, 2006).

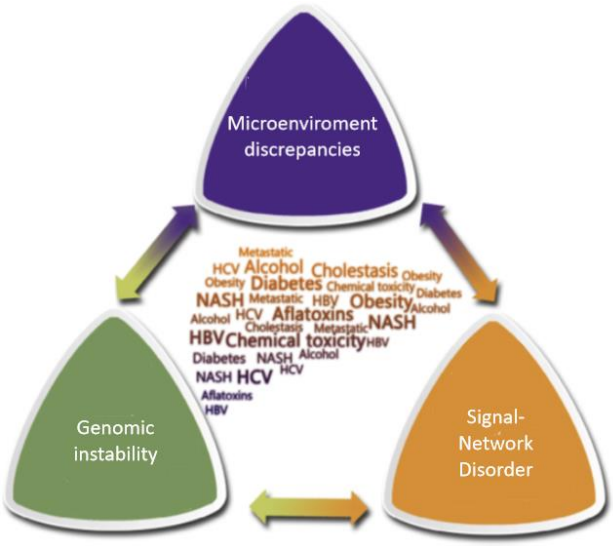


Figure 7 - A multi-step process of liver carcinogenesis emerging from the accumulation of genetic and epigenetic changes, microenvironment and signal network alterations with different risk factors leading to heterogeneity of cancer. Adapted from (Li and Wang, 2016).

Treatment of HCC depends on assessment of the tumour stage using the Barcelona Clinic Liver Cancer (BCLC) stratification, an algorithm that classifies HCC into five stages: 0, A, B, C and D (Attwa and El-Etreby, 2015). Patients with early-stage HCC (0, A and B) are treated by partial liver resection (removal of a portion of the liver), liver transplantation or ablation which induces tumour necrosis by injection of chemicals (e.g., ethanol, acetic

acid) or temperature modification (microwave, laser or cryoablation) (Forner, Reig and Bruix, 2012; Li and Wang, 2016). For more advanced stages (C and D), radiation therapy and systemic chemotherapy are the most common treatments. In systemic therapy, sorafenib, a kinase inhibitor, is currently considered as the first drug and the only approved therapy that might improve survival in patients with advanced HCC (Attwa and El-Etreby, 2015).

Lung cancer

Lung cancer was estimated to be most incident and the deadliest cancer for both men and women worldwide in 2018 (Bray *et al.*, 2018).

There are two major subtypes of lung cancer: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), accounting for 15% and 85% of all lung cancers, respectively. NSCLC can be classified into three histologic subtypes: squamous-cell carcinoma, adenocarcinoma and large-cell carcinoma (Herbst, Heymach and Lippman, 2008; Zappa and Mousa, 2016). Squamous-cell carcinoma (25-30% of all lung cancer cases) starts from precursors of squamous cells, localized in the airway epithelial cells from the bronchial tubes, in the centre of the lungs. Smoking is highly related to the presence of this subtype of NSCLC (Zappa and Mousa, 2016). Adenocarcinoma is the most common type in smokers and non-smokers, accounting for 40% of all lung cancers. Its origin is in type II alveolar cells that secret mucus and other substances. Compared to other types of lung cancer, adenocarcinoma has a tendency to grow slower, thus having a better chance of being discovered at earlier stages (Masayuki *et al.*, 1995; Zappa and Mousa, 2016). Finally, large-cell carcinoma (5-10% of all lung cancers) is a very heterogeneous group and presents no evidence of squamous or glandular maturations, so is commonly diagnosed by exclusion of other possibilities (Zappa and Mousa, 2016). This type of lung cancer can occur in any part of the lung and tends to grow and spread quickly (Mansinhos, 2017) and is highly correlated with smoking (Zappa and Mousa, 2016).

Lung cancer is highly correlated with smoking since 85-90% of patients with this type of cancer have a history of smoking (Vineis, 2005). Besides smoking, there are other risk factors, such as passive smoking, radon exposure, occupational exposure to asbestos, silica or uranium, previous radiotherapy to the lungs, decreased fruit and vegetable consumption, extreme air pollution and genetic and family factors (Teh and Belcher, 2014). There are several mechanisms that might be involved in the development of lung tumourigenesis, such as activation of growth factor signalling proteins (e.g. EGFR, KRAS,

BRAF, MEK-1, HER2, MET, ALK and RET), as well as inactivation of tumour suppressor genes (e.g. *TP53*, *PTEN*) (Larsen and Minna, 2011). Activation of growth factor signalling proteins can occur by gene amplification or other genetic alterations, including point mutations and structural rearrangements, leading to uncontrolled signalling of oncogenic pathways (Larsen and Minna, 2011).

The most frequent treatments for lung cancer are surgery, laser ablation of malignant lesions, chemotherapy, radiation therapy and photodynamic therapy, according to cancer stage (Larsen and Minna, 2011). However, there is still a noticeable need to try to discover more cost-effective and minimally invasive therapies.

Prostate cancer

Prostate cancer (PCa) is the second most frequently diagnosed type of cancer in men worldwide, with 1.3 million new cases and 362 thousand deaths predicted for 2018 (Bray *et al.*, 2018). In Portugal, PCa is the most incident type of cancer and the third cause of death by cancer, since of the 6609 new cases diagnosed in 2018, 1879 resulted in death (World Health Organization, 2019).

Prostate diseases are chronic and progressive, needing years to develop. The most frequent lesion associated with the development of PCa is the high-grade prostatic intraepithelial neoplasia (HGPIN), which according to the previously postulated arises from the low-grade form of prostatic intraepithelial neoplasia (LGPIN). Evidence shows that most genes expression alterations involved in the PCa occur from the normal epithelium to HGPIN and not from HGPIN to carcinoma. Figure 8 represents a proposed model for the progression from normal epithelium to PCa. The development of PCa is long and followed by several molecular processes and alterations in important pathways, specific for each stage (Joshua *et al.*, 2008; Sciarra *et al.*, 2008; Shen and Abate-Shen, 2010).

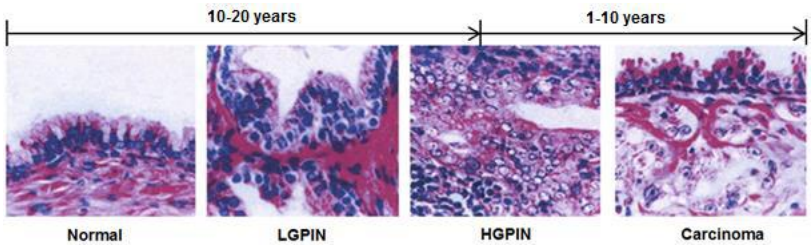


Figure 8 – Evolution from normal epithelium to carcinoma. Adapted from (Sciarra *et al.*, 2008).

PCa is a very heterogeneous and multifocal type of cancer and the most frequent type is adenocarcinoma accounting for 95% of all cases (Shen and Abate-Shen, 2010).

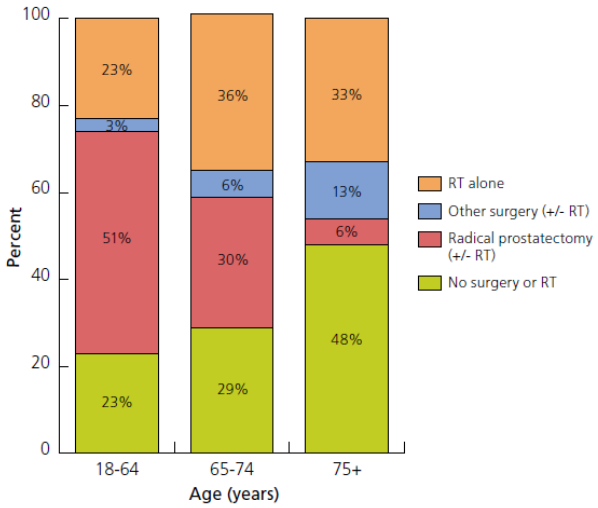
The risk factors associated with PCa, contrary to other types of cancer, remain mostly unknown (Grönberg, 2003). However, the development of PCa is associated with three main risk factors which are age, race/ethnics, and family predisposition (Gann, 2002), being age the most ruling factor. With aging, the incidence rate drastically increases until it reaches its maximum peak around 80 years. Although, the relationship between aging and increase PCa incidence is not completely understood, it might be due to genes expression alterations occurring in the prostate with aging, mostly genes associated with inflammation, senescence and oxidative stress (Shen and Abate-Shen, 2010; Cardoso, 2013).

Another determinant factor is family predisposition, which is responsible for 5-10% of all cases of this pathology. Men whose first-degree relatives have been diagnosed with PCa, before 60 years-old, have a four-fold increase risk of developing the disease when compared to the population in general (Adjakly *et al.*, 2015). Hereditary conditions are associated with Lynch Syndrome and with mutations in *BRCA1*, *BRCA2* and *HOXβ13* genes. Mutations in *BRCA1* and *BRCA2* have been associated with more aggressive prostatic carcinomas in younger individuals, which are characterized by a high rate of ganglionic metastasis and high mortality rates (Attard *et al.*, 2015; Hussein, Satturwar and Van Der Kwast, 2015). *HOXβ13* mutations are also associated with PCa, however, the exact mechanism in which they interfere is not well established, knowing only that this gene interacts with androgen receptor (AR), promoting the development and differentiation of normal prostate tissue into carcinoma (Attard *et al.*, 2015; Hussein, Satturwar and Van Der Kwast, 2015).

Incidence of PCa has increased in the last decade, however, mortality rate decreased. This is due not only to the early detection and diagnose, but also to the arising of more effective therapeutic strategies. Early PCa diagnosis increases the number of therapeutic options available (Marques Figueira, 2016). The most common early diagnostic tests are prostate-specific antigen (PSA) screening and rectal examination (Attard *et al.*, 2015). Transrectal ultrasound imaging (TRUS) is also used enabling visualization of prostate size and anatomy, allowing to detect PCa lesions, which usually appear denser relative to normal tissue.

In terms of treatment, there are many options available (Siegel *et al.*, 2012). Figure 9 highlights the most common treatment approaches for PCa, used depending on age. Hormonal therapy (androgen-deprivation) and chemotherapy are used in more advance PCa cases, being chemotherapy used as last resource when all the other therapies fail

(Cardoso, 2013). The use of docetaxel in combination with prednisone appears to be the most suitable treatment (Heidenreich *et al.*, 2014). More recently, a vaccine, Sipuleucel-T (Sip-T) was approved, only in the United States, by the FDA (Food and Drug Administration) for the treatment of asymptomatic or minimally symptomatic advanced, relapsing, and castration-resistant prostate cancer (CRPC). This vaccine contains an autologous cellular immunotherapy, with autologous CD54+ cells, which stimulates the immune system. Sip-T has proven to increase the overall survival in 4.1 months, however, its concomitant use with chemotherapy and immunosuppressive drugs has not been studied yet (Kantoff *et al.*, 2010; Heidenreich *et al.*, 2014).



Radical prostatectomy = removal of the prostate along with nearby tissues;
RT = radiation therapy.

Figure 9 - Primary treatments for PCa, according to age in 2016. Printed from (Miller, Siegel and Jemal, 2016).

Steroids and their derivatives

Steroids belong to a class of natural or synthetic organic compounds, whose basic molecular structure consists of 17 carbon atoms arranged in four rings (*A, B, C* and *D*) bonded to 28 hydrogen atoms (Figure 10). Steroidogenesis is a multistep process for the biochemical synthesis of more complex steroid molecules involving conversion of cholesterol (C27) into progestins (C21) followed by androgens (C19) and finally into estrogens (C18) recurring to various enzymes (Miller and Auchus, 2011; Gupta, Kumar and Negi, 2013). In fact, steroids are very important in our organism since they regulate series of biological processes. They can easily penetrate cell membranes and bind to the nuclear and membrane receptors. Considering this, steroidal molecules are attracting a lot of attention among researchers not only due to their fascinating structure but also due to their wide range of pharmacological properties, since a small change in the steroid skeleton can prompt an extensive biological response (Gupta, Kumar and Negi, 2013)**.**

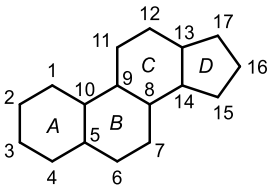


Figure 10 – General structure of a steroid.

Biomedical applications

Since their discovery in 1935, steroids have played a significant role in the treatment of several diseases in the most variable areas of medicine (Ericson-Neilsen and Kaye, 2014). There are several types of steroids, however, the ones used in clinical practice are steroid hormones (sex hormones) such as progesterone and estradiol both used in birth control pills, anabolic steroids used to help fighting the effects of chronic wasting disease, late stages of AIDS and to help with the symptoms of osteoporosis (Rasheed and Qasim, 2013). They are also used to increase muscle mass. Lastly, the most prescribed class of steroids in medicine is corticosteroids, which include glucocorticoids and mineralocorticoids. Glucocorticoids regulate metabolism and supress inflammation, being used as a treatment for a variety of inflammatory and autoimmune diseases. Mineralocorticoids regulate sodium and water levels by maintaining blood volume and controlling renal excretion of electrolytes (Shaikh *et al.*, 2012; Rasheed and Qasim, 2013).

Steroids are widely used as anti-inflammatory agents to treat several conditions such as arthritis, psoriasis and asthma (Rasheed and Qasim, 2013). Their anti-inflammatory properties have been associated with their inhibitory effects on phospholipase A2, an enzyme responsible for producing inflammatory molecules (Ericson-Neilsen and Kaye, 2014). Prednisone, prednisolone and methylprednisolone are examples of steroids currently used as anti-inflammatory agents in clinics (Rasheed and Qasim, 2013). Additionally, steroids also present remarkable antitumour activity in several types of cancer which will be addressed in the section below.

Steroids as antitumour agents

Cancer development related to steroid hormones is mainly due to rapid cell proliferation where metabolizing enzymes and steroidal receptors play major roles (Gupta, Kumar and Negi, 2013). It is commonly known that estrogens play an essential role in cell proliferation, however, over-expression of this molecule leads to an excessive proliferation of hormone-sensitive cells, which ultimately leads to several types of hormone-dependent cancers such as breast, uterine, ovarian, prostate and endometrial cancers (Chen, Zeng and Tse, 2008). Notwithstanding, steroids have also been investigated as anti-hormonal drugs. For example, some studies reported that changing the hormonal balance of the body by administering sex hormones produces useful remission from cancer (Biglia *et al.*, 2005; Drudge-Coates, 2005).

To reduce the hormonal response of cancer cells, steroids can act as cytotoxic and as cytostatic (antiproliferative) antitumour agents against several types of cancers. A brief description of some steroidal anticancer agents is presented in Figure 11. Among cytostatic steroids, there are the ones that inhibit the biosynthesis of hormones through enzyme inhibition, such as steroid sulfatase inhibitors (STSI), aromatase inhibitors (AIs) and hydroxy-steroid dehydrogenase inhibitors (17β-HSDI); and the ones that inhibit the hormone function, such as antiestrogens and antiprogestins. Although cytotoxic steroids may or may not have anti-hormonal effects, naturally occurring and semisynthetic steroids belong to the cytotoxic steroids category and usually follow different modes of action through non-hormonal targets (Gupta, Kumar and Negi, 2013). Semi-synthesized steroidal molecules are the main topic of this study.

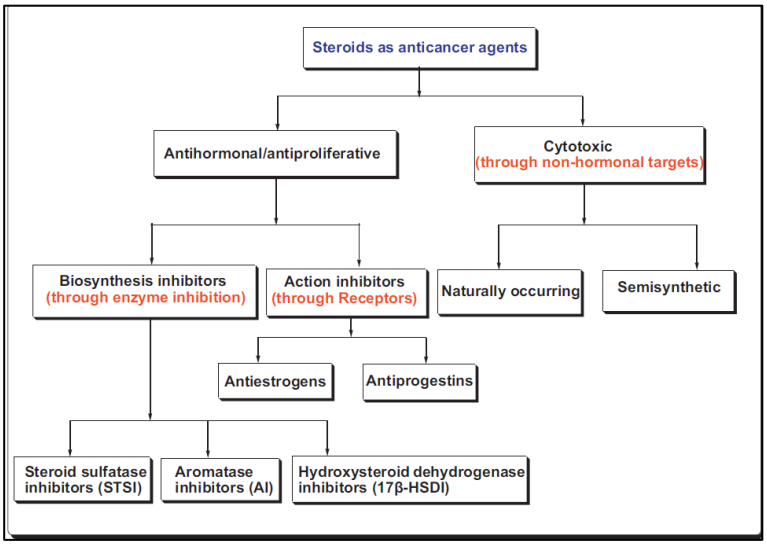


Figure 11 - Classification of steroids as anticancer agents. Adapted from (Gupta, Kumar and Negi, 2013).

As it was mentioned before, currently, steroidal derivatives are one of the most diversified therapeutic group of compounds. Most of the steroidal anticancer drugs have been developed as enzyme inhibitors and cytotoxic drugs. There are several steroidal compounds being used as adjuvants in cancer chemotherapy, such as prednisone, useful in the treatment of malignant lymphoma and acute leukemia in children (Toksöz *et al.*, 2004), cyproterone acetate for PCa (Berrevoets, Umar and Brinkmann, 2002) and AI's, such as exemestane used in the treatment of postmenopausal breast cancer (Amaral *et al.*, 2015), among others (Bansal and Acharya, 2014). More recently, some steroidal molecules have been investigated as potential anticancer agents, such as the novel steroidal imidazole, a dual inhibitor of AR/CYP17 that showed promising antiproliferative effects in LNCap cells (half maximal inhibitory concentration, IC_{50}=23 μM) (Hou *et al.*, 2019). Two steroids icogenin aglycone analogs were also synthesized and evaluated in several cell lines presenting IC_{50} ranging from 2.40 to 9.05 μM in HeLa (cervical cancer), KB (nasopharyngeal carcinoma), BGC-823 (stomach cancer), A549 (lung cancer) and HCT-8 (colon cancer) cell lines (Guan, Li and Lei, 2017).

To conclude, a reasonable number of steroidal compounds are being used in the medicine field and a lot more are being evaluated and shown to display interesting chemotherapeutic activities including tissue-selective cytotoxicity to achieve reduced peripheral toxicities, target specificity to produce cytotoxicity in the tumour cells, and implication in the treatment of inoperable malignancies (Bansal and Acharya, 2014). Therefore, the development of steroidal derivatives as safer and target specific antitumoural agents seems to be an excellent and promising future approach.

Steroid derivative compounds: oximes and epoxides

The relationship between chemical structure and biological function is of great interest when it comes to trying to discover new pharmaceutical compounds. Some steroid molecules have shown antitumour properties and chemical modifications of the steroid skeleton have been implemented to synthesize new potent anticancer agents. Epoxides and oximes are also two structural features frequently associated with anticancer activity.

Structure and general properties

Epoxides are ubiquitous and occur in a significant variety of natural products, in the environment, in industry and *in vivo* arising from biochemical transformations (Manson, 1980; Marco-Contelles, Molina and Anjum, 2004; Thibodeaux, Chang and Liu, 2012). They are cyclic ethers with an oxygen atom in a saturated three-membered ring. Epoxides can be formed through the oxidation of alkenes with a peroxycarboxylic acid. Peroxycarboxylic acids contain an electrophilic oxygen that is added to the double bond (epoxidation of the double bond). An example of a peroxycarboxylic acid used in laboratory is performic acid, which is obtained by mixing formic acid (HCO_2H) with hydrogen peroxide (H_2O_2). For the reaction to proceed, the use of an inert solvent such as chloroform, dichloromethane or benzene is required (Cepa *et al.*, 2008; Vollhardt and Schore, 2011). Steroidal epoxides are steroid molecules containing an epoxide group and an example of a steroidal epoxide formation is presented in Figure 12.

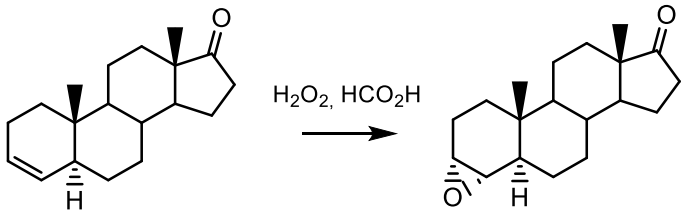


Figure 12 - Schematic representation of a steroidal epoxide formation using H_2O_2, HCO_2H.

Oximes are a chemical group with the general formula $R^1R^2C=N\text{-}OH$ and they exist as colorless crystals and are poorly soluble in water (Aakeröy *et al.*, 2013; Canario *et al.*, 2018). Aldehydes and ketones condense with various amine derivatives to form imine products that are very crystalline and have sharp melting points which means these

molecules are easily obtained in the laboratory (Vollhardt and Schore, 2011). Steroidal oximes can be formed through the reaction of a steroid, containing a ketone or an aldehyde group, with a particular amine derivative such as hydroxylamine (NH_2OH) (Cepa *et al.*, 2008; Vollhardt and Schore, 2011). An example of the formation of a steroidal oxime is presented in Figure 13.

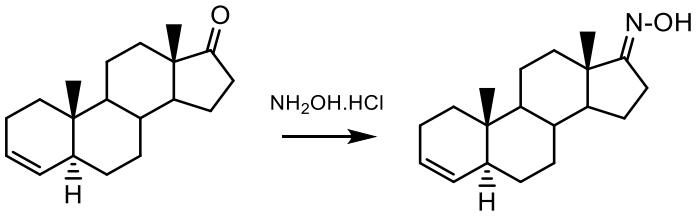


Figure 13 - Schematic representation of a steroidal oxime formation using $NH_2OH.HCl$.

Antitumour activity of steroidal oximes and epoxides

In 1997, scientists discovered several steroidal molecules with very interesting and unusual structures isolated from *Cinachyrella* marine sponges such as (6*E*)-hydroximinocholest-4-en-3-one (**1**) and its 24-ethyl analogue (**2**) (Rodriguez *et al.*, 1997). Both of these molecules represented in Figure 14, have the particularity of having an oxime group and have shown relevant antiproliferative activity against several types of cancer cells (Deive, Rodríguez and Jiménez, 2001; Canario *et al.*, 2018).

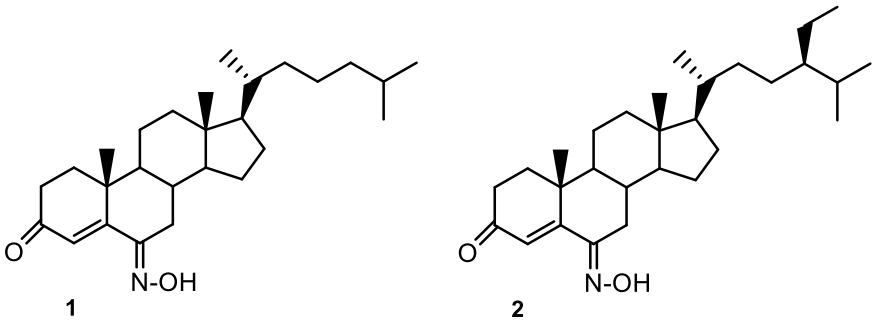


Figure 14 - Structure of steroidal oximes (6E)-hydroxyiminocholest-4-en-3-one (1) and (6E)-hydroxyimino-24-ethylcholestan-4-en-3-one (2) isolated from marine sponges. Adapted from (Rodriguez *et al.*, 1997).

After this, a lot of steroidal oximes have been designed and proved to have antitumoural activity. For example, the compound in Figure 15 showed cytotoxicity against PC3 cancer cells (IC_{50} = 49.8 nmol) (Cui *et al.*, 2009) and the one in Figure 16 demonstrated apoptotic effects on cervicouterine cancer cell line but not in normal cells (Sánchez-Sánchez *et al.*, 2016). Dhingra and collaborators (Dhingra *et al.*, 2011) developed a series

of 17-oximino-5-androsten-3β-yl esters and evaluated their antiproliferative activities against a PCa cell line (DU-145). The compounds revealed to have good antiproliferative activity presenting IC_{50} ranging from 2.3 to 6.5 μM. In a study by Dimas and collaborators (Dimas *et al.*, 2014) several steroidal cardiac inhibitors were synthesized and their anticancer activity *in vitro* and *in vivo* was explored, with the compounds showing potent antitumour activity in multiple cell lines from different tumours. The most promising compound, presented in Figure 17, demonstrated remarkable potencies (as measured by half growth inhibition - GI_{50}, total growth inhibition concentration - TGI and half lethal concentration - LC_{50} values) in most cells *in vitro* (Dimas *et al.*, 2014). Furthermore, other studies about the same compound and also about istaroxime (Figure 18) reported promising results, mostly, in PCa cells (Honisch *et al.*, 2014; Alevizopoulos *et al.*, 2016; Stagno *et al.*, 2017).

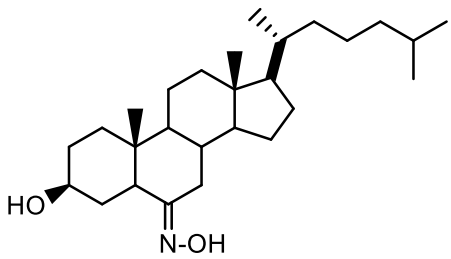


Figure 15 - Structure of (6*E*)-Hydroximinocholestan-3β-ol. Adapted from (Cui *et al.*, 2009).

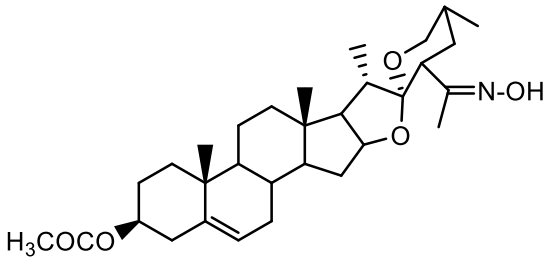


Figure 16 - Structure of the steroidal oxime (23*R*)-acetyldiosgenin acetate. Adapted from (Sánchez-Sánchez et al., 2016).

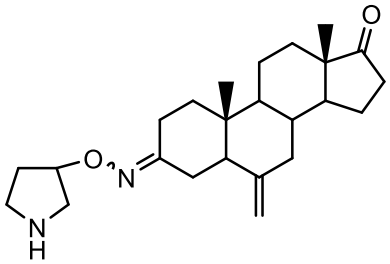


Figure 17 – Structure of the 3-[(*R*)-3-pyrrolidinyl] oxime derivative. Adapted from (Dimas *et al.*, 2014).

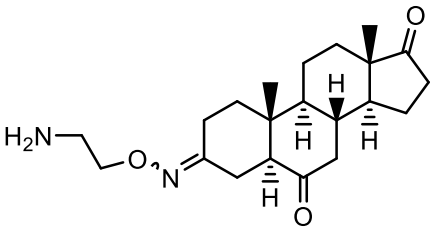


Figure 18 – Structure of istaroxime. Adapted from Gobbini *et al*., 2008.

Steroidal oximes are not the only interesting molecules when it comes to antitumour compounds. In fact, it has also been proven that the introduction of an epoxide group in a steroid skeleton, can originate compounds with anticancer properties. Given this, a lot of steroidal epoxides have been synthesized. For example, in a study made with breast cancer cells, it was shown that the steroidal epoxide molecule, shown in Figure 19, exerted antiproliferative and cytotoxicity activities in the referred cells (Cepa *et al.*, 2008). When

assessing cell cycle, an increase in G_0/G_1 and a decrease in S and G_2/M phases was found. Moreover, thymidine incorporation results together with the fraction of cells in S phase suggested that cell proliferation may continue, however, with a reduction in growth rate (Cepa *et al.*, 2008).

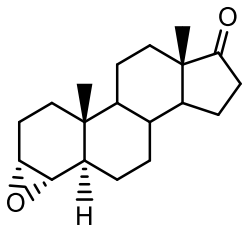


Figure 19 - Structure of the steroidal epoxide 3α ,4α-epoxy-5α-androstan-17-one. Adapted from (Cepa *et al.*, 2008).

Moreover, epoxide derivatives of exemestane were synthesized, namely 6β-spirooxiranandrosta-1,4-diene-3,17-dione (Figure 20, compound **3**) and 1α,2α-epoxy-6 methylenandrost-4-ene-3,17-dione (Figure 20, compound **4**) (Varela *et al.*, 2014). The substitution of the C-6 exocyclic and the C-1 double bonds, respectively, by epoxide groups, led to very potent derivatives in MCF-7aro cells. Compound **3** presented an IC_{50} of 0.73 µM in MCF-7aro cells, and compound **4** presented an IC_{50} of 1.18 µM and in the same cells. Both compounds induced a decrease in cell viability mainly in an aromatase dependent manner. (Varela *et al.*, 2014).

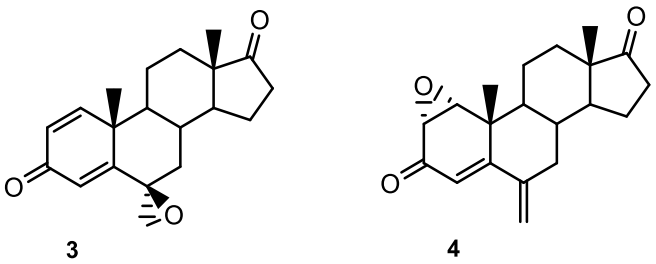


Figure 20 - Structure of 6β-spirooxiranandrosta-1,4-diene-3,17-dione (3) and 1α,2α-epoxy-6-methylenandrost-4-ene-3,17-dione (4). Adapted from (Varela *et al.*, 2014).

5α,6α-Epoxides presented in Figure 21, were synthesized and evaluated by Santafé and collaborators (Santafé *et al.*, 2002). Both compounds demonstrated a pronounced cytotoxicity against PC3 tumour cells (IC_{50}=1-5 µg/mL for **5** and 1 µg/mL for **6**).

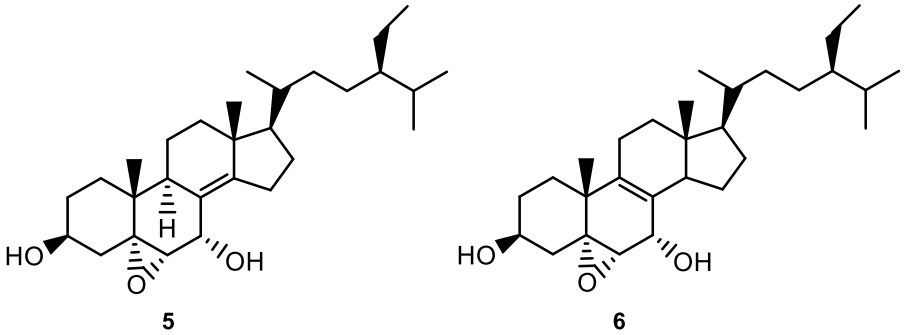


Figure 21 - Structure of 5α,6α-epoxy-24*R**-ethylcholest-8(14)-en-3β,7α-diol (**5**) and 5α,6α-epoxy-24*R**-ethylcholest-8-en-3β,7α-diol (**6**). Adapted from (Santafé *et al.*, 2002).

Finally, 6α,7α-Epoxy-17-oxa-ɒ-homoandrosta-1,4-diene-3,16-dione (Figure 22) demonstrated a very strong cytotoxic activity against PC3 cells (IC_{50}= 2.2±0.04 μM) and lower cytotoxicity in other two cell lines, MCF-7 and MDA-MB-231 (IC_{50}>100 μM for both cancer cell lines) (Djurendić *et al.*, 2008). In parallel, this steroid lacked cytotoxicity against the MRC5 (normal fetal lung fibroblasts) (Djurendić *et al.*, 2008). This study enlightened, once again, that the introduction of an epoxide function in the androstane derivatives had tremendous effects on the cytotoxic activity of these compounds.

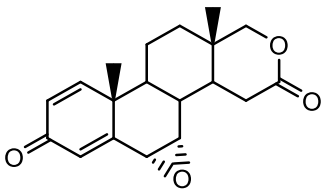


Figure 22 - Structure of 6α,7α-epoxy-17-oxa-D-homoandrosta-1,4-diene-3,16-dione. Adapted from (Djurendić *et al.*, 2008).

Mechanisms of action

One of the mechanisms described for the antitumour activity of the steroidal oximes is enzyme inhibition. This is the case of the 17α-hydroxylase/C17-20-lyase (P450 17, CYP 17) and 5α-reductase, which are two enzymes involved in androgen biosynthesis and are targets for the treatment of prostatic diseases (Hartmann *et al.*, 2000). The steroidal oximes have the capacity of forming a coordinate bond with the heme iron group of 5α-reductase (Aggarwal *et al.*, 2010). Steroidal oximes have also been associated with inhibition of steroid sulfatase (STS) (Hejaz *et al.*, 1999). High activity of this enzyme is highly associated with the progression of several hormone-dependent cancers (e.g. breast and endometrium),

therefore its inhibition might be a useful strategy to stop the proliferation of cancer cells with increase STS activity (Gupta, Kumar and Negi, 2013). Furthermore, aromatase (Holland *et al.*, 1992; Cepa *et al.*, 2008; Bansal *et al.*, 2010) and 17β-hydroxysteroid dehydrogenase (Fischer *et al.*, 2005; Allan *et al.*, 2006) are other enzymes that can be inhibited by some steroidal oximes.

Another possible mechanism of action of steroidal oximes responsible for their antitumour activity was demonstrated by Sánchez-Sánchez *et al.*, 2016 in which the steroidal oximes synthesized interfered with the cell cycle, ultimately, causing cell death, which was demonstrated by morphological changes, such as DNA contraction, cell shrinking and apoptotic bodies formation. This apoptosis might be triggered through a caspase-dependent process, since their findings revealed the presence of active caspase-3 in the two cell lines studied, HeLa and CaSki, meaning that both compounds activated caspase-3 which, ultimately, resulted in activation of cytoplasmic DNAse which, in turn, is responsible for DNA fragmentation (Sánchez-Sánchez *et al.*, 2016).

Steroidal oximes have been associated with Na^+/K^+-ATPase inhibition. The sodium potassium pump (Na^+/K^+ ATPase) is a transmembrane protein complex composed by multiple isoform combinations of catalytic α, regulatory β and modulatory γ subunits, acting as a key energy driver maintaining ionic and osmotic balance (Dimas *et al.*, 2014). It has been implicated in several signalling pathways but most recently, Na^+/K^+ ATPase has been also investigated as a possible target in a number of cancers such as prostate, lung, colorectal and others (Sakai *et al.*, 2004; Mijatovic *et al.*, 2007; Dimas *et al.*, 2014). In fact, aberrant expression of some subunits of the Na^+/K^+ ATPase has been reported in cancer cells (Newman *et al.*, 2008). Following this line, a promising class of novel, non-sugar containing, steroidal compounds emerged and among them are some steroidal oximes (Micheletti *et al.*, 2002; Gobbini *et al.*, 2008; Shah *et al.*, 2009; Dimas *et al.*, 2014; Honisch *et al.*, 2014; Alevizopoulos *et al.*, 2016; Stagno *et al.*, 2017).

Relatively to steroidal epoxides and like steroidal oximes, one of the mechanisms described for their antitumour activity is enzyme inhibition, namely, aromatase inhibition as it was described in several studies (Cepa *et al.*, 2005; Cepa *et al.*, 2008; Varela *et al.*, 2012; Amaral *et al.*, 2013a; Varela *et al.*, 2014; Roleira *et al.*, 2019).

Moreover, other mechanisms by which steroidal epoxides can act include cell cycle arrest at G_0/G_1 phase (Cepa *et al.*, 2008; Amaral *et al.*, 2015) or at G_2/M phase (Choi *et al.*, 2006; Amaral *et al.*, 2017) accompanied by induction of apoptosis through different pathways (Qi *et al.*, 2011; Rah *et al.*, 2012; Peng *et al.*, 2017). Steroidal epoxides were also associated with a decrease in the mitochondrial transmembrane potential ($\Delta\Psi_m$) (Qi *et al.*,

2011; Amaral *et al.*, 2015; Amaral *et al.*, 2017) and an increase in ROS production (Amaral *et al.*, 2015; Peng *et al.*, 2017; Han *et al.*, 2018).

Some steroidal epoxides have also been described as Na^+-K^+-ATPase inhibitors (Yeh *et al.*, 2003; Qi *et al.*, 2011; Lu *et al.*, 2017; Peng *et al.*, 2017; Dai *et al.*, 2018), which as it was referred earlier in this dissertation, is being investigated as a promising anticancer feature.

Although there are already some studies, reported to date, about the anticancer properties of steroidal oximes and epoxides, there is still a lot to learn about these group of compounds. This is proven by the fact that, currently, many of the synthesized compounds do not reach pharmaceutical market, due to several factors such as lack of selectivity towards the desired target, the target might not have been well-identified or there might be more than one target, among others. So, in line with this, it is essential to continue to explore this type of compounds to try to design new drugs with fewer side effects and higher cytotoxicity towards cancer cells. Moreover, performing more *in vitro* and *in vivo* studies is crucial, not only to better explore the mechanisms of action of steroidal oximes and epoxides but also to confirm the efficiency of both families of compounds in clinical practice.

OBJECTIVES

Objectives

The previous revision of the literature highlights the fact that steroidal oximes and epoxides deserve to be deeply studied as potential anticancer agents. In line with this, the main purpose of this work was to synthesize steroidal oximes and epoxides, combining in the same structure steroids with epoxides, steroids with oximes and finally, steroids with an epoxide and an oxime group and then evaluate their biological activity and antitumour capacities. The synthesized steroidal epoxides and oximes were evaluated in a set of several types of cancer cell lines, which include PCa cells (PC3), CRC cells (WiDr), NSCLC cells (H1299) and HCC cells (HepG2) for their antiproliferative activity. For the most promising compounds (**3,4 – OLOX** and **4,5 – OLOX**), additional studies were performed in PC3 and WiDr cells, in order to understand their mechanisms of action. The assays performed included analysis of cell cycle, cell viability and cell death mechanisms (BAX/BCL-2 expression and mitochondrial membrane potential analysis), oxidative stress and haemolysis quantification.

MATERIALS AND METHODS

Synthesis of steroidal epoxides and oximes

Chemistry

Melting points (Mp) were determined on a Reichert Thermopan hot block apparatus. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Unity 400 operating at 400 MHz (^{1}H) and at 100 MHz (^{13}C). Chemical shifts were recorded in δ values expressed in parts per million (ppm) downfield from tetramethylsilane as an internal standard. All *J*-values are expressed in Hz. Analytical samples for physicochemical and biological assays were obtained by crystallization using methanol as solvent (5α-androst-3-en-17-one oxime) and by column chromatography in the case of androst-4-en-17-one oxime (silica gel 60 with petroleum ether 60-80ºC and ethyl acetate mixtures). Compound 3α,4α-epoxy-5α-androstan-17-one oxime and compound 4α,5α-epoxyandrostan-17-one oxime were obtained immediately after filtration without further purification. Reagents and solvents were used as purchased from the manufacturers without further purification. All the precursor compounds were obtained from testosterone by the routes previously reported (Cepa *et al.*, 2008; Varela *et al.*, 2012).

5α-Androst-3-en-17-one oxime (3,4 – OLOX)

To a solution of 5α-androst-3-en-17-one **(3,4 – OL)** (31.7 mg; 0.12 mmol) in methanol (2 mL), hydroxylamine hydrochloride (10.54 mg; 0.153 mmol) and $CH_3COONa.3H_2O$ (19.6 mg; 0.144 mmol) were added. The reaction was stirred at 40ºC for 2 h and 25 min until all the starting material has been consumed. In order to control the reaction, thin-layer chromatography in silica gel TLC plates was performed (hexane:ethyl acetate, 8:2). After water addition, the precipitated formed was removed from suspension by filtration under reduced pressure. The resulting steroidal oxime was purified by crystallization from methanol. The crystals were then washed with hexane and dried yielding the pure compound 5α-androst-3-en-17-one oxime **(3,4 – OLOX)** (10 mg, 30%). Melting point, ^{1}H NMR and ^{13}C NMR were previously described (Cepa *et al.*, 2008).

3α,4α-Epoxy-5α-androstan-17-one oxime (3,4 – EPOX)

To a solution of 3α,4α-epoxy-5α-androstane-17-one **(3,4 – EP)** (29.7 mg; 0.104 mmol) in methanol (2 mL), hydroxylamine hydrochloride (10.8 mg; 0.157 mmol) and $CH_3COONa.3H_2O$ (0.136 mmol) were added. The reaction was stirred at 40ºC for 4 h until all the starting material has been consumed. In order to control the reaction, thin-layer chromatography in silica gel TLC plates was performed (hexane:ethyl acetate, 7:3). After water addition, the precipitated formed was removed from suspension by filtration under reduced pressure and dried yielding the pure compound 3α,4α-epoxy-5α-androstan-17-one oxime **(3,4 – EPOX)** (17.9 mg, 57%). Melting point, ^{1}H NMR and ^{13}C NMR were previously described (Cepa *et al.*, 2008).

Androst-4-en-17-one oxime (4,5 – OLOX)

To a solution of androst-4-en-17-one **(4,5 – OL)** (30.0 mg; 0.11 mmol) in methanol (2 mL), hydroxylamine hydrochloride (11.4 mg; 0.162 mmol) and $CH_3COONa.3H_2O$ (21.4 mg; 0.157 mmol) were added. The reaction was stirred at 40ºC for 4 h until all the starting material has been consumed. In order to control the reaction, thin-layer chromatography in silica gel TLC plates was performed (hexane:ethyl acetate, 8:2). After water addition, the precipitated formed was removed from suspension by filtration under reduced pressure. The residue was then purified by column chromatography (petroleum ether 60-80ºC:ethyl acetate, 95:5) affording the pure compound androst-4-en-17-one oxime **(4,5 – OLOX)** (26.3 mg, 64%). $Mp_{(petroleum\ ether\ 60\text{-}80ºC/ethyl\ acetate)}$: 151-153ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.84 (3H, *s*, 19-H_3), 0.99 (3H, *s*, 18-H_3), 5.26 (1H, *t*, $J_{4,3}$ =3.81), 10.04 (1H, *s*, NOH); ^{13}C NMR (100 MHz, DMSO-*d*6) δ: 17.1 (C-19), 18.7 (C-18), 19.0, 20.5, 22.7, 24.7, 25.1, 31.8, 32.2, 34.1, 34.8, 36.7, 37.1, 43.0, 53.1, 54.0, 118.9 (C-4), 143.9 (C-5), 167.8 (C-17).

4α,5α-Epoxyandrostan-17-one oxime (4,5 – EPOX)

To a solution of 4α,5α-epoxyandrostan-17-one **(4,5 – EP)** (60.5 mg; 0.14 mmol) in methanol (4 mL), hydroxylamine hydrochloride (22.6 mg; 0.322 mmol) and $CH_3COONa.3H_2O$ (42.8 mg; 0.314 mmol) were added. The reaction was stirred at 40ºC overnight until all the starting material has been consumed. In order to control the reaction, thin-layer chromatography in silica gel TLC plates was performed (hexane:ethyl acetate, 7:3). After water addition, the precipitated formed was removed from suspension by filtration under reduced pressure and dried yielding the pure compound 4α,5α-epoxyandrostan-17-

one oxime **(4,5 – EPOX)** (37.8 mg, 90%). $Mp_{(methanol)}$: 204-206ºC. ^{1}H NMR (400 MHz, DMSO-*d*6) δ: 0.832 (3H, *s*, 19-H_3), 0.949 (3H, *s*, 18-H_3), 2.87 (1H, *d*, $J_{4\beta,3H}$ =3.96), 10.06 (1H, *s*, NOH); ^{13}C NMR (100 MHz, DMSO-*d*6) δ: 14.8 (C-19), 17.1 (C-18), 19.0, 20.4, 22.8, 23.1, 24.7, 29.4, 29.5, 30.8, 33.89, 33.92, 35.9, 43.0, 46.00, 53.0, 60.1, 64.6, 167.8 (C-17).

Evaluation of the synthesized compounds *in vitro*

Cell culture

In this study four cancer cell lines namely, WiDr (ATCC® CCL-218™), H1299 (ATCC® CRL-5803™), HepG2 (ATCC® HB-8065™) and PC3 (ATCC® CRL-1435™) cancer cells and two normal cell lines, namely normal human colon epithelial cell line, CCD841 CoN (ATCC® CRL1790™) and normal human lung cell line, MRC5 (ATCC® CCL-171™) were used. All cell lines, excepted for CCD841 CoN, were acquired from American Type Collection Culture (ATCC, USA) and were maintained in optimal conditions at 37ºC in 5% CO_2 atmosphere following the repository instructions (ATCC). CCD-841 CoN cells were kindly provided by Instituto de Investigación Sanitaria de la Fundación Jiménez Díaz (Madrid, Spain). The cell lines WiDr, H1299, HepG2, CCD841 CoN and MRC5 were cultured in *Dulbecco's Modified Eagle Medium*, DMEM (Sigma, D5648) and PC3 cell line was cultured in *Roswell Park Memorial Institute Medium*, RPMI-1640 (Sigma, R4130). Both mediums were supplemented with 5% heat-inactivated fetal bovine serum, FBS (Sigma, F7524, except for CCD841 CoN and MRC5 that were supplemented with 15% FBS), antibiotics (100 U/mL of penicillin and 10 µg/mL streptomycin; Sigma, A5955) and 0.25 mM sodium pyruvate (Gibco, 11360) for DMEM and 1 mM for RPMI.

Given that all cell lines used in this study were maintained in adherent culture, it was necessary to detach the cells from the flasks. For that, a solution of *TrypLE™ Express* (Gibco, 12605-028) was used to dissociate the cellular monolayer. After adding the reagent, cells were incubated for 5 to 10 min at 37ºC in 5% CO_2, followed by inactivation with the appropriated culture medium for each cell line. Afterwards, a suspension of each cell line stained with trypan blue (Sigma, 302643) was counted with the aid of a Neubauer chamber and an inverted microscope (Nikon, Eclipse TS 100) with a 100x amplification.

Treatment with the compounds synthesized

Initially, solutions of 15 mM of each compound dissolved in dimethylsulfoxide (DMSO, Sigma, D4540) were prepared. Before treatment of each cell line, solutions of each compound were prepared by diluting the original solution so that the volume to be added to the cultures was up to 1% of the culture medium volume in each well or flask.

To start, the cytotoxic effect of each compound was evaluated in the four cancer cell lines previously described (WiDr, H1299, HepG2 and PC3). The cells were seeded in 48 well-plates (Sarstedt, Germany) at a density of 100.000 cells/mL in a volume of 500 µL per well. After incubation overnight, cells were treated with steroids **3,4 – OL**; **3,4 – EP**; **4,5 – OL**; **4,5 – EP** and **3,4 – OLOX**; **3,4 – EPOX**; **4,5 – OLOX** and **4,5 – EPOX** at different concentrations ranging from 1 to 75 µM for 72 h.

For further studies, WiDr and PC3 cells and compound **3,4 – OLOX** and **4,5 – OLOX** were selected since they were the ones with better results.

To test the selectivity of **3,4 – OLOX** and **4,5 – OLOX**, CCD841 CoN and MRC5 non-tumour cell lines were seeded in 48-well plates (Sarstedt, Germany) at a density of 100.000 cells/mL in a volume of 500 µL per well. After incubation overnight, cells were treated with steroids **3,4 – OLOX** and **4,5 – OLOX** at different concentrations ranging from 1 to 75 µM for 72 h.

WiDr and PC3 cells were seeded in 75 cm^2 flasks (SPL Life Sciences, Korea) at a density of $4x10^6$ cells/flask. After overnight incubation, cells were treated with compounds **3,4 – OLOX** and **4,5 – OLOX** at a concentration corresponding to the IC_{50} and a concentration higher than the IC_{50} values obtained for both compounds, as represented in Table 1 and 2. The following assays were then performed by flow cytometry: detection of different types of cell death, analysis of cell cycle, analysis of the expression of intracellular proteins BAX and BCL-2 and evaluation of the mitochondrial membrane potential.

For the reactive oxygen species assay measured by fluorimetry, WiDr and PC3 cells were plated at a density of $5x10^6$ cells/flaks and, after incubation overnight, they were treated with the two compounds at a concentration corresponding to the IC_{50} and a concentration higher than the IC_{50} value, which are represented in Table 1 and 2.

Lastly, haemolytic activity of **3,4 – OLOX** and **4,5 – OLOX** was evaluated. The concentrations used were the same for both compounds (10 µM and 75 µM). Two controls were used: a positive control, triton X-100, which promotes cellular lysis and a negative control, DMSO corresponding to the solvent.

The selection of the concentrations used in all the studies relies on the results obtained from the cytotoxicity assay.

Controls of untreated cells were performed, and results are expressed as a percentage of the cells treated with the solvent (DMSO) for the cytotoxicity assay and as a percentage of the control cells for the other studies.

Table 1 – Concentrations of **3,4 – OLOX** and **4,5 – OLOX** tested in WiDr for each assay.

Cell line	Assay		IC_{50}		$>IC_{50}$	
			3,4 - OLOX	**4,5 - OLOX**	**3,4 - OLOX**	**4,5 - OLOX**
WiDr	**Fluorimetry**	DHE	9.1 µM	16.1 µM	15 µM	30 µM
		DCF				
		GSH				
	Flow Cytometry	AN/IP	9.1 µM	16.1 µM	50 µM	50 µM
		JC-1				
		BAX/BCL2				
		Cell cycle			40 µM	40 µM

Table 2 - Concentrations of **3,4 – OLOX** and **4,5 – OLOX** tested in PC3 for each assay.

Cell line	Assay		IC_{50}		$>IC_{50}$	
			3,4 - OLOX	**4,5 - OLOX**	**3,4 - OLOX**	**4,5 - OLOX**
PC3	**Fluorimetry**	DHE	13.8 µM	14.5 µM	20 µM	25 µM
		DCF				
		GSH				
	Flow Cytometry	AN/IP	13.8 µM	14.5 µM	50 µM	50 µM
		JC-1				
		BAX/BCL2				
		Cell cycle			40 µM	40 µM

Antiproliferative effect

In order to understand the antiproliferative effects of the steroidal derivatives previously synthesized, the MTT assay was used to evaluate cell metabolic activity and flow cytometry was used to assess cell cycle alterations.

Assessment of metabolic activity by the MTT assay

Evaluation of metabolic activity was performed by the colorimetric assay MTT, 3-(4,5-dimethylthiazolyl-2)2,5-diphenyltetrazolium bromide. MTT, a yellow tetrazole reagent, is reduced forming purple formazan crystals in the presence of dehydrogenase enzymes located mainly in the viable mitochondria, *i.e*., in metabolically active cells (Mosmann, 1983). Metabolic activity was evaluated as a measure of cell proliferation.

After an incubation time of 72 h, the medium was removed and the cells were washed with phosphate buffer saline, (PBS, 137 mM NaCl [S7653, Sigma, USA], 2.7 mM KCl [P9333, Sigma, USA], 10 mM $Na_2HPO_4.2H_2O$ [S5011, Sigma, USA]) and 2 mM KH_2PO_4 [P0662, Sigma, USA], pH=7.4). Then, 150 µL of an MTT solution (Sigma, USA) at 0.5 mg/mL and pH=7.4 was added to each well and cells were incubated for about 3 h in the dark at 37ºC. Next, 150 µL of isopropanol (Sigma, USA) at 0.04 M was added and the plates were agitated to dissolve the purple formazan crystals. Absorbance values were measure at 570 and 620 nm in an ELISA spectrophotometer (Synergy HT, Biotek, USA).

The percentual data obtained allowed to establish dose-response curves and to determine the half maximal inhibitory concentration of the compounds, used in this study (IC_{50}), using the GraphPad software 6.0.

Cell cycle analysis by flow cytometry

The effect of the two compounds in cell cycle progression (G_0/G_1 phase, G_2/M phase and S phase) was evaluated through flow cytometry by staining with a solution of propidium iodide (PI) with RNase, 72 h after treatment with **3,4 – OLOX** and **4,5 – OLOX**. As it was mentioned above, PI can intercalate with DNA and RNA bases. Therefore, this experiment requires incubation with RNase to obtain a specific staining only for DNA. The dye binds stoichiometrically to the cells' DNA allowing to obtain an histogram of the distribution of the different cell populations in each of the cell cycle phases (Deitch, Law and White, 1982). This is possible due to the fact that S phase cells present more DNA content than the cells

in G_0/G_1 phase and the cells in G_2/M phase present twice the amount of DNA than cells in G_0/G_1 phase. It is also possible to identify an apoptotic peak, with less DNA amount.

To each assay, $1x10^6$ cells were detached from the flasks and centrifuged at 1300xG for 5 min in a Heraeus centrifuge (Multifuge 1L-R, Kendro Laboratory Products, Germany). To fix the cells, 200 µL of ethanol at 70% was added to the sediments in agitation and then the samples were incubated for 30 min in the dark at 4ºC. After incubation time, the samples were centrifuged at 1300xG for 5 min, washed with 2 mL of PBS and to the resulting pellet, 200 µL of a PI/RNase solution (Immunostep, PI/RNase) was added and incubated for 15 min in the dark at room temperature. The analysis was performed in a FACSCalibur flow cytometer with excitation and emission wavelengths of 488 and 640 nm, respectively. Results were expressed as a percentage of the cell in each subpopulation: pre-G_0/G_1, G_0/G_1, S and G_2/M.

Analysis of cell viability and cell death mechanisms

To assess if the cytotoxic effect of **3,4 – OLOX** and **4,5 – OLOX** is mediated by cell death pathways, cell viability and cellular death mechanisms were evaluated. Posteriorly, in order to characterize cell death pathways induced by both compounds, several parameters were evaluated such as BAX and BCL-2 expression and changes in mitochondrial membrane potential ($\Delta\Psi_m$).

Analysis of viability and cell death by flow cytometry

Cell viability and the different induced types of cell death were assessed by flow cytometry, 72 h after treatment with **3,4 – OLOX** and **4,5 – OLOX**, by double staining the cells with annexin V (AV) stained with the fluorescein isothiocyanate fluorochrome, FITC, and PI.

Alteration of the plasmatic membranes' architecture in which there is a redistribution of several phospholipids species is one of the modifications associated with apoptosis. One of these species is phosphatidylserine that suffers a translocation from the inner to the outer layer of the plasmatic membrane at the beginning of apoptosis process (Vermes *et al.*, 1995). Therefore, the high affinity of AV to phosphatidylserine allows discriminating between viable and apoptotic cells (Vermes, Haanen and Reutelingsperger, 2000). On the other hand, the plasmatic membranes' rupture which indicates late apoptosis/necrosis, allows the entry of PI to the intercellular space where PI intercalates with DNA and emits fluorescence

(Abrantes *et al.*, 2010). Thereby, the double staining provides a way to distinguish four populations of cells: the population of live cells, negative for both staining; the population of early apoptotic cells, positive for staining with AV-FITC and negative for staining with PI; the population of late/necrotic cells, positive for both AV-FITC and PI and the population of necrotic cells, negative for the AV-FITIC staining and positive for the PI staining (Vermes, Haanen and Reutelingsperger, 2000).

To carry out this evaluation, $1x10^6$ cells per assay were detached from the flasks. Cell suspensions were, then, centrifuged at 1300xG for 5 min and the obtained pellets were resuspended in 1 mL of PBS and centrifuged again in the same conditions. Cells were incubated with 100 µL of binding buffer (0.01 M Hepes [H7523, Sigma, USA], 0.14 M NaCl and 0.25 mM of $CaCl_2$ [C4901, Sigma, USA]), 2.5 µL of AV-FITC (ANXVKF, Immunostep) and 1 µL of PI (ANXVKF, Immunostep) during 15 min at room temperature in the dark. After incubation time, 400 µL of binding buffer was added. Analysis was performed in a cytometer (FACSCalibur, Becton Dickinson, USA) using excitation wave lengths of 488 nm and emission wave lengths of 530 nm for AV-FITC and 640 nm for PI. Results are expressed as the percentage of identified cells in each subpopulation.

Analysis of intracellular proteins BAX and BCL-2

Intracellular proteins BCL-2 (B-cell lymphoma-2) and BAX (BCL-2 associated X protein) play a major role in the cellular balance between survival and mitochondrial apoptosis (Edlich, 2018). Evaluation of these two proteins was fulfilled 72 h after treatment with **3,4 – OLOX** and **4,5 – OLOX** by flow cytometry.

For each assay, $1x10^6$ cells were detached from the flasks and centrifuged at 1300xG for 5 min. After washing with PBS, cells were centrifuged at the same conditions and the pellet was fixed with 100 µL of fixation solution (Immunostep, Intracell Kit, Spain) for 15 min in the dark and at room temperature. After the incubation, cells were washed with 2 mL of PBS and centrifuged at 1300xG for 5 min. The pellet was incubated with 100 µL of a permeabilization solution (Intracell Kit, Immunostep, Spain) and then, 2.5 µL of anti-BAX-PE antibody (sc-20067 PE, Santa Cruz Biotechnology) and 2.5 µL of anti-BCL-2-FITC antibody (sc-509 FITC, Santa Cruz Biotechnology) were added and the samples were incubated for 15 min in the dark at room temperature. After incubation time, 2 mL of PBS was added, and the cell suspension was centrifuged at 1300xG for 5 min. Finally, the pellet was resuspended in 400 µL of PBS. The analysis was performed in a FACSCalibur cytometer with excitation and emission wavelengths of 585 nm for BAX and 530 nm for BCL-2.

Evaluation of the mitochondrial membrane potential

The $\Delta\Psi_m$ is a crucial event in terms of progression of apoptosis. It is measured using the fluorescence dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide). JC-1 is a lipophilic and cationic dye that enters in the mitochondria in the form of monomers (M) and under mitochondria polarization conditions forms aggregates (A) that emit fluorescence in the red zone. With the decrease of the $\Delta\Psi_m$, JC-1 is excluded from the mitochondria and is dispersed in the cytoplasm in the form of monomers that emit fluorescence in the green zone. The ratio between green fluorescence and red fluorescence (M/A) gives us an estimation of the $\Delta\Psi_m$, regardless of mitochondrial mass (Cossarizza *et al.*, 1993). Analysis of mitochondrial membrane potential was performed 72 h after treatment.

To carry out this assay, $1x10^6$ cells were detached from the flasks, centrifuged at 1300xG for 5 min and then 1 mL of PBS was added. The samples were centrifuged again at the same conditions and incubated with JC-1 (Sigma, T4069) at a concentration of 5 mg/mL for 15 min at 37ºC. Then, 2 mL of PBS was added to each sample and centrifuged once again at 1300xG for 5 min. Finally, 400 µL of PBS was added and the detection was performed in a FACSCalibur cytometer with excitation wavelengths of 530 nm for the monomers and 590 nm for the aggregates. Results are expressed as the ratio variation of the fluorescence intensities of monomers/aggregates (M/A), regarding the control.

Reactive oxygen species (ROS)

ROS are generated not only during oxidative metabolism but also in response to foreign agents to the cellular microenvironment which ultimately, induces protein modifications (Moldogazieva, Lutsenko and Terentiev, 2018). The assays to measure ROS production were performed 24 h after treatment with **3,4 – OLOX** and **4,5 – OLOX** by fluorimetry. After treatment, $1x10^6$ cells were detached from the flasks and centrifuged for 5 min at 1300xG. After washing the cells with PBS, they were centrifuged again at the same conditions and 1 mL of PBS was added to each sample.

Analysis of intracellular content of peroxides

Cells were incubated with 5 µM of $DCFH_2$-DA dye (2',7'- dichlorodihydrofluorescein diacetate, [Invitrogen, USA]) for 45 min at 37ºC in the dark. Next, cells were centrifuged for

5 min at 1300xG and the analysis was performed in a spectrophotometer (Synergy HT, Biotek, USA) with excitation and emission wavelengths of 485 and 528 nm, respectively.

Analysis of intracellular content of the superoxide anion

Cell suspension was incubated with 2 µM of DHE dye (*dihydroethidium*, [D7008, Sigma, USA]) for 15 min at 37ºC, in the dark. Then, the cells were centrifuged for 5 min at 1300xG and the reading was performed in a spectrophotometer (Synergy HT, Biotek, USA) with excitation and emission wavelengths of 530 and 645 nm, respectively.

Antioxidant defences

The cellular antioxidant defence system is fundamental to maintain the cellular redox balance when in the presence of oxidant agents. In particular, reduced glutathione (GSH) acts in the detoxification of hydrogen peroxides. The assays to measure GSH were performed 24 h after treatment and evaluated by fluorometry.

Analysis of intracellular levels of reduced glutathione

After treatment, $1x10^6$ cells were detached from the flasks and centrifuged for 5 min at 1300xG. After washing with PBS, cells were centrifuged once again at the same conditions and 1 mL of PBS was added. Each cell suspension was incubated with 1 µM of orange mercury (M7750, Sigma, USA) for 15 min at 37ºC, in the dark. Cells were, then, washed with PBS and centrifuged at 1300xG. The analysis was conducted using a spectrophotometer (Synergy HT, Biotek, USA) with excitation and emission wavelengths of 485 and 590 nm, respectively.

Haemolysis evaluation

Erythrocytes are very abundant cells present in the blood and its mechanical stability represents a very good parameter for *in vitro* assessment of hemocompatibility, since the membrane of erythrocytes can undergo remarkable changes in its structural properties upon drug treatment (Chazin *et al.*, 2018). Therefore, *in vitro* screening of hemocompatibility represents a very important parameter of pre-clinical studies for a new compound to assess

if it can induce lesions in the erythrocytes' membrane or even to evaluate if the compound can be administered intravenously (Naahidi *et al.*, 2014).

The assay performed relies on the measurement of haemoglobin release and was based on a study by Oliveira and collaborators (De Oliveira *et al.*, 2011). The greater amount of haemoglobin release, the greater the lesion in the erythrocytes' membrane and, consequently, the minor the probability of its administration intravenously.

Initially, 1 mL of human blood was collected and diluted in 30 volumes of NaCl 0.85% containing $CaCl_2$ 10 mM. After washing 3 times by centrifugation (1300xG/3 min) a pellet of erythrocytes was obtained. This pellet was resuspended in 2% NaCl 0.85% containing $CaCl_2$ 10 mM and the resultant cell suspension was distributed in 96 well-plates. **3,4 – OLOX** and **4,5 – OLOX** were added to the wells at a concentration of 10 μM and 75 μM. Triton X-100 (1%) was used as a positive control and DMSO was used as a negative control. Cells were incubated for 1 h under constant agitation at room temperature. The samples were, then, centrifuged at 4000 xG for 5 min and the supernatant was transferred to a new 96-well plate. The absorbance was read using a spectrophotometer (Synergy HT, Biotek, USA) at a wavelength of 540 nm. Haemolysis was estimated by quantifying the haemoglobin released according to the following equation:

$$\%haemolysis = \frac{Abs\ (compound)}{Abs\ (positive\ control) - Abs\ (negative\ control)} \times 100$$

Statistical analysis

Statistical analysis was performed using the IBM® SPSS® Statistics software, version 24.0 (IBM Corporation, Armonk, USA). For all comparisons, a significance level of 5% was used and normal distribution was assessed using Shapiro-Wilk test and variance of the quantitative variables using Levene test.

By default, non-parametric tests were used when n<10. For comparisons with control conditions, one-sample T test or Wilcoxon test were used, for parametric or non-parametric analysis, respectively. For comparisons of two conditions, Mann-Whitney test was used or T student test in case of parametric analysis. In case of normal distribution, differences between more than two conditions were performed using the parametric one-factor analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis otherwise,

followed by pos-hoc analysis using Tukey correction (for equal variances assumed) or Games-Howel (for equal variances not assumed).

In the cell proliferation analysis by the MTT assay, the experimental values obtained were adjusted, using the GraphPad software 6.0., to a dose-response sigmoidal model represented in the following equation:

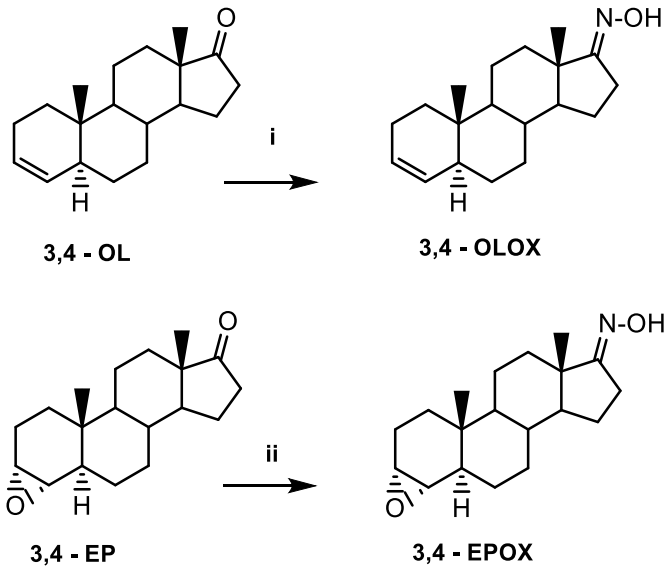
$$Proliferation\ (\%) = A1 + \frac{A2 - A1}{1 + 10^{(log x_0 - x)p}}$$

where A1 and A2 correspond to the plateaus and x_0 corresponds to the half maximal inhibitory concentration (IC_{50}). Significant differences were accounted for when the 95% confident intervals of the compounds did not intersect.

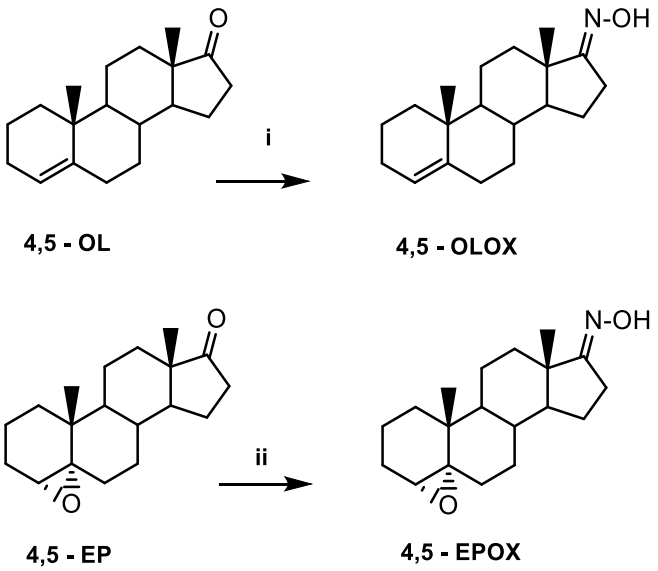
RESULTS

Chemistry

Reaction of compound **3,4 – OL** and **3,4 – EP** with hydroxylamine hydrochloride and sodium acetate in methanol gave the steroidal oxime derivatives **3,4 – OLOX** and **3,4 – EPOX** with 30% and 57% yield, respectively (Scheme 1). Treatment of compound **4,5 – OL** and **4,5 – EP** in methanol with hydroxylamine hydrochloride and sodium acetate yielded the steroidal oximes **4,5 – OLOX** and **4,5 – EPOX** in 64% and 90%, respectively (Scheme 2). NMR studies revealed, unequivocally, that the products of the reactions represented in Scheme 2 were indeed, the planned compounds **4,5 – OLOX** and **4,5 – EPOX**. ^{1}H NMR and ^{13}C NMR spectra for the products of the reactions represented in Scheme 1 had already been reported as it was mentioned before. Chemical properties such as the LogP theoretical values were assessed and are represented in Table 3. These values were obtained by the ChemDraw software.



Scheme 1 - Synthesis of steroidal oximes. Reagents and conditions (i) $NH_2OH.HCl$, $CH_3COONa.3H_2O$, methanol, 40ºC, 2h and 25 min (30%); (ii) $NH_2OH.HCl$, $CH_3COONa.3H_2O$, methanol, 40ºC, 4 h (57%).



Scheme 2 - Synthesis of steroidal oximes. Reagents and conditions (i) $NH_2OH.HCl$, $CH_3COONa.3H_2O$, methanol, 40ºC, 4 h (64%); (ii) $NH_2OH.HCl$, $CH_3COONa.3H_2O$, methanol, 40ºC, Overnight (90%).

Table 3 –LogP theoretical values obtained by the ChemDraw software.

	3,4 – OLOX	**3,4 - EPOX**	**4,5 – OLOX**	**4,5 - EPOX**
LogP	5.48	4.32	5.33	4.35

Antiproliferative effect assessment

The first cytotoxicity study performed in this work allowed to evaluate the effect of increasing concentrations of the synthesized compounds on the metabolic activity of four tumour cell lines previously described. To evaluate the selectivity of the compounds, the metabolic activity of two normal cell lines previously described (CCD841 CoN and MRC5) was also assessed.

Metabolic activity

Metabolic activity after treatment with compounds **3,4 – OLOX**, **3,4 – EPOX**, **4,5 – OLOX** and **4,5 – EPOX**, assessed through the MTT assay, 72 h after treatment at concentrations ranging from 1 to 75 µM, was determined relatively to cell cultures treated with DMSO, to which was assigned a metabolic activity of 100%. Analysis of the results allowed the establishment of the best fit sigmoid curves for each cell line, represented in Figures 23, 24, 25 and 26. Dose-response curves allowed determining IC_{50} values, which are shown in Table 4.

Figure 23 shows the dose-response curves of WiDr cell line treated with all compounds synthesised. In general, there was a dose-dependent decrease in cell proliferation for all compounds with the exception of **3,4 – OL**. However, **3,4 – OLOX** and **4,5 – OLOX** showed a more pronounced decrease. Furthermore, the IC_{50} values calculated (Table 4) demonstrated that, in fact, **3,4 – OLOX** (IC_{50}=9.1 µM) and **4,5 – OLOX** (IC_{50}=16.1 µM) were the ones with better antiproliferative activity, showing significant differences compared to the other compounds [**3,4 – EPOX** (IC_{50}=75 µM) and **4,5 – EPOX** (IC_{50}=53.4 µM)].

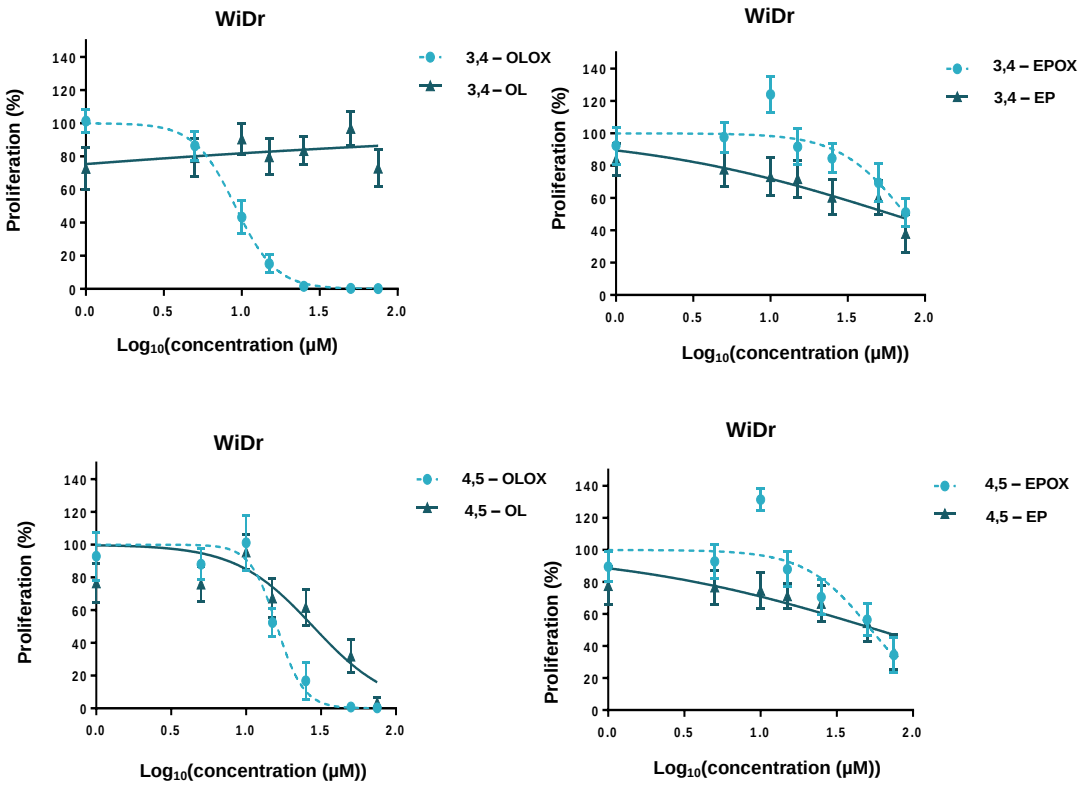


Figure 23 - Dose response curves of WiDr cell line after treatment with compounds **3,4 – OL; 3,4 – EP; 4,5 – OL** and **4,5 – EP** and **3,4 – OLOX; 3,4 – EPOX; 4,5 – OLOX** and **4,5 – EPOX** for 72 h. Results are presented as the percentage of cell proliferation as a function of compounds' concentration (Log_{10}(concentration)) and express the mean and standard deviation of, at least, four independent experiments, in triplicate.

The effect of all compounds in H1299 cell proliferation is represented in Figure 24. The compounds induced different responses in H1299 cell proliferation being that **3,4 – OLOX**, **4,5 – OLOX** and **4,5 – OL** induced a significant dose-dependent decrease cell proliferation, comparing to **3,4 – EPOX**, **3,4 – EP**, **4,5 – EPOX** and **4,5 – EP** that failed to decrease cell proliferation. Moreover, the IC_{50} values (Table 4) of **3,4 – OLOX** (IC_{50}=18.6 µM), **4,5 – OLOX** (IC_{50}=19.2 µM) and **4,5 – OL** (IC_{50}=21.5 µM) were significant different when comparing to **3,4 – EPOX**, **3,4 – EP**, **4,5 – EPOX** and **4,5 – EP**.

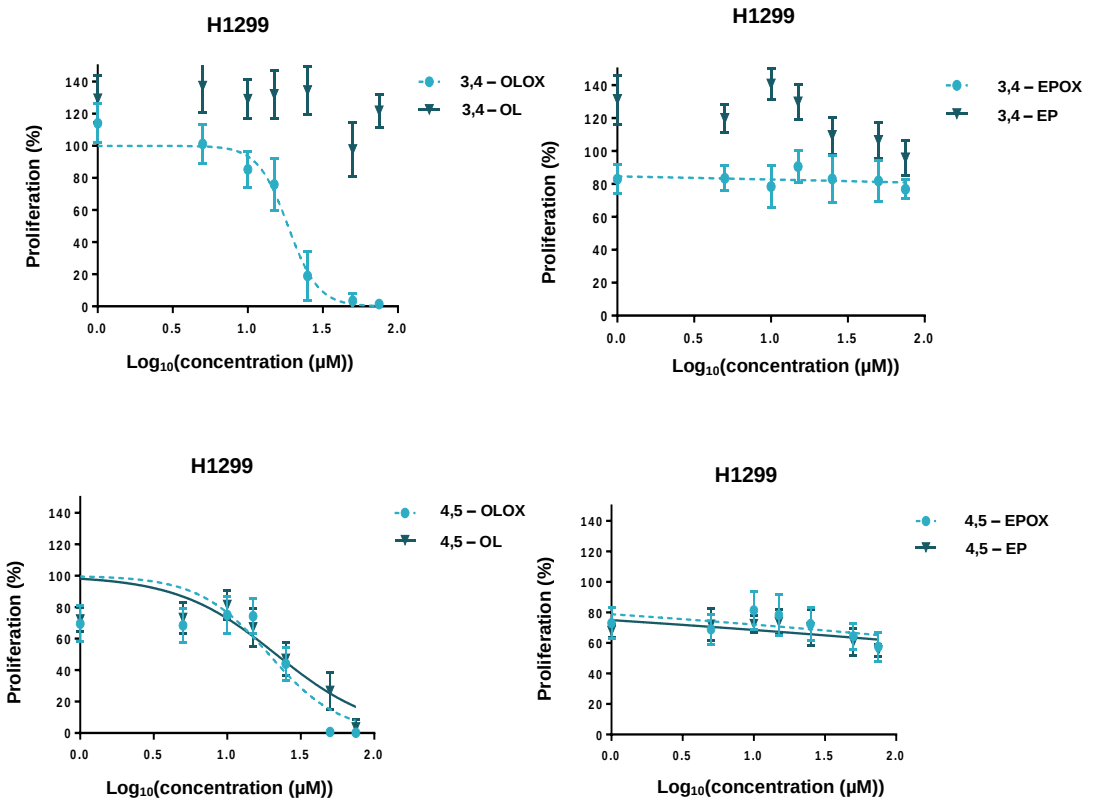


Figure 24 - Dose response curves of H1299 cell line after treatment with compounds **3,4 – OL; 3,4 – EP; 4,5 – OL** and **4,5 – EP** and **3,4 – OLOX; 3,4 – EPOX; 4,5 – OLOX** and **4,5 – EPOX** for 72 h Results are presented as the percentage of cell proliferation as a function of compounds' concentration (Log_{10}(concentration)) and express the mean and standard deviation of, at least, four independent experiments, in triplicate.

In general, all compounds induced a dose-dependent decrease in PC3 cell proliferation as it can be observed in Figure 25. However, **3,4 – OLOX** and **4,5 – OLOX** demonstrated a more accentuated decrease when comparing to the other compounds. Moreover, the compound with the best antiproliferative activity was **3,4 – OLOX**, which is demonstrated by its IC_{50} value (IC_{50}=13.8 µM) (Table 4) that was significantly lower when comparing to all compounds, except **4,5 – OLOX** (IC_{50}= 14.5 µM), the second compound with better antiproliferative activity.

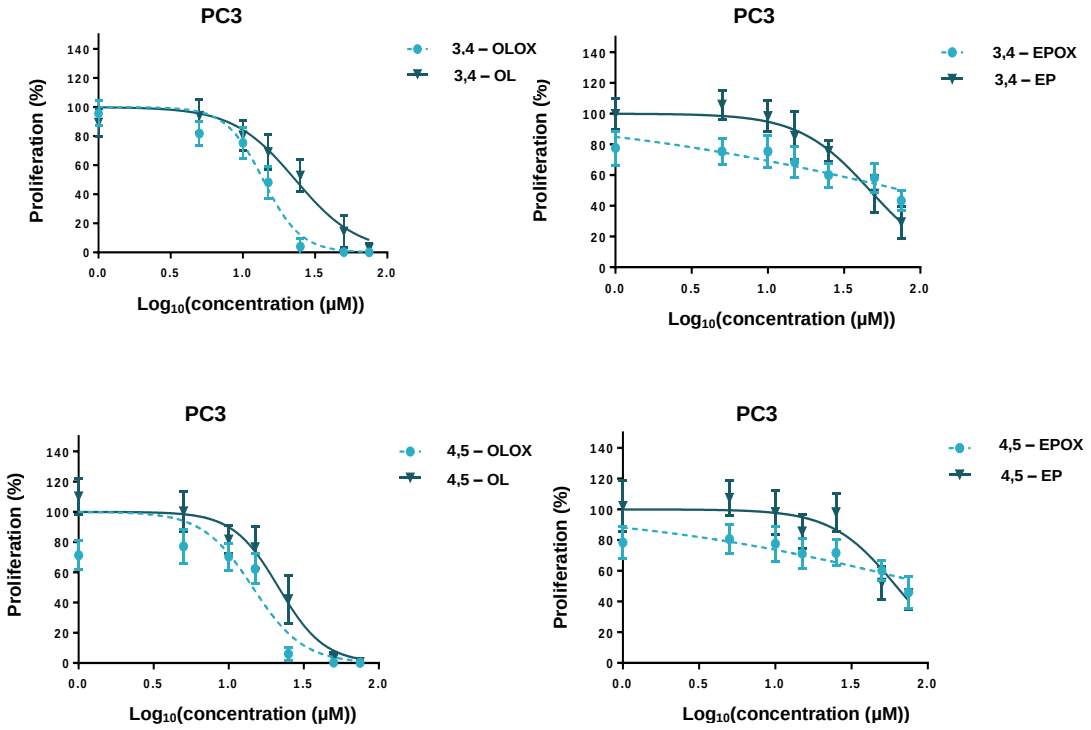


Figure 25 - Dose response curves of PC3 cell line after treatment with compounds **3,4 – OL; 3,4 – EP; 4,5 – OL** and **4,5 – EP** and **3,4 – OLOX; 3,4 – EPOX; 4,5 – OLOX** and **4,5 – EPOX** for 72 h. Results are presented as the percentage of cell proliferation as a function of compounds' concentration (Log_{10}(concentration)) and express the mean and standard deviation of, at least, four independent experiments, in triplicate.

Figure 26 represents the dose-response curves of HepG2 cell line treated with all compounds. Generally, all compounds caused a dose-dependent decrease in HepG2 cell proliferation. IC_{50} values presented in Table 4, were very similar between the two oximes, **3,4 – OLOX** (IC_{50}=23.9 μM) and **4,5 – OLOX** (IC_{50}=18.2 μM) and their parent compounds, **3,4 – OL** (IC_{50}=15.0 μM) and **4,5 – OL** (IC_{50}=17.7 μM), respectively. **3,4 – EPOX** (IC_{50}=64.3 μM) and **4,5 – EPOX** (IC_{50}=52.7 μM) and their parent compounds, **3,4 – EP** (IC_{50}=32.6 μM) and **4,5 – EP** (IC_{50}=42.4 μM), respectively also showed similar IC_{50} values among them.

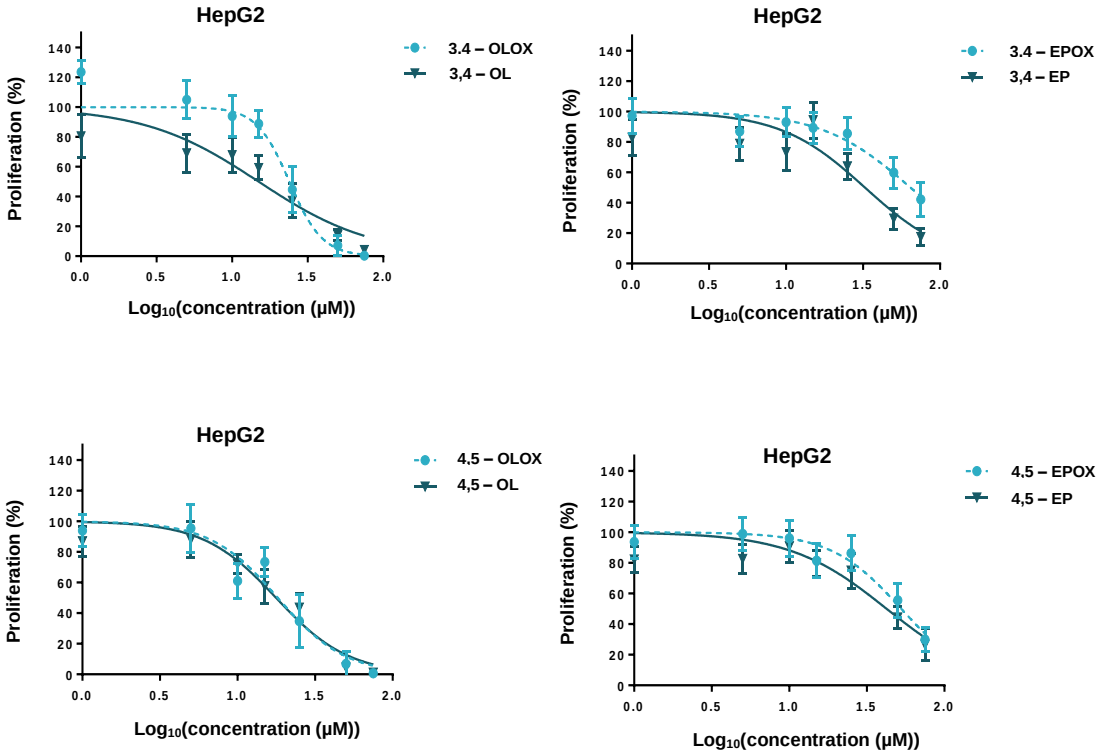


Figure 26 - Dose response curves of HepG2 cell line after treatment with compounds **3,4 – OL; 3,4 – EP; 4,5 – OL and 4,5 – EP** and **3,4 – OLOX; 3,4 – EPOX; 4,5 – OLOX** and **4,5 – EPOX** for 72 h. Results are presented as the percentage of cell proliferation as a function of compounds' concentration (Log_{10}(concentration)) and express the mean and standard deviation of, at least, four independent experiments, in triplicate.

The most active compound in all cell lines was **3,4 – OLOX,** evidenced by the decrease of metabolic activity with the increase of its concentration. WiDr was the most sensitive cell line to compound **3,4 – OLOX** presenting an IC_{50} value of 9.1 µM, significantly lower than in PC3 (IC_{50}=13.8 µM), H1299 (IC_{50}=18.6 µM) and HepG2 (IC_{50}=23.9 µM). Compound **4,5 – OLOX** also exhibited an interesting antiproliferative effect as we can see by the decrease of metabolic activity with the increase in concentration. In this case, PC3 was the most sensitive cell line with an IC_{50} value of 14.5 µM however, the differences were not significant.

Compounds **3,4 – EPOX** and **4,5 – EPOX** did not show good antiproliferative activity as, in general, the metabolic activity does not decrease with the increase of concentration and the differences between IC_{50} in each cell line were not significant. In fact, in H1299, compounds **3,4 – EPOX** and **4,5 – EPOX** totally failed to decrease metabolic activity as it can be seen in Figure 24.

Moreover, compounds **3,4 – OL**, **3,4 – EP**, **4,5 – OL** and **4,5 – EP** were also evaluated, in order to trying to understand if the introduction of an oxime group influenced or not their activity. Results demonstrate that for compound **3,4 – OLOX** and **4,5 – OLOX**

the introduction of an oxime group in their precursors seems to increase their cytotoxic activity as it can be seen by comparing the IC_{50} values of each compound (Table 4). In WiDr and H1299 cancer cell lines, this effect is more pronounced with a significant difference between the IC_{50} values of **3,4 – OL** and **3,4 – OLOX**. Relatively to compounds **4,5 – OL** and **4,5 – OLOX**, although the differences are not significant, IC_{50} values of all cell lines with the exception of HepG2, are lower for compound **4,5 – OLOX**.

Table 4 – IC_{50} values obtained after incubation of WiDr, PC3, H1299 and HepG2 cell lines with compounds **3,4 – OL; 3,4 – EP; 4,5 – OL** and **4,5 – EP** and **3,4 – OLOX; 3,4 – EPOX; 4,5 – OLOX and 4,5 – EPOX** for 72 h. R^2 values and the 95% confidence intervals (95% CI) are also presented.

	WiDr			H1299			PC3			HepG2		
Compound	**IC_{50} (μM)**	**R^2**	**95% CI**	**IC_{50} (μM)**	**R^2**	**95% CI**	**IC_{50} (μM)**	**R^2**	**95% CI**	**IC_{50} (μM)**	**R^2**	**95% CI**
3,4 - OLOX	9.1	0.99	[8.8;9.4]	18.6	0.98	[15.6;22.0]	13.8	0.97	[11.4;16.8]	23.9	0.96	[18.6;30.9]
3,4 - OL	-	-	-	-	-	-	23.1	0.97	[18.6;28.7]	15.0	0.89	[9.2;24.4]
3,4 - EPOX	75.0	0.75	-	-	-	-	77.9	0.83	-	64.3	0.94	[48.6;85.1]
3,4 - EP	60.9	0.85	-	-	-	-	46.8	0.98	[40.5;54.1]	32.6	0.79	[18.6;56.9]
4,5 - OLOX	16.1	0.97	[13.6;19.1]	19.2	0.72	[9.8;37.7]	14.5	0.81	[9.0;23.2]	18.2	0.94	[13.0;25.4]
4,5 - OL	28.0	0.78	[15.6;50.2]	21.5	0.77	[11.4;40.6]	21.5	0.98	[17.9;25.9]	17.7	0.96	[13.4;23.5]
4,5 - EPOX	53.4	0.73	[29.1;98.2]	-	-	-	-	-	-	52.7	0.95	[41.9;66.3]
4,5 - EP	59.1	0.77	-	-	-	-	62.1	0.90	[45.0;85.8]	42.4	0.84	[27.0;66.8]

Finally, as compound **3,4 – OLOX** and **4,5 – OLOX** revealed better antiproliferative effect, their selectivity towards cancer cell lines was evaluated by analysing its effects in two normal cell lines, CCD841 CoN and MRC5. As so, metabolic activity was assessed through the MTT assay 72 h after treatment with compounds **3,4 – OLOX** and **4,5 – OLOX**, with different concentrations of each compound ranging from 1 to 75 μM. Metabolic activity was determined exactly the same as for the tumour cells and is represented in Figure 27. IC_{50} values of the two normal cells lines and the respective tumour cells, for terms of comparison, are presented in Table 5.

Results demonstrate that there was a decrease in the metabolic activity of both non-tumour cell lines with the increase of compounds concentration. However, this decrease was less pronounced compared to the results obtained for cancer cell lines.

Compound **3,4 – OLOX** in CCD841 CoN cell line presents an IC_{50} of 31.7 μM which is significantly higher than the IC_{50} value presented by WiDr cell line (IC_{50}=9.1 μM). Moreover, IC_{50} value obtained for the same cell line treated with **4,5 – OLOX** (Table 5) was higher than the maximum concentration used in the study and so, significantly different from

those obtained in WiDr cell line. Relatively to MRC5 cell line, the IC_{50} of **3,4 – OLOX** (IC_{50}=29.5 µM) was higher comparatively to the IC_{50} in H1299 (IC_{50}=18.6 µM) however, this difference was not significant. IC_{50} values of **4,5 – OLOX** were very similar for MRC5 (IC_{50}=19.8 µM) and H1299 (IC_{50}=19.2 µM).

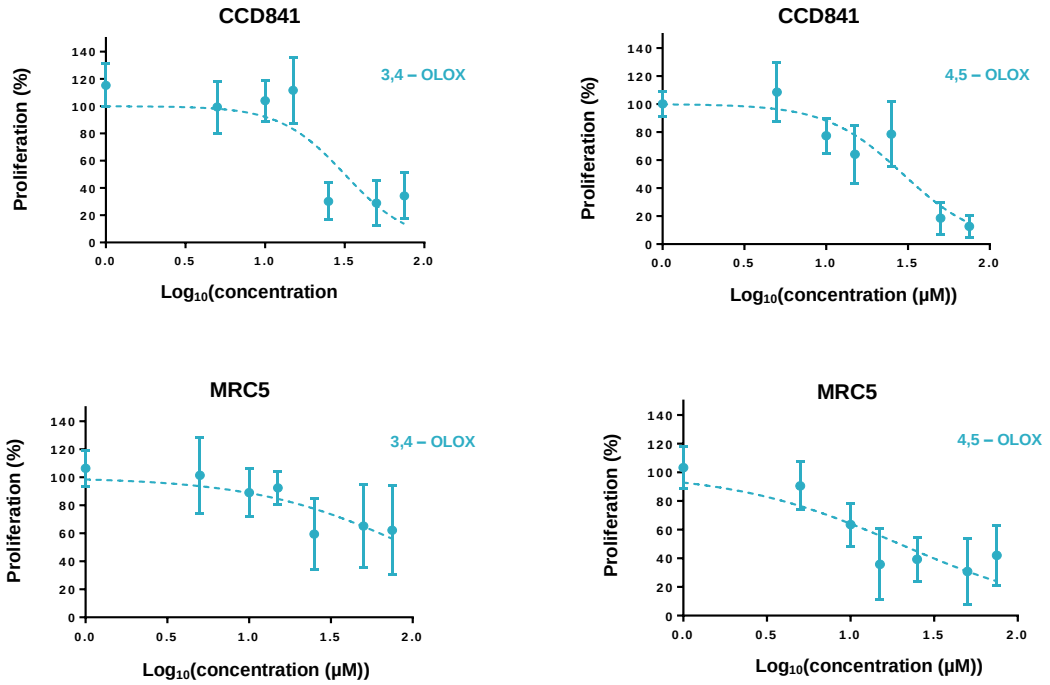


Figure 27 - Dose response curves of CCD841 and MRC5 cell lines after treatment with compounds **3,4 – OLOX** and **4,5 – OLOX** for 72 h. Results are presented as the percentage of cell proliferation as a function of compounds' concentration (Log_{10}(concentration)) and express the mean and standard deviation of, at least, three independent experiments, in triplicate.

Table 5 - IC_{50} values obtained after incubation of CCD841 CoN and MRC5 cell lines with compounds **3,4 – OLOX** and **4,5 – OLOX** for 72 h. R^2 values and the 95% confidence intervals (95% CI) are also represented.

	3,4 - OLOX			4,5 - OLOX		
Cell line	**IC_{50} (µM)**	**R^2**	**95% CI**	**IC_{50} (µM)**	**R^2**	**95% CI**
CCD841	31.7	0.74	[14.8;67.7]	>75	0.76	-
WiDr	9.1	0.99	[8.8;9.4]	16.1	0.97	[13.6;19.1]
MRC5	29.5	0.88	[18.1;48.1]	19.8	0.79	[8.8;44.1]
H1299	18.6	0.98	[15.6;22.0]	19.2	0.72	[9.8;37.7]

Cell cycle

Analysis of DNA content of cellular populations allows to distribute them by the different cell cycle phases. Thereby, the effects induced by the treatment with **3,4 – OLOX** and **4,5 – OLOX**, at the level of cell cycle of PC3 and WiDr cell lines are presented in Figure 28.

In PC3 cell line, **3,4 – OLOX** at a concentration correspondent to the IC_{50}, induced a cell cycle arrest in G_0/G_1 phase which resulted in a significant increase of 74.67±1.05% to 84.33±2.49% cells (p=0.003) comparatively to the control. It is also important to highlight the presence of an apoptotic peak in the highest concentration of **3,4 – OLOX** used, with a significant 10-fold increase (p=0.006) in the percentage of cells, in relation to the control. Regarding the **4,5 – OLOX**, it is evident the presence of an apoptotic peak shown by the significant 26-fold increase (p=0.02) in the percentage of cells comparatively to the control, in the highest concentration. There was also a significant increase of the apoptotic peak between the two concentrations (p=0.010) of this compound.

In WiDr cells, **3,4 – OLOX** induced a cell cycle arrest in G_2/M phase for the highest concentration analysed, noted by a significant increase in the percentage of cells in the G_2/M (p=0.016) phases comparatively to the control. There was also an apoptotic peak in the highest concentration, which can be seen by a 21-fold significant increase (p=0.002) comparing to the control. A significant increase can also be seen between the two concentrations used (p=0.002). Compound **4,5 – OLOX** led to a significant increase of the apoptotic peak, comparing to the control, of 1.17±0.31% to 16.33±2.88% (p<0.001) in the highest concentration. Furthermore, between the two concentrations studied there was a significant increase in the percentage of cells in G_0/G_1 phase (p=0.001) and in the apoptotic peak (p<0.001).

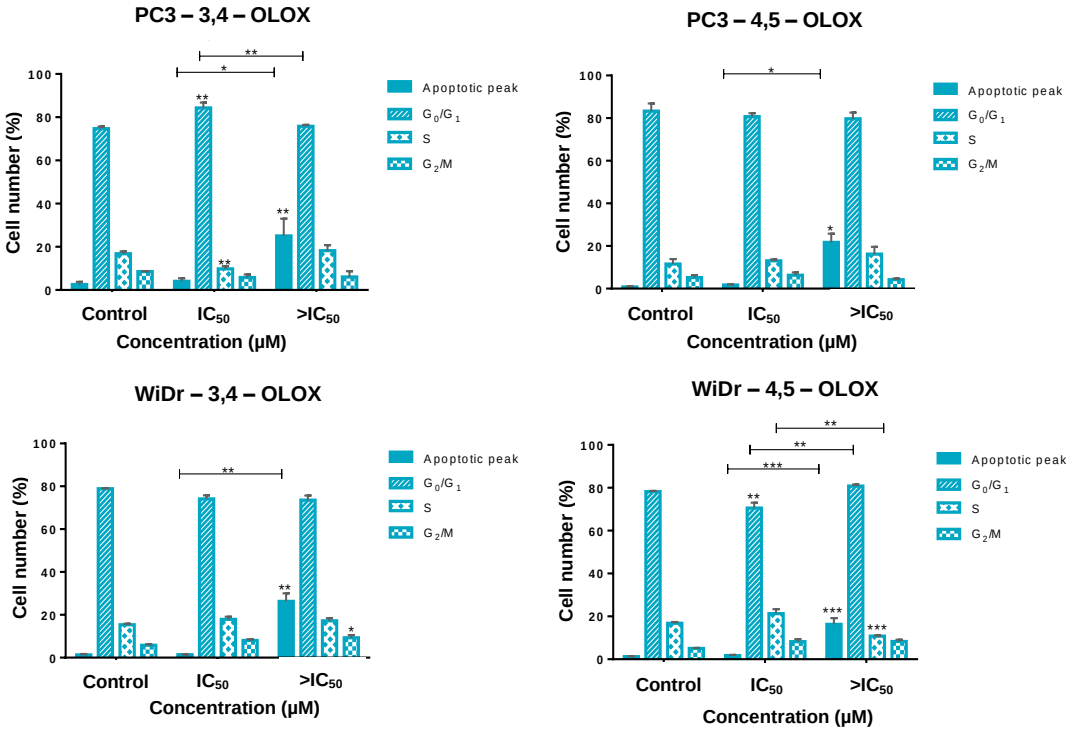


Figure 28 – Cell cycle analysis of PC3 and WiDr cell lines 72 h after treatment with **3,4 – OLOX** and **4,5 – OLOX**. Results are presented as a percentage (%) of cells in apoptotic peak (Pre-G_0/G_1 peak) and in phase G_0/G_1, S and G_2/M and express the mean±SEM of, at least, three independent experiments, in duplicate. Significant differences are denoted by *p<0.05, ** p<0.01 and *** p<0.001.

Cell viability and cell death mechanisms

To assess if the decrease of cell proliferation after the treatment with **3,4 – OLOX** and **4,5 – OLOX** was accompanied by an increase of cell death, cell viability and the types of cell death induced were analysed by flow cytometry using the double staining with AV and IP. In order to study the active cell death pathways, the expression of BAX and BCL-2 and the alterations of the mitochondrial membrane potential (Δψm) were evaluated by flow cytometry. All these experiments were performed 72 h after treatment with **3,4 – OLOX** and **4,5 – OLOX** and the concentrations used correspond to the IC_{50} value of each compound for each cell line and to a concentration higher than the IC_{50} value (>IC_{50}) that was the same for both compounds and cell lines, according to the Materials and Methods section (Table 1 and 2).

Viability and cell death

The different cell populations identified in each studied condition is presented in Figure 29. Cell viability was influenced by the presence of both compounds in a concentration-dependent manner given that with the increase of **3,4 – OLOX** and **4,5 – OLOX** concentration, cell viability decreases in both cell lines.

In detail, the most sensitive cell line to both compounds was PC3 with a significant decrease in cell viability, compared to the control. The decrease was from 84.88±1.92% to 12.14±2.19% with **3,4 – OLOX** and from 87.13±0.55% to 11.67±2.42% with **4,5 – OLOX**, in the highest concentration (p<0.001 for both compounds). Furthermore, there were also significant differences between the two concentrations used for both compounds in the percentage of viable cells (p<0.001 for both compounds).

In WiDr cells, there was also a significant decrease in cell viability, however, these cells were more resistant to the two compounds than PC3. When compared to the control, the highest concentration induced a significant decrease from 81.50±2.20% to 33.83±5.79% for **3,4 – OLOX** and from 78.67±1.38% to 40.00±4.29% for **4,5 – OLOX** (p<0.001 for both compounds). In this cell line, it was also noteworthy the significant difference in the percentage of viable cells (p<0.001), between the two concentrations of **3,4 – OLOX** used.

The reduction in cell viability after treatment with the highest concentration of **3,4 – OLOX** in PC3 was accompanied by a significant increase in late apoptotic/necrotic cells from 1.13±0.13% to 19.14±2.77% (p=0.002) and in necrotic cells from 9.63±2.12% to 59.57±2.08% (p<0.001). Between the two concentrations studied there were also significant differences in the same conditions mentioned above (p=0.002 for the late apoptotic/necrotic cells and p<0.001 for the necrotic cells). In relation to **4,5 – OLOX**, the reduction in cell viability after treatment with both concentrations of these compound was accompanied by a significant increase in apoptotic cells from 3.63±0.53% to 9.88±0.85% (IC_{50}, p<0.001) and from 3.63±0.53% to 36.00±8.62% (>IC_{50}, p=0.03) compared to control. Moreover, there was a significant increase in late apoptotic/necrotic cells from 1.38±0.18% to 21.33±1.73% relatively to control, in the highest concentration (p<0.001). There is also a tendency to an increase of necrotic cells, in the highest concentration; however, it does not reach significance.

In WiDr there was a significant predominance of necrotic cells (49.67±7.71%, p=0.012) with the highest concentration of **3,4 – OLOX** when comparing to the control. Moreover, there was also a significant difference between the two concentrations used of **3,4 – OLOX** in the percentage of necrotic cells (p=0.002). **4,5 – OLOX** induced a significant

predominance of late apoptotic/necrotic cells (10.83±0.75%, *p*<0.001) and of necrotic cells (44.17±3.24%, *p*=0.001) in the highest concentration compared to the control.

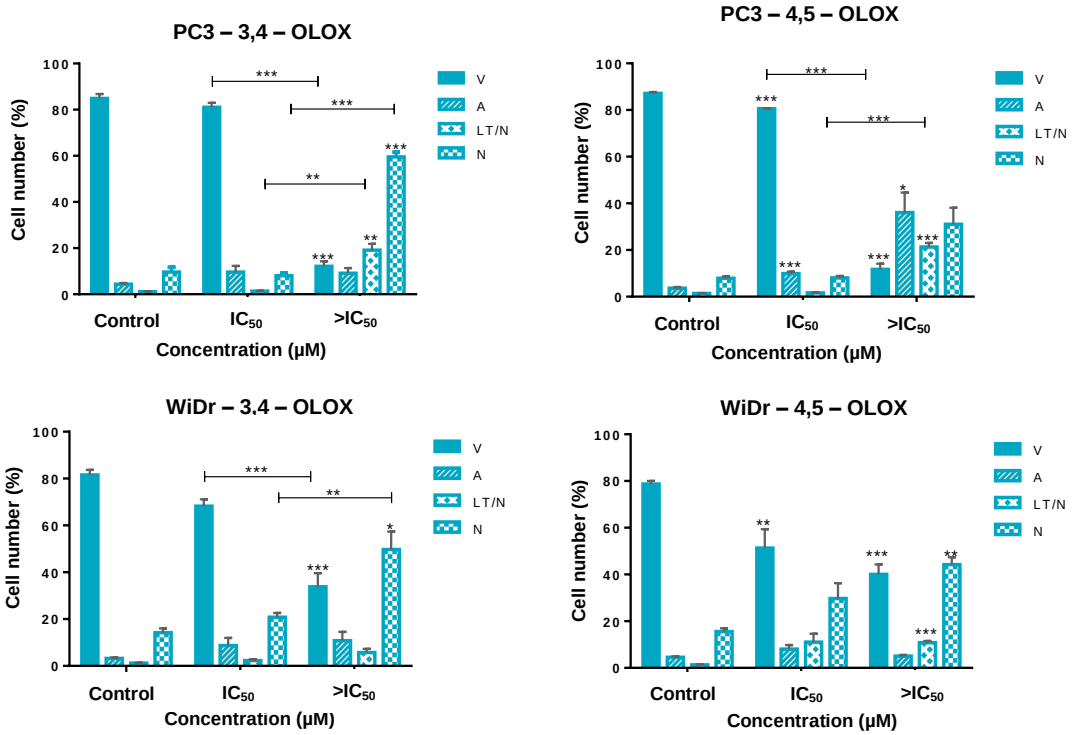


Figure 29 – Cell viability and types of cell death induced in PC3 and WiDr cell lines 72 h after treatment with **3,4 – OLOX** and **4,5 – OLOX**. Results are presented as a percentage (%) of viable cells (V), in early apoptosis (AI), in late apoptosis/necrosis (LT/N) and necrosis (N) and express the mean±SEM of, at least, three independent experiments, in duplicate. Significant differences are denoted by **p*<0.05, ** *p*<0.01 and *** *p*<0.001.

Expression of intracellular proteins BAX and BCL-2

Figure 30 presents the levels of basal protein expression of BAX and BCL-2 in PC3 and WiDr cell lines. In both cell lines treated with both compounds, there was a concentration-dependent increase of BAX/BCL-2 ratio.

In PC3 cell line, there was a significant increase of the BAX/BCL-2 ratio, for both compounds (165.70±13.14%, *p*=0.018 for **3,4 – OLOX** and 150.81±12.77%, *p*=0.017 for **4,5 – OLOX**), in the highest concentration compared to control. Significant differences between the two concentrations used for both compounds were also noted (*p*=0.008 for **3,4 – OLOX** and *p*=0.041 for **4,5 – OLOX**).

For the WiDr cell line, there was a significant increase of the BAX/BCL-2 ratio, in the highest concentration used of **3,4 – OLOX** (267.74±35.76%, *p*=0.008) and a significant decrease in the IC_{50} concentration used of **4,5 – OLOX** (81.88±1.88%, *p*=0.027) when comparing to the control. Between the two concentrations used for both compounds, there were also significant differences (*p*<0.001 for **3,4 – OLOX** and *p*=0.013 for **4,5 – OLOX**).

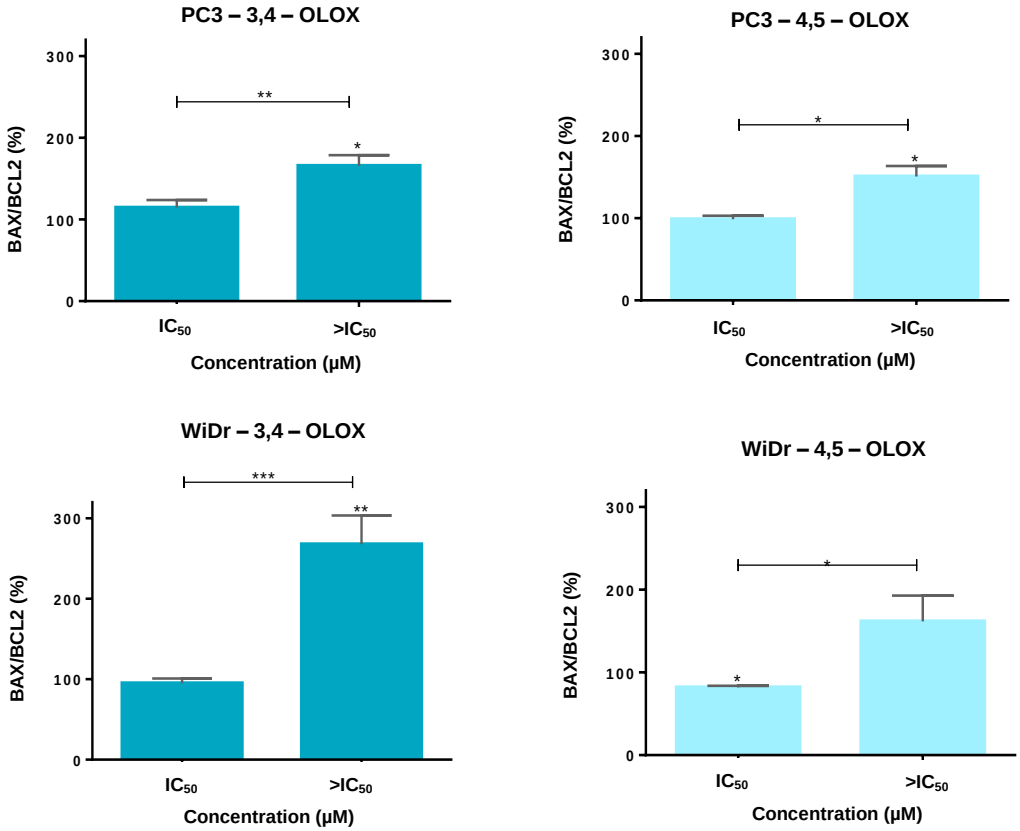


Figure 30 – Protein expression of BAX and BCL2 in PC3 and WiDr cell lines 72 h after treatment with **3,4 – OLOX** and **4,5 – OLOX**. Expression levels are presented as a ratio between BAX/BCL2 in relation to control. Results express the mean±SEM of, at least, three independent experiments, in duplicate. Significant differences are denoted by *p<0.05, ** p<0.01 and *** p<0.001.

Mitochondrial membrane potential ($\Delta\Psi_m$)

To assess the role of mitochondria in cell death induced by **3,4 – OLOX** and **4,5 – OLOX**, the changes in the $\Delta\psi_m$ of PC3 and WiDr cell lines 72 h after treatment were evaluated (Figure 31). It is important to point out that the higher the M/A ratio, the lower the mitochondrial membrane potential, which indicates mitochondrial dysfunction.

The exposure of PC3 cells to the IC_{50} concentration of **3,4 – OLOX** induced a significant decrease in the M/A ratio comparing to the control (*p*=0.02). With the highest concentration, there is a tendency to an increase in the M/A ratio, however, it does not reach

significance. Relatively to the effect of **4,5 – OLOX** in the same cell line there was a significant increase from 100 to 365.84±46.13%, in the M/A ratio, in the highest concentration (p=0.028) comparing to the control, meaning that there was a significant decrease of the $\Delta\psi_m$. Furthermore, there was also a significant difference between the two concentrations studied (p=0.001).

In WiDr cell line, the IC_{50} of **3,4 – OLOX** induced a significant increase from 100 to 126.63±7.41% in the M/A ratio, comparatively to the control (p=0.028). The highest concentration used shows, clearly, a tendency to an increase in the M/A ratio despite this increase not being significant. Significant differences (p=0.004) were also appointed between the two concentrations of this compound, which means that the $\Delta\psi_m$ decreased significantly from the IC_{50} concentration to the higher one. Finally, the IC_{50} of **4,5 – OLOX** induced a significant decrease in the M/A ratio, when comparing to the control. However, comparing both treatments, there was a significant increase (p=0.025), in the highest concentration, showing a decrease of the $\Delta\psi_m$.

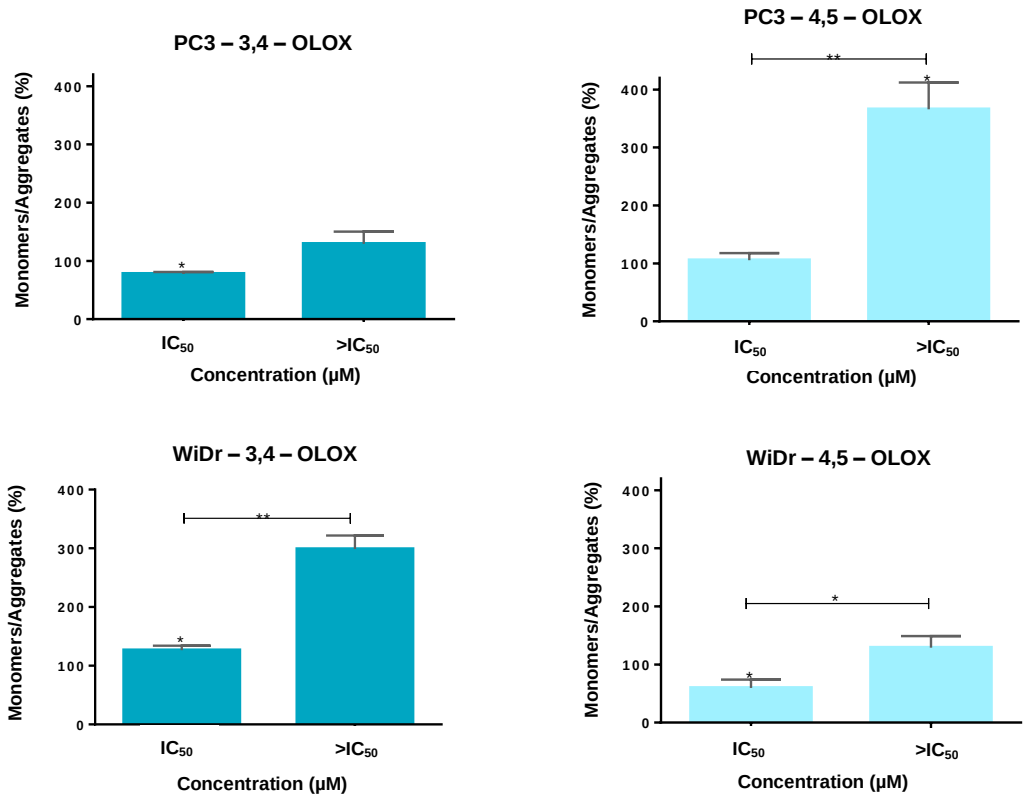


Figure 31 – Mitochondrial membrane potential ($\Delta\psi_m$) of PC3 and WiDr cell lines 72 h after treatment with **3,4 – OLOX** and **4,5 – OLOX**. Results are presented as a ratio of monomers/aggregates (M/A) for each condition relatively to control. The increase of M/A ratio is directly correlated with the decrease of $\Delta\psi_m$. Results express the mean±SEM of, at least, three independent experiments, in duplicate. Significant differences are denoted by *p<0.05 and ** p<0.01.

Reactive oxygen species

The influence of **3,4 – OLOX** and **4,5 – OLOX** was assessed in the cell redox environment by quantifying the intracellular production of ROS, namely the superoxide anion (using DHE probe) and peroxides (using DCF probe), 24 h after treatment with the referred compounds. The concentrations used correspond to the IC_{50} value of each compound obtained for each cell line and to a concentration higher than the IC_{50} value (>IC_{50}) that was the same for all compounds and cell lines, which were previously described in the Materials and Methods section (Table 1 and 2).

Intracellular production of peroxides and superoxide anion

Figure 32 shows the effects of **3,4 – OLOX** and **4,5 – OLOX** in the intracellular production of peroxides (R-O-O-R) and of the superoxide anion (O_2^-). As it can be seen, the effects of both compounds in the production of the two ROS were concentration-dependent for both cell lines, inducing, in general, an increase in ROS comparatively to the control.

In PC3 cell line, the IC_{50} concentration of **3,4 – OLOX** induced a 1.24-fold significant increase in the intracellular production of the superoxide anion (p=0.018) and 1.14-fold for the peroxides (p=0.008), both relatively to the control. The highest concentration used also reported a 1.49-fold significant increase for superoxide anion (p=0.012) and 1.35-fold for peroxides (p=0.008), relative to the control. Significant differences between the two concentrations for both ROS were also appointed (p=0.008 for superoxide anion and p=0.004 for peroxides). Both **4,5 – OLOX** concentrations tested induced a significant increase in the intracellular production of peroxides and superoxide anion comparatively to the control. In detail, the IC_{50} concentration increased significantly 1.22 times the production of superoxide anion (p=0.005) and 1.85 times the production of peroxides (p=0.012) in relation to the control. The highest concentration increased significantly 1.17 times the production of superoxide anion (p=0.005) and 1.31 times the production of peroxides (p=0.008) when compared to the control. Furthermore, there was also denoted significant differences between the two concentrations studied (p=0.001) in the intracellular production of peroxides.

Regarding the WiDr cell line, it is possible to observe that **3,4 – OLOX** increased significantly the production of both ROS, being this increase 1.53 times higher than the control for superoxide anion (p=0.002) and 1.23 times higher than the control for peroxides (p=0.002), in the IC_{50} concentration. The highest concentration also reported a 1.79-fold significant increase for superoxide anion (p=0.002) and 1.41-fold (p<0.001) for peroxides

comparatively to the control. There was also a significant increase in the highest concentration (p=0.005) when compared to the IC_{50} concentration. The IC_{50} concentration of **4,5 – OLOX** induced a significant increase of 1.09 times for superoxide anion (p=0.043) and 1.23 times for peroxides (p=0.018) relatively to control. The same was observed for the highest concentration, with a significant increase of 1.27 times comparatively to the control (p=0.028). Moreover, between the two concentrations regarding the production of peroxides, a significant decrease can be observed (p<0.001).

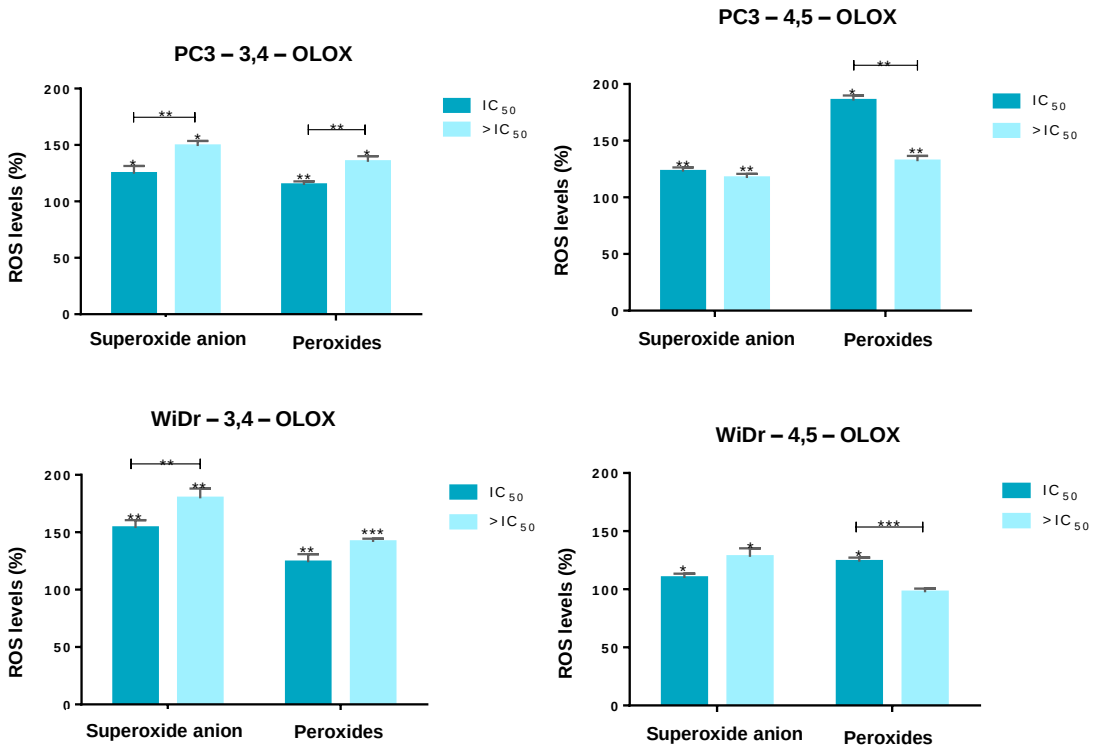


Figure 32 – Intracellular production of ROS, superoxide anion and peroxides, by PC3 and WiDr cell lines 24 h after treatment with **3,4 – OLOX** and **4,5 – OLOX**. Results are normalized to control and express the mean±SEM of, at least, four independent experiments, in quadruplicate. Significant differences are denoted by *p<0.05, ** p<0.01 and *** p<0.001.

Antioxidant defences

Intracellular levels of reduced glutathione

The influence of **3,4 – OLOX** and **4,5 – OLOX** in the cell antioxidant activity was assessed by quantifying the intracellular production of GSH, as presented in Figure 33. For that, two different concentrations were used: the IC_{50} concentration of each compound for

each cell line and a concentration higher than the IC_{50} value (>IC_{50}) that was the same for all compounds and cell lines.

In general, both compounds did not induce any notorious differences in the intracellular expression of GSH in PC3 and WiDr cell lines, except for the IC_{50} concentration of **3,4 – OLOX** in PC3 cells. In this case, there was a significant decrease from 100 to 92.74±1.85% (p=0.018) in the GSH intracellular levels comparing to the control. For the highest concentration of the same compound in WiDr cells, there was a significant increase from 100 to 108.07±4.54% (p=0.009) comparatively to the control. Moreover, there was also a significant increase in the highest concentration when compared to the IC_{50} concentration (p=0.002).

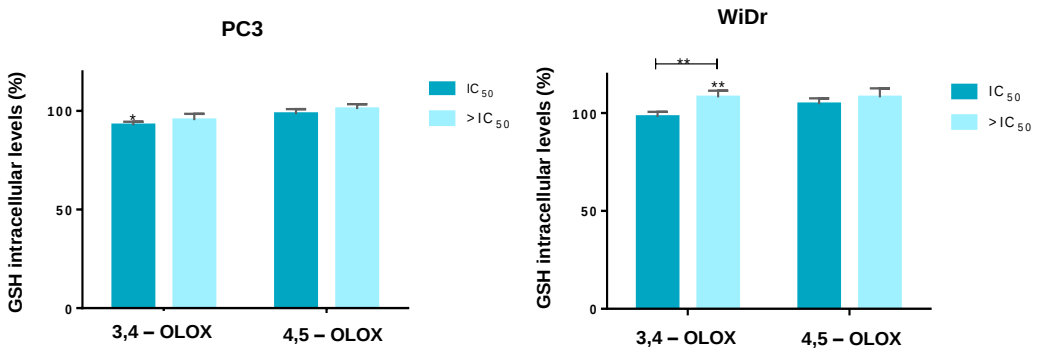


Figure 33 – Intracellular expression of GSH by PC3 and WiDr cell lines 24 h after treatment with **3,4 – OLOX** and **4,5 – OLOX**. Results are normalized to control and express the mean±SEM of, at least, four independent experiments, in quadruplicate. Significant differences are denoted by *p<0.05 and ** p<0.01.

Hemocompatibility

The alterations in the erythrocytes' membrane integrity induced by **3,4 – OLOX** and **4,5 – OLOX** were evaluated by assessing haemoglobin release. If the compounds compromise the integrity of the cells' membrane, the haemoglobin is released to the medium and then is easily quantified.

For this study, there were tested two concentrations of both compounds (10 µM and 75 µM). Different samples of erythrocytes derived from human blood were also used. Figure 34 represents the percentage values of haemoglobin release after treatment with **3,4 – OLOX** and **4,5 – OLOX**.

Results demonstrated that both **3,4 – OLOX** and **4,5 – OLOX** did not induce significant haemolysis in the erythrocytes. More specifically, the percentage of haemoglobin released by the positive control (94.32±3.92%) was significantly higher compared to the compounds-treated cells. This percentage was about 18 times higher (p<0.001) than the obtained with the two concentrations of **3,4 – OLOX** and 18 and 9 times higher than the obtained with 10 µM and 75 µM of **4,5 – OLOX** (p<0.001), respectively.

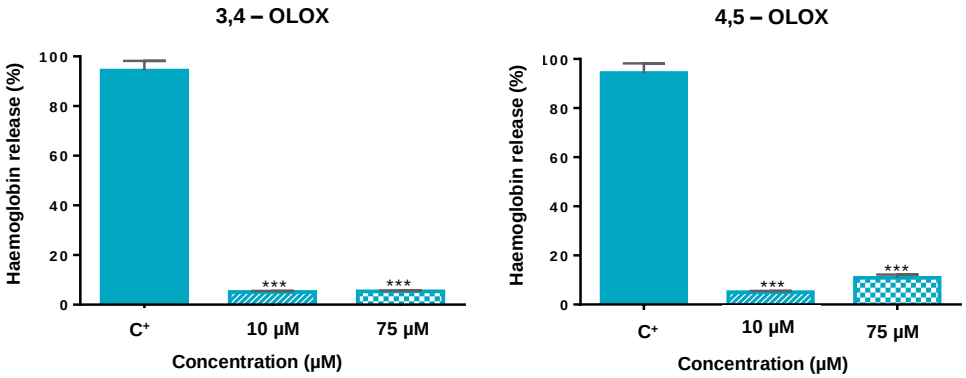


Figure 34 – Quantification of haemoglobin release expressed in percentage in relation to the positive control lesser the negative control after the incubation of erythrocytes with two concentrations of **3,4 – OLOX** and **4,5 – OLOX**. Results express the mean±SEM of, at least, three independent experiments, in triplicate. Significant differences are denoted by *** p<0.001.

DISCUSSION

Tumour cells are well known for their abnormal proliferation and for their ability to overcome the usual control mechanisms, which distinguish them from the normal cells (Knowles and Selby, 2005). Cancer cells can overcome the referred mechanisms by deregulating the production and release of growth-promoting signals that are responsible for telling the cells if they should undergo division or not. This allows the uptake and metabolism of nutrients that are able to promote cell survival and growth, resulting in the formation of a tumour (Hanahan and Weinberg, 2011; Courtnay *et al.*, 2015).

The main goal of this dissertation was to synthesize steroidal epoxides and oximes (compounds **3,4 – OLOX, 3,4 – EPOX, 4,5 – OLOX** and **4,5 – EPOX**) and then evaluate them in several cancer cells lines (WiDr, H1299, PC3 and HepG2), due to the interesting anticancer properties demonstrated by some steroidal epoxides (Amaral *et al.*, 2013b; Peng *et al.*, 2017) and oximes (Dhingra *et al.*, 2011; Canario *et al.*, 2018) in previous studies. Since very few is known about these compounds, and some, are even new, which is the case of compound **4,5 – OLOX** and **4,5 – EPOX**, the decision was to do a screening in different types of cancer. The precursors of each synthesized compound were also evaluated in the same cell lines to assess the influence of the oxime group in the compounds' activity. The most active compounds (**3,4 – OLOX** and **4,5 – OLOX**) were, then, selected for further studies in two cell lines, PC3 and WiDr.

The steroidal epoxides and oximes (four compounds) were successfully synthesized according to the following rational: the precursor compounds **3,4 – OL** and **3,4 – EP** which contain an olefin and an epoxide group at position 3,4, respectively, originated **3,4 – OLOX** and **3,4 – EPOX** both with an oxime group at position 17 as it is exemplified in Figure 10; the synthesis of these two compounds was already reported in a previous study (Cepa *et al.*, 2008); the precursor compounds **4,5 – OL** (olefin at position 4,5) and **4,5 – EP** (epoxide at position 4,5) originated two compounds, **4,5 – OLOX** and **4,5 – EPOX**, both bearing an oxime at C-17 position, which synthesis has never been reported before. All precursor compounds had already been synthesized prior to the beginning of this dissertation. As it was mentioned above, epoxides and oximes were chosen based on their previously demonstrated anticancer properties. Interestingly, epoxides have been identified for many years as biologically harmful compounds due to their toxicity, and for that reason have not been considered a good approach when designing new drugs (Manson, 1980; Besse and Veschambre, 1994; Hughes, Miller and Swamidass, 2015). However, their cytotoxicity is useful when talking about anticancer drugs being that several epoxides have been tested for their anticancer activities. The steroidal epoxides synthesized in this dissertation, surprisingly, did not show significant cytotoxicity in any of the cell lines studied, demonstrating poor antiproliferative activity, in general. On the other hand, compounds

bearing an olefin group at position 3,4 (**3,4 – OLOX**) and 4,5 (**4,5 – OLOX**) presented good antiproliferative activity against all types of cancer cell studied with IC_{50} values ranging from 9.1 µM to 19.2 µM. Given these results, it is possible to infer that the studied olefins are more prompt to cell proliferation inhibition than the studied epoxides in the considered cell lines.

One of the main objectives of this dissertation was to assess if the introduction of an oxime group in the steroidal scaffold influenced the cytotoxic activity of the molecule. Results demonstrated that the compounds bearing an epoxide moiety in 3,4 and 4,5 position, **3,4 – EPOX** and **4,5 – EPOX**, respectively, and an oxime at position C-17, were not good antiproliferative agents against the four cancer cell lines studied. The anticancer activity of compounds **3,4 – EP** and **4,5 – EP** was also evaluated, and results showed that activity has remained very similar between compounds in all cell lines, demonstrating that the oxime did not interfere with it. These results seem to be contradictory with the current literature that points out the anticancer activity of steroidal epoxides. In fact, in a study by Amaral and co-authors the parent compound of **4,5 – EPOX**, compound **4,5 – EP** showed good antiproliferative activity against MCF-7aro, a breast cancer cell line (IC_{50}=8.5 µM) (Amaral *et al.*, 2013a). The parent compound of **3,4 – EPOX**, compound **3,4 – EP** also inhibited breast cancer cells proliferation (Cepa *et al.*, 2008). The differences accounted might be due to the type of cell lines used since both compounds are good aromatase inhibitors as it was reported by (Varela *et al.*, 2012), and breast cancer cells are rich in aromatase.

On the other hand, compounds presenting an olefin group at the same positions mentioned before (**3,4 – OLOX** and **4,5 – OLOX**) demonstrated good antiproliferative activity in the four cancer cell lines studied (Figure 23, 24, 25 e 26), being that WiDr and PC3 were the ones with the lower IC_{50} values (Table 4). **3,4 – OLOX** had an IC_{50} value of 9.1 µM in WiDr and 13.8 µM in PC3. On the other hand, the IC_{50} values of **4,5 – OLOX** were 16.1 µM in WiDr and 14.5 µM in PC3. The two parent compounds of **3,4 – OLOX**, compound **3,4 – OL**, and **4,5 – OLOX**, compound **4,5 – OL**, were tested to assess the effect of the introduction of an oxime group in the compounds' activity. As we can see in Figures 23, 24, 25 and 26 the compounds bearing an oxime in the C-17 position were, generally, more active in all cell lines, except for HepG2, compared to their parent compounds, especially for compound **3,4 – OLOX** where the differences were more evident. It was clear for **3,4 – OLOX** and **4,5 – OLOX** that the introduction of an oxime moiety in the compounds' backbone had a tremendous effect in their activity by enhancing it. Several studies reported similar results with different steroidal compounds, where the presence of an oxime group improves their antitumour activity against several cancer cell lines (Berényi *et al.*, 2013;

Sánchez-Sánchez *et al.*, 2016; Bu *et al.*, 2017). For example, a steroidal oxime previously synthesized (Carvalho *et al.*, 2011) showed promising activities against colorectal HT-29 cancer cells (IC_{50}=11.9 µM), HepG2 cancer cells (IC_{50}=13.2 µM) and PC3 cancer cells (IC_{50}=15.0 µM). All these values are very similar to the ones obtained in this study, which are presented in Table 4. Another study of (Dubey, Jindal and Piplani, 2005) showed that C-17 oximes synthesized had relevant activity (there was no cell growth) in a lung cancer cell line. A C-17 oxime very similar to **3,4 – OLOX** and **4,5 – OLOX** was previously synthesized (Merlani, Amiranashvili and Mulkidzhanyan, 2006). This compound exhibited a relevant antiproliferative activity on melanoma and kidney cancer cells (concentration of the compound causing 50% suppression of cell growth, TGI_{50}=0.2 and 0.1 µM, respectively). All these results obtained by other researchers, together with the IC_{50} values obtained in this study, bespeak the potential antiproliferative effect of **3,4 – OLOX** and **4,5 – OLOX**.

The comparison between the IC_{50} values obtained with the conventional cytostatic drugs used in clinical practice with the ones obtained with the two oximes (**3,4 – OLOX** and **4,5 – OLOX**), for the same incubation time and cell lines, points out the promising anticancer activity of these two compounds (Table 6). In detail, the IC_{50} values of 5-FU (IC_{50}=10.5 µM), oxaliplatin (IC_{50}=10.9 µM) and irinotecan (IC_{50}=13.9 µM) in WiDr cells (Pires *et al.*, 2018) are all higher or similar to the ones obtained for **3,4 – OLOX** and **4,5 – OLOX**. Regarding the PC3 cell line, the IC_{50} values of cisplatin (IC_{50}=18.35 µM) and flutamide (IC_{50}=57.99 µM) (Mamede *et al.*, 2013), two drugs used in the treatment of PCa, are higher than the IC_{50} values of **3,4 – OLOX** and **4,5 – OLOX**. Carboplatin (IC_{50}>500 µM) and etoposide (IC_{50}=229.27 µM) (Ribeiro da Silva, 2012) drugs used in the treatment of lung cancer, present IC_{50} values higher than the ones obtained for **3,4 – OLOX** (IC_{50}=18.6 µM) and **4,5 – OLOX** (IC_{50}=19.2 µM) in H1299 cell line. Finally, the IC_{50} values obtained for **3,4 – OLOX** (IC_{50}= 23.9 µM) and **4,5 – OLOX** (IC_{50}= 18.2 µM) were similar to the one of 5-FU (IC_{50}=18.98 µM) (Brito *et al.*, 2013), one of the drugs used in the treatment of HCC.

Table 6 – IC_{50} values of some chemotherapeutic drugs used for the treatment of different types of cancer. Adapted from (Ribeiro da Silva, 2012; Brito *et al.*, 2013; Mamede *et al.*, 2013; Pires *et al.*, 2018).

Cell line	Drug/Compound	IC_{50} (μM)
WiDr	**3,4 - OLOX**	9.1
	4,5 - OLOX	16.1
	5-FU	10.50
	Oxaliplatin	10.90
	Irinotecan	13.90
PC3	**3,4 - OLOX**	13.8
	4,5 - OLOX	14.5
	Cisplatin	18.35
	Flutamide	57.99
H1299	**3,4 - OLOX**	18.6
	4,5 - OLOX	19.2
	Cisplatin	14.54
	Carboplatin	>500
	Etoposide	229.7
HepG2	**3,4 - OLOX**	23.9
	4,5 - OLOX	18.2
	5-FU	18.98

Olefins revealed to be better at inhibiting proliferation comparing to epoxides in all cell lines, regardless of their position. Moreover, position 3,4 is more suitable for olefins than position 4,5 for all cell lines, except for HepG2, as it can be seen by the IC_{50} values presented in Table 4. As for epoxides, it seems that there is no difference regardless of the position, except for WiDr and HepG2 cell lines, where it seems that position 4,5 makes the compound more prone to inhibition (Table 4). The different chemical properties of olefins and epoxides might be the explanation for the different activity of both types of compounds. Despite having similar LogP values (Table 3), which indicates identical lipophilicity and passage through the cellular membrane (Waring, 2010), olefins and epoxides have different chemical functions and different reactivities (Vollhardt and Schore, 2011). Moreover, the target-interaction, which is still unknown for these types of molecules, might be more

effective for the compounds presenting an olefin instead of an epoxide. More studies are required in order to unravel the action mechanisms behind this type of compounds.

Achieving selectivity is one of the main goals in the discovery and optimization of a compound on the path towards developing a drug. In drug design, the proper balance of escaping undesirable targets and covering one or more targets of interest is a continual drug development challenge (Huggins, Sherman and Tidor, 2012). In line with this, selectivity of the two best compounds, **3,4 – OLOX** and **4,5 – OLOX**, was assessed. For that, two normal cell lines were used, namely CCD841 CoN, a normal colon cell line and MRC5, a normal lung cell line. Results point out to a selectivity towards WiDr cancer cell line compared to CCD841 CoN, for both compounds. This is evidenced by the differences encountered in the IC_{50} values of CCD841 CoN (31.7 µM for **3,4 – OLOX** and IC_{50}>75 µM for **4,5 – OLOX**) and WiDr (9.1 µM for **3,4 – OLOX** and 16.1 µM for **4,5 – OLOX**) (Table 5). This was, indeed, a very interesting outcome and another step towards the discovery of a good anticancer agent, in this case, for colorectal cancer. In relation to the MRC5 cell line, the compounds induced a more pronounced cytotoxicity with IC_{50} values similar to the ones obtained for H1299 (Table 5). Similar results were previously stated (Berényi *et al.*, 2013) where one of the oximes synthesized influenced the viability of the MRC5 cell line (IC_{50}=6.94 µM). It would also be interesting to analyse **3,4 – OLOX** and **4,5 – OLOX** in a normal prostate cell line given the good results obtained for PC3 cell line.

Cell cycle inhibition is a known target for the treatment of cancer (McDonald and El-Deiry, 2000; Buolamwini, 2005). Proliferation and cell death must be regulated aiming to maintain the tissues' homeostasis. This regulation is achieved by the coupling between the cell cycle progression and controlled cell death (Hughes and Mehmet, 2003). Accordingly, the effect of **3,4 – OLOX** and **4,5 – OLOX** in WiDr and PC3 cell lines, the two selected cell lines where the referred compounds showed better IC_{50} values, was studied in the above-mentioned processes. Different concentrations of **3,4 – OLOX** induce different responses in the cell cycle of PC3 cells. As it can be observed, there was a cell cycle arrest in G_0/G_1 phase in the IC_{50} concentration and an apoptotic peak in the highest concentration. The G_0/G_1 phase corresponds to the phase where the cells are preparing to replicate the genetic material, where there is protein and RNA synthesis and, consequently, an increase in cellular size (Funk, 1999). What might happen in this case is that the stimuli can be so exaggerated that there is a blockage before the cells can synthesize their DNA, probably due to the G_1 checkpoint, responsible for detecting DNA damage and for verifying if the cell is in condition of undergoing division. If lesions are repaired, cell cycle progresses until division, but if these lesions persist, programmed cell death is induced. This clearly happens when PC3 cells are treated with the highest concentration of **3,4 – OLOX**, fact proved by

the presence of the apoptotic peak. For WiDr cell line, we can also detect a cell cycle arrest in G_2/M for the highest concentration. Blocking in this phase might be related with G_2 checkpoint, which prevents cells from entering phase M when DNA is damaged, providing an opportunity for repair and stopping proliferation of damaged cells. In these situations, cell cycle stops and there is lesion repair or, in case, of irreparable damage, cell undergoes apoptosis (Stark and Taylor, 2004). Therefore, blockade in G_2/M phase is related to death by apoptosis and with an increase in the apoptotic peak which is observed in WiDr cells in the highest concentration. Similar results were reported by (Berényi *et al.*, 2013) in HeLa cells where some steroidal oximes induced a cell cycle arrest in G_2/M accompanied by an apoptotic peak with a concentration of 30 µM which was very similar to the highest concentration used in this study (40 µM). On the other hand, **4,5 – OLOX** induced an apoptotic peak in both cell lines for the highest concentration.

Apoptosis is a programmed cell death mechanism, extremely ordained and regulated that occurs to eliminate damaged cells (Grivicich, Regner and Rocha, 2007). On the other hand, necrosis is a type of cell death associated with inflammatory reactions which occurs, normally, when the cells are damaged by external factors which cause irreparable lesions (Syntichaki and Tavernarakis, 2002). Apoptosis is regarded as “programmed cell death” while necrosis is considered as “unprogrammed” due to deregulated activity (Dhuriya and Sharma, 2018). More recently, a new type of cell death has been discussed where an apoptotic pathway was inhibited, exhibiting morphological features of both apoptosis and necrosis, namely necroptosis (Degterev *et al.*, 2005; Dhuriya and Sharma, 2018). The final mode of cell death will depend on the cell type, cell microenvironment, and initial inducers (Chen, Kang and Fu, 2018).

In line with this, the type of cell death induced by **3,4 – OLOX** and **4,5 – OLOX** was evaluated using the AV/IP double staining. Results obtained in this dissertation demonstrated that both **3,4 – OLOX** and **4,5 – OLOX** induced a dose-dependent decrease in cell viability, which was accompanied by a dose-dependent increase in cell death in both PC3 and WiDr cell lines. However, the type of death induced was different. In PC3 cells there was an apoptotic peak with both concentrations used of **3,4 – OLOX**. Moreover, for the highest concentration used, this was accompanied by an increase in the number of cells in late apoptosis/necrosis and an even higher increase in the number of cells in necrosis, which might indicate cell death by necrosis. Furthermore, for the lowest concentration used (IC_{50}) in the same cell line, there was a tendency to an occurrence of cellular death by apoptosis, which is demonstrated by an increase in the number of cells in apoptosis. Although this increase was not significant, it might have biological value. Both concentrations of **3,4 – OLOX** also induced an apoptotic peak in WiDr cell line, which was

accompanied by a significant increase of necrotic cells only for the highest concentration used, which might indicate cell death by necrosis. Similar to what happened in PC3 cell line, in WiDr the IC_{50} concentration of **3,4 – OLOX** also demonstrated a tendency to an increase in the number of cells in apoptosis. Several studies reported that other steroidal oximes induced apoptosis in PC3 cells (Huang *et al.*, 2012), in MCF-7 cells (Ajduković *et al.*, 2015) and in HeLa and CaSki cells, two cervicouterine cell lines (Sánchez-Sánchez *et al.*, 2016), all studies using concentrations lower than 20 µM, which is similar to the ones used in this study for PC3 (13.8 µM) and WiDr (9.1 µM). Based on these results, it is possible to assess that the type of death induced by **3,4 – OLOX** is dose-dependent since for lower concentrations this compound induced apoptosis (mainly in PC3 cells) and with higher concentrations the type of death induced is necrosis. Clearly, higher concentrations of this oxime induced more severe lesions which is a determinant factor in the type of cell death.

On the other hand, **4,5 – OLOX**, induced different responses depending on the cell line studied. In PC3, this compound induced cell death, probably, by apoptosis, which is proven by an increase in the percentage of apoptotic and late apoptotic/necrotic cells and with the apoptotic peak verified. This process is more notorious in the highest concentration, however, in the IC_{50} concentration, we can also observe an increase in apoptotic cells. In WiDr cell line, **4,5 – OLOX** mostly likely induced cell death by necrosis. In this cell line, these alterations only occurred for the highest concentration used.

Maintaining homeostasis is crucial and for that, there is a delicate balance between cell death and survival. Uncontrolled cell proliferation and escape from cell death have been recognized as *hallmarks* of cancer cells (Hanahan and Weinberg, 2011). Several strategies have been studied in order to stop or limit cancer spreading among them inducing cells to cell-cycle arrest (McLaughlin, Finn and La Thangue, 2003), target apoptosis-related proteins and more recently, necroptosis-associated proteins and ROS-based therapies (Chen, Kang and Fu, 2018). Therefore, the mechanisms of cell death were studied in more detail through the analysis of BAX and BCL-2 expression, mitochondrial membrane potential ($\Delta\psi_m$) and oxidative stress.

A dysregulation between the expression of pro- and anti-apoptotic proteins might keep the cells from correctly respond to apoptotic stimuli (Petros, Olejniczak and Fesik, 2004). The BCL-2 family plays a crucial role in regulating apoptosis, necrosis and autophagy, in such a way that alterations in its expression and function contribute to the pathogenesis and progression of numerous types of cancer (Reed, 2006, 2008). BAX, a pro-apoptotic protein and BCL-2, an anti-apoptotic protein, belong to this cell death regulatory protein family. Over-expression of anti-apoptotic proteins induce resistance to anti-tumoural therapies insofar as they act as apoptosis repressors, blocking the release of

cytochrome C while pro-apoptotic members act like apoptosis promoters. These effects can be quantified though the balance between BAX and BCL-2 expression (Petros, Olejniczak and Fesik, 2004; Gogvadze, Orrenius and Zhivotovsky, 2008).

The effect of **3,4 – OLOX** and **4,5 – OLOX** in the BAX and BCL-2 expression was analysed through the BAX/BCL-2 ratio presented in Figure 30 where we can see a dose-dependent increase for both compounds in both cell lines. The most evident increase was in WiDr cells treated with **3,4 – OLOX**. In light with these results, we can infer that apoptosis and necroptosis might be the types of cell death responsible for decreasing cell viability and for inducing cellular death. These two cellular death types may occur simultaneously (Zhang *et al.*, 2015) or mutually transform because of the interconnection of the downstream death signalling pathways (Chen, Kang and Fu, 2018). More specifically, cells can commit to necrotic cell death when apoptosis is blocked (Leist and Jäättelä, 2001) and oxidative stress-induced necrotic cell death involves the activation of an apoptosis-associated pathway (Chen, Kang and Fu, 2018). Another study (Mandal *et al.*, 2014) also reported this relationship between apoptosis and necroptosis.

Control of mitochondrial membrane permeabilization is a critical event positive and negatively regulated by specific proteins in the intracellular matrix (Knowles and Selby, 2005; Lopez and Tait, 2015). Although several molecular mechanisms are not well known yet, it has been described that BAX mediates the inner mitochondrial membrane permeabilization which results in the dissipation of the $\Delta\psi_m$. The $\Delta\psi_m$ can be assessed as the charge difference between the mitochondrial matrix and cytoplasm which has implications in the induction of apoptosis (Ly, Grubb and Lawen, 2003). The energy potential associated with the Δψm is used by the complex V of the respiratory chain to synthesize ATP (Pires, 2016). Therefore, the collapse of the Δψm results in surcease of ATP synthesis, release of cytochrome C and, consequently, in cell death (Knowles and Selby, 2005).

In this study, both compounds in both cell lines induced a dose-dependent increase in the percentage of the monomers/aggregates ratio, which means a decrease in the $\Delta\psi_m$, consistent with apoptosis induction. In both cell lines treated with **3,4 – OLOX** there was a dose-dependent decrease of the $\Delta\psi_m$, evidenced by the increase in the percentage of the monomers/aggregates ratio. This is another evidence of cell death by necroptosis. As it was mentioned before, a decrease in the $\Delta\psi_m$ and an increase in the BAX/BCL2 ratio are both related to apoptosis induction. This means that cells are activating apoptosis-related signalling pathways, which culminate in necrosis. Apoptosis is a regulated process, genetically programmed and dependent on the availability of ATP and, consequently, of mitochondrial function (Pires, 2016). High levels of intracellular ATP often favours

apoptosis, whereas a low level often promotes necrosis (Leist *et al.*, 1997; Chen, Kang and Fu, 2018). In this way, extreme consumption of intracellular ATP or the inhibition of ATP synthesis may cause a switch from apoptosis to necrosis (Eguchi and Shimizu, 1997). The mitochondria is the major site that generates ATP, thus, mitochondria dysfunction can trigger necrosis by ATP depletion. Moreover, excessive mitochondrial ROS formation and the onset of the mitochondrial permeability transition are also causally linked to the shift from apoptosis to necroptosis (Denecker *et al.*, 2001; Chen, Kang and Fu, 2018). In relation to **4,5 – OLOX** there was also a dose-dependent decrease in the $\Delta\psi_m$, seen by the dose-dependent increase in the monomers/aggregates ratio in both cell lines. In PC3 cells, the $\Delta\psi_m$ suffered a much more accentuated decrease, evidenced by a significant increase in the monomers/aggregates ratio. This is consistent with an occurrence of cell death by apoptosis, seen by the percentage of cells in apoptosis in the cell viability studies and with the apoptotic peak verified in the cell cycle analysis. WiDr cells showed a tendency to a decrease in the $\Delta\psi_m$, however, this was not significant and goes accordingly to the predominance of cell death by necroptosis/necrosis.

Physiologically, the main ROS include the anion superoxide ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2). Physiologic concentrations of these radical species are maintained in a very narrow range, due to the existing balance between the production and removal rate by enzymatic antioxidants like SOD, GPx or catalase or by non-enzymatic antioxidants like GSH (Koskenkorva-Frank *et al.*, 2013).

As previously discussed, necroptosis is probably, the main type of cell death induced by **3,4 – OLOX** (in both cell lines) and **4,5 – OLOX** (in WiDr cell line) and ROS production is proposed to be an executioner of necroptosis (Christofferson and Yuan, 2010). Thence, the production of ROS and the antioxidant defence, GSH, were evaluated. Fluorescence analysis with DHE dye allowed to quantify the intracellular production of superoxide anion, and DCF dye allowed to quantify the intracellular content of peroxides, after PC3 and WiDr cells exposure to both compounds. **3,4 – OLOX** induced a dose-dependent increase in both peroxides and in superoxide in both cell lines. For **4,5 – OLOX**, there was an increase relatively to the control of both ROS in both cell lines, however, non-dependent on the concentration of compounds. In fact, peroxides levels after treatment with **4,5 – OLOX** decreased between both concentrations used. This might be due to the conversion of these species in other ROS (Gáspár, 2011). Olefins, as it was mentioned earlier, are a very reactive group, thus, the olefin moiety present in **3,4 – OLOX** and **4,5 – OLOX** might be reacting with the peroxides, generating radical species. Finally, the antioxidant defence GSH did not show relevant differences except the increase observed for **3,4 – OLOX** in WiDr cell lines. The absence of a relationship between the intracellular levels of GSH and

the intracellular levels of peroxides might be justified by the involvement of other reactive species reacting with the DCF dye, such as the nitric oxide, the peroxynitrite, hypochlorite and other reactive nitrogen species (RNS) (Rhee *et al.*, 2010). On the other hand, it can also reveal that the intracellular peroxides converted in other species and were not detoxified by GSH (Nechifor, Neagu and Manda, 2012).

Haemolysis is a term used to designate destruction of red blood cells by lysis. The phenomenon is caused by intrinsic abnormalities of the red blood cells or by the external environment. Haemolysis in cancer has been observed in association with disseminated malignancies treated with a variety of chemotherapeutic agents (Rytting, Worth and Jaffe, 1996). For example, tamoxifen, one of the most prescribed anti-estrogen drugs in chemotherapy for breast cancer, induces changes in the framework of the erythrocyte membrane and its cytoskeleton, which results in haemolysis and, consequently, in hemolytic anemia (Cruz Silva *et al.*, 2000; De Oliveira *et al.*, 2011).

In view of that, a hemocompatibility study was performed to assess if **3,4 – OLOX** and **4,5 – OLOX** induced lysis in the red blood cells which, ultimately allows predicting whether an IV administration of the compounds is possible or not, or if anemia can be a preventable side effect with these new compounds. Both compounds did not induce haemoglobin release, *i.e*., the erythrocytes membrane did not suffer alterations allowing the haemoglobin to stay inside them. In line with this, it is possible to infer that IV administration of both compounds might be possible. Also, and bearing in mind that anemia is usually a well-known side effect of some anticancer drugs, the non-induction of haemoglobin release by **3,4 – OLOX** and **4,5 – OLOX** constitutes an advantage towards the conventional chemotherapy drugs.

CONCLUSIONS AND FUTURE PERSPECTIVES

Nowadays and despite the numerous therapeutic approaches for the treatment of cancer, the medical need remains still largely unmet. Hence, the design and development of safer, target specific, and effective anticancer agents is crucial.

In this work, four steroidal oximes were synthesized being that two of them (**4,5 – OLOX** and **4,5 – EPOX**) were synthesized for the first time. Cytotoxicity studies demonstrated that among the four oxime compounds only the ones bearing an olefin group (**3,4 – OLOX** and **4,5 – OLOX**) showed good antiproliferative activity in all cell lines. General SARs analysis points out that the presence of an olefin group either at position 3,4 or 4,5 is important for the cytotoxicity of the compounds being that position 3,4 seems to be more favourable in all cell lines, except for HepG2 cell line. The presence of an epoxide group does not influence the cytotoxicity of the compounds since **3,4 – EPOX** and **4,5 – EPOX** showed poor antiproliferative activity. Moreover, the presence of an oxime group also seems to be important for the cytotoxicity of **3,4 – OLOX** and **4,5 – OLOX** since the compounds without this group (**3,4 – OL** and **4,5 – OL**), in general, presented higher IC_{50} values.

So, in line with this, it was possible to infer that **3,4 – OLOX** and **4,5 – OLOX** showed very promising anticancer properties such as cell cycle arrest, induction of cell death by apoptosis/necroptosis and increased ROS production in PC3 and WiDr cell lines. Furthermore, the hemocompatibility assay reinforced the previous conclusions, since both compounds did not induce release of haemoglobin meaning that **3,4 – OLOX** and **4,5 – OLOX** did not damage the erythrocytes' membrane which makes it possible for them to be administered intravenously.

The high cytotoxicity against prostate and colorectal cancer cells and selectivity towards colorectal cancer cells shown by **3,4 – OLOX** and **4,5 – OLOX**, in an early stage of *in vitro* evaluation, allowed to conclude that these compounds might serve as a possible approach as anticancer drugs for PCa and CRC cancer. These results encourage future studies in order to discover the right mechanisms behind the action of these steroidal oximes. In fact, very little is known about how this type of molecules act and exactly which is/are their target/targets. Further studies might include assessment of several parameters such as DNA damage through the comet assay, protein expression of proteins associated with cell death by western blot and most importantly approaches for discovering the protein target of these molecules, which include direct biochemical methods, genetic interaction methods and computational inference methods. Moreover, *in vivo* studies are also an important step towards showing the efficacy of **3,4 – OLOX** and **4,5 – OLOX** as promising anticancer drugs.

REFERENCES

Aakeröy CB, Sinha AS, Epa KN, Chopade PD, Smith MM, Desper J(2013) 'Structural chemistry of oximes', *Crystal Growth and Design*, 13(6), pp. 2687–2695.

Abrantes AM, Serra MES, Gonçalves AC, Rio J, Oliveiros B, Laranjo M, Rocha-Gonsalves AM, Sarmento-Ribeiro AB, Botelho MF (2010) 'Hypoxia-induced redox alterations and their correlation with 99mTc-MIBI and 99mTc-HL-91 uptake in colon cancer cells', *Nuclear Medicine and Biology*. Elsevier Inc., 37(2), pp. 125–132.

Adjakly M, Ngollo M, Dagmedir A, Judes G, Pajon A, Karsli-Ceppioglu S, Penault-Llorca F, Boiteux JP, Bignon YJ, Guy L, Bernard-Gallon D (2015) 'Prostate cancer: The main risk and protective factors-Epigenetic modifications', *Annales d'Endocrinologie*. Elsevier Masson SAS, 76(1), pp. 25–41.

Aggarwal S, Thareja S, Verma A, Bhardwaj TR, Kumar M (2010) 'An overview on 5α-reductase inhibitors', *Steroids*, 75(2), pp. 109–153.

Ajduković JJ, Gaši KM, Jakimov DS, Klisurić OR, Jovanović-Šanta S, Sakač MN, Aleksić LD, Djurendić EA (2015) 'Synthesis, structural analysis and antitumor activity of novel 17α-picolyl and 17(E)-picolinylidene A-modified androstane derivatives', *Bioorganic and Medicinal Chemistry*. Elsevier Ltd, 23(7), pp. 1557–1568. doi: 10.1016/j.bmc.2015.02.001.

Alevizopoulos K, Dimas K, Papadopoulou N, Schmidt EM, Tsapara A, Alkahtani S, Honisch S, Prousis KC, Alarifi S, Calogeropoulou T, Lang F, Stournaras C (2016) 'Functional characterization and anti-cancer action of the clinical phase II cardiac Na^+/K^+ ATPase inhibitor istaroxime: *in vitro* and *in vivo* properties and cross talk with the membrane androgen receptor', *Oncotarget*, 7(17).

Allan GM, Lawrence HR, Cornet J, Bubert C, Fischer DS, Vicker N, Smith A, Tutill HJ, Purohit A, Day JM, Mahon MF, Reed MJ, Potter BVL (2006) 'Modification of estrone at the 6, 16, and 17 positions: Novel potent inhibitors of 17β-hydroxysteroid dehydrogenase type 1', *Journal of Medicinal Chemistry*, 49(4), pp. 1325–1345.

Amaral C, Varela C, Azevedo M, Tavares da Silva E, Roleira FMF, Chen S, Correia-da-Silva G, Teixiera N (2013a) 'Effects of steroidal aromatase inhibitors on sensitive and resistant breast cancer cells: Aromatase inhibition and autophagy', *Journal of Steroid Biochemistry and Molecular Biology*. Elsevier Ltd, 135(1), pp. 51–59.

Amaral C, Varela C, Borges M, Tavares da Silva E, Roleira FMF, Correia-da-Silva G, Teixeira N (2013b) 'Steroidal aromatase inhibitors inhibit growth of hormone-dependent breast cancer cells by inducing cell cycle arrest and apoptosis', *Apoptosis*, 18(11), pp. 1426–1436.

Amaral C, Lopes A, Varela CL, Tavares da Silva E, Roleira FMF, Correia-da-Silva G,

Teixeira N (2015) 'Exemestane metabolites suppress growth of estrogen receptor-positive breast cancer cells by inducing apoptosis and autophagy: A comparative study with Exemestane', *International Journal of Biochemistry and Cell Biology*. Elsevier Ltd, 69, pp. 183–195.

Amaral C, Varela CL, Maurício J, Sobra AF, Costa SC, Roleira FMF, Tavares da Silva E, Correia-da-Silva G, Teixeira N (2017) 'Anti-tumor efficacy of new 7α-substituted androstanes as aromatase inhibitors in hormone-sensitive and resistant breast cancer cells', *Journal of Steroid Biochemistry and Molecular Biology*. Elsevier, 171, pp. 218–228.

Aran V, Victorino AP, Thuler LC, Ferreira CG (2016) 'Colorectal Cancer: Epidemiology, Disease Mechanisms and Interventions to Reduce Onset and Mortality', *Clinical Colorectal Cancer*. Elsevier Inc., 15(3), pp. 195–203.

Armaghany T, Wilson JD, Chu Q, Mills G (2012) 'Genetic alterations in colorectal cancer', *Gastrointestinal Cancer Research*, 5(1), pp. 19–27.

Attard G, Parker C, Eeles RA, Schröder F, Tomlins SA, Tannock I, Drake CG, Bono JS (2015) 'Prostate cancer', *Lancet*, 387, pp. 114–120.

Attwa MH, El-Etreby SA (2015) 'Guide for diagnosis and treatment of hepatocellular carcinoma', *World Journal of Hepatology*, 7(12), pp. 1632–1651.

Bansal R, Guleria S, Ries C, Hartmann RW (2010) 'Synthesis and antineoplastic activity of O-alkylated derivatives of 7-hydroximinoandrost-5-ene steroids', *Archiv der Pharmazie*, 343(7), pp. 377–383.

Bansal R, Acharya PC (2014) 'Man-made cytotoxic steroids: Exemplary agents for cancer therapy', *Chemical Reviews*, 114(14), pp. 6986–7005.

Berényi Á, Minorics R, Iváni Z, Ocsovszki I, Ducza E, Thole H, Messinger J, Wölflin J, Mótyán G, Mernyák E, Frank É, Schneider G, Zupkó I (2013) 'Synthesis and investigation of the anticancer effects of estrone-16-oxime ethers in vitro', *Steroids*, 78(1), pp. 69–78.

Berrevoets CA, Umar A, Brinkmann AO (2002) 'Antiandrogens: Selective androgen receptor modulators', *Molecular and Cellular Endocrinology*, 198(1–2), pp. 97–103.

Besse P, Veschambre H (1994) 'Chemical and Biological Synthesis of Chiral Epoxides', *Tetrahedron*, 50(30), pp. 8885–8927.

Biglia N, Sgro L, Defabiani E, De Rosa G, Ponzone R, Marenco D, Sismondi P (2005) 'The influence of hormone replacement therapy on the pathology of breast cancer', *European Journal of Surgical Oncology*, 31(5), pp. 467–472.

Bosetti C, Turati F, La Vecchia C (2014) 'Hepatocellular carcinoma epidemiology', *Best*

Practice and Research: Clinical Gastroenterology. Elsevier Ltd, 28(5), pp. 753–770.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A (2018) 'Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries', *CA Cancer J Clin*, 68(11), pp. 394–424.

Brenner H, Kloor M, Pox C (2014) 'Colorectal cancer', *The Lancet*. Elsevier Ltd, 383(9927), pp. 1490–1502.

Brito AF, Abrantes AM, Pinto-Costa C, Gomes AR, Mamede AC, Casalta-Lopes J, Gonçalves AC, Sarmento-Ribeiro AB, Tralhão JG, Botelho MF (2013) 'Hepatocellular carcinoma and chemotherapy: The role of p53', *Chemotherapy*, 58(5), pp. 381–386.

Bu M, Cao T, Li H, Guo M, Yang BB, Zeng C, Zhou Y, Zhang N, Hu L (2017) 'Synthesis and biological evaluation of novel steroidal 5α,8α-epidioxyandrost-6-ene-3β-ol-17-(O-phenylacetamide)oxime derivatives as potential anticancer agents', *Bioorganic and Medicinal Chemistry Letters*. Elsevier Ltd, 27(16), pp. 3856–3861.

Buolamwini, J. (2005) 'Cell Cycle Molecular Targets in Novel Anticancer Drug Discovery', *Current Pharmaceutical Design*, 6(4), pp. 379–392.

Canario C, Silvestre S, Falcão AC, Alves G (2018) 'Steroidal Oximes: Useful Compounds with Antitumor Activities', *Current medicinal chemistry*, 25(6), pp. 660–686.

Cardoso MRJ '*The role of HOXB13 in prostate cancer', Master Thesis,* Universidade do Porto, 2013.

Carvalho JFS, Silva MMC, Moreira JN, Simões S, Melo MLS (2011) 'Selective cytotoxicity of oxysterols through structural modulation on rings A and B. Synthesis, in vitro evaluation, and SAR', *Journal of Medicinal Chemistry*, 54(18),

Cepa M, Correia-da-Silva G, Tavares da Silva E, Roleira FMF, Borges M, Teixeira N (2008) 'New steroidal aromatase inhibitors: Suppression of estrogen-dependent breast cancer cell proliferation and induction of cell death', *BMC Cell Biology*, 9, pp. 1–14.

Cepa M, Tavares da Silva E, Correia-da-Silva G, Roleira FMF, Teixeira N (2008) 'Synthesis and biochemical studies of 17-substituted androst-3-enes and 3,4-epoxyandrostanes as aromatase inhibitors', *Steroids*, 73(14), pp. 1409–1415.

Cepa MMDS, Tavares da Silva E, Correia-da-Silva G, Roleira FMF, Teixeira N (2005) 'Structure−Activity Relationships of New A,D-Ring Modified Steroids as Aromatase Inhibitors: Design, Synthesis, and Biological Activity Evaluation', *Journal of Medicinal Chemistry*, 48(20), pp. 6379–6385.

Chazin EDL, Terra L, Júnior WTV, Sanches PS, Pinto LC, Martins T, Souza MVN, Gomes

CRB, Montenegro RC, Joffily A, Castro HC, Vasconcelos TRA (2018) 'Evaluation of 1,3-benzoxathiol-2-one and 1,3-benzothiazole derivatives as potential anticancer and antifungal agents', *Medicinal Chemistry*, 1, pp. 1–8.

Chen GG, Zeng Q, Tse GM (2008) 'Estrogen and Its Receptors in Cancer', *Medicinal Research Reviews*, 28(6), pp. 954–974.

Chen Q, Kang J, Fu C (2018) 'The independence of and associations among apoptosis, autophagy, and necrosis', *Signal Transduction and Targeted Therapy*. Springer US, 3(1).

Choi JK, Murillo G, Su BN, Pezzuto JM, Kinghorn AD, Mehta RG (2006) 'Ixocarpalactone A isolated from the Mexican tomatillo shows potent antiproliferative and apoptotic activity in colon cancer cells', *FEBS Journal*, 273(24), pp. 5714–5723.

Christofferson DE, Yuan J (2010) 'Necroptosis as an alternative form of programmed cell death', *Current Opinion in Cell Biology*. Elsevier Ltd, 22(2), pp. 263–268.

Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C (1993) 'A new method for the cytofluorimetric analysis of mitochondrial membrane potencial using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1)', *Biochemical and Biophysical Research Communications*, 197(1), pp. 40–45.

Courtnay R, Ngo DC, Malik N, Ververis K, Tortorella SM, Karagiannis TC (2015) 'Cancer metabolism and the Warburg effect: the role of HIF-1 and PI3K', *Molecular biology reports*, 42(4), pp. 841–851.

Cruz Silva MM, Madeira VMC, Almeira LM, Custódio JBA (2000) 'Hemolysis of human erythrocytes induced by tamoxifen is related to disruption of membrane structure', *Biochimica et Biophysica Acta - Biomembranes*, 1464(1), pp. 49–61.

Cui J, Fan L, Huang Y, Xin Y, Zhou A (2009) 'Synthesis and evaluation of some steroidal oximes as cytotoxic agents: Structure/activity studies (II)', *Steroids*, 74(12), pp. 989–995.

Dai G, Zheng D, Guo WC, Yang J, Cheng AY (2018) 'Cinobufagin Induces Apoptosis in Osteosarcoma Cells Via the Mitochondria-Mediated Apoptotic Pathway', *Cellular Physiology and Biochemistry*, 46(3), pp. 1134–1147.

Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J (2005) 'Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury', *Nature Chemical Biology*, 1(2), pp. 112–119.

Deitch AD, Law H, White RD (1982) 'A Stable Propidium Iodide Staining Procedure for Flow Cytometry', *Journal of Histochemistry and Cytochemistry*, 30, pp. 967–972.

Deive N, Rodríguez J, Jiménez C (2001) 'Synthesis of cytotoxic 6E-hydroximino-4-ene

steroids: Structure/activity studies', *Journal of Medicinal Chemistry*, 44(16), pp. 2612–2618.

Denecker G, Vercammen D, Steemans M, Berghe TV, Brouckaert G, Loo GV, Zhivotovsky B, Fiers W, Grooten J, Declercq W, Vandenabeele P (2001) 'Death receptor-induced apoptotic and necrotic cell death: Differential role of caspases and mitochondria', *Cell Death and Differentiation*, 8(8), pp. 829–840.

Dhingra N, Bhardwaj TR, Mehta N, Mukhopadhyay T, Kumar A, Kumar M (2011) '17-Oximino-5-androsten-3β-yl esters: Synthesis, antiproliferative activity, acute toxicity, and effect on serum androgen level', *Medicinal Chemistry Research*, 20(7), pp. 817–825.

Dhuriya YK, Sharma D (2018) 'Necroptosis: A regulated inflammatory mode of cell death', *Journal of Neuroinflammation*. Journal of Neuroinflammation, 15(1), pp. 1–9.

Dienstmann R, Salazar R, Tabernero J. (2014) 'The Evolution of Our Molecular Understanding of Colorectal Cancer: What We Are Doing Now, What the Future Holds, and How Tumor Profiling Is Just the Beginning', *American Society of Clinical Oncology Educational Book*, 34, pp. 91–99.

Dimas K, Papadopoulou N, Baskakis C, Prousis KC, Tsakos M, Alkahtani S, Honisch S, Lang F, Calogeropoulou T, Alvizopoulos K, Stournaras C (2014) 'Steroidal Cardiac Na^+/K^+ ATPase Inhibitors Exhibit Strong Anti-Cancer Potential in vitro and in Prostate and Lung Cancer Xenografts in vivo', *Anti-Cancer Agents in Medicinal Chemistry*, 14(5), pp. 762–770.

Djurendić EA, Sakač MN, Zaviš MP, Gaković AR, Čanadi JJ, Andrić SA, Klisurić OR, Kojić VV, Bogdanović GM, Gaši KMP (2008) 'Synthesis and biological evaluation of some new A,B-ring modified steroidal d-lactones', *Steroids*, 73(6), pp. 681–688.

Drudge-Coates L (2005) 'Prostate Cancer and the Principles of Hormone Therapy Treatment', *British Journal of Nursing*, 14(7), pp. 368–375.

Dubey S, Jindal DP, Piplani P (2005) 'Synthesis and antineoplastic activity of some 16-benzylidene substituted steroidal oximes', *Indian Journal of Chemistry - Section B Organic and Medicinal Chemistry*, 44(10), pp. 2126–2137.

Edlich F (2018) 'BCL-2 proteins and apoptosis: Recent insights and unknowns', *Biochemical and Biophysical Research Communications*. Elsevier Ltd, 500(1), pp. 26–34.

Eguchi Y, Shimizu S, Tsujimoto Y (1997) 'Intracellular ATP Levels Determine Cell Death Fate By Apoptosis or Necrosis', *Cancer Research*, 57.

Encarnação JC, Abrantes AM, Pires AS, Botelho MF (2015) 'Revisit dietary fiber on colorectal cancer: butyrate and its role on prevention and treatment', *Cancer and Metastasis Reviews*, 34(3), pp. 465–478.

Ericson-Neilsen W, Kaye AD (2014) 'Steroids: pharmacology, complications, and practice delivery issues.', *The Ochsner journal*, 14(2), pp. 203–7.

Farazi PA, DePinho RA (2006) 'Hepatocellular carcinoma pathogenesis: From genes to environment', *Nature Reviews Cancer*, 6(9), pp. 674–687.

Fearon ER (2016) 'Molecular genetics of colorectal cancer', *Seminars in Colon and Rectal Surgery*, 27(4), pp. 172–175.

Fearon ER, Vogelstein B (1990) 'A Genetic Model for Colorectal Tumorigenesis', *Cell*, 61, pp. 759–767.

Fischer DS, Allan GM, Bubert C, Vicker N, Smith A, Tutill HJ, Purohit A, Wood L, Packham G, Mahon MF, Reed MJ, Potter BVL (2005) 'E-ring modified steroids as novel potent inhibitors of 17β- hydroxysteroid dehydrogenase type 1', *Journal of Medicinal Chemistry*, 48(18), pp. 5749–5770.

Fleming M, Ravula S, Tatishchev SF, Wang HL (2012) 'Colorectal carcinoma : Pathologic aspects Key words Histologic variants', *J Gastrointest Oncol*, 3(3), pp. 1–15.

Forner A, Reig M, Bruix J (2012) 'Hepatocellular carcinoma', *The Lancet*. Elsevier Ltd, 379, pp. 1245–1255. doi: 10.1016/S0140-6736(18)30010-2.

Funk J (1999) 'Cancer cell cycle control', *Anticancer Research*, 19(6A).

Gann PH (2002) 'Risk Factors for Prostate Cancer', 4, pp. 3–10.

Gáspár S (2011) 'Detection of superoxide and hydrogen peroxide from living cells using electrochemical sensors', *ACS Symposium Series*, 1083, pp. 289–309.

Gobbini M, Armaroli S, Banfi L, Benicchio A, Carzana G, Fredizzi G, Ferrari P, Giacalone G, Giubileo M, Marazzi G, Micheletti R, Moro B, Pozzi M, Scotti PE, Torri M, Cerri A (2008) 'Novel Analogues of Istaroxime , a Potent Inhibitor of Na + , K + -ATPase : Synthesis and Structure', *Society*, pp. 4601–4608.

Gogvadze V, Orrenius S, Zhivotovsky B (2008) 'Mitochondria in cancer cells: what is so special about them?', *Trends in Cell Biology*, 18(4), pp. 165–173.

Gonzalez-Pons M, Cruz-Correa M (2015) 'Colorectal Cancer Biomarkers : Where Are We Now ?', *BioMed Research International*, 2015.

Grivicich I, Regner A, Rocha AB (2007) 'Morte Celular por Apoptose', *Revista Brasileira de Cancerologia*, 53(3), pp. 335–343.

Grönberg H (2003) 'Prostate cancer epidemiology', *The Lancet*, 361, pp. 859–864.

Guan, Y. Y., Li, S. Z. and Lei, P. S. (2017) 'Synthesis and cytotoxic activity of two steroids:

icogenin aglycone analogs', *Journal of Asian Natural Products Research*. Taylor & Francis, 19(5), pp. 481–488.

Gupta A, Kumar BS, Negi AS (2013) 'Current status on development of steroids as anticancer agents', *Journal of Steroid Biochemistry and Molecular Biology*. Elsevier Ltd, 137, pp. 242–270.

Han Q, Ma Y, Wang H, Dai Y, Chen C, Liu Y, Jing L, Sun X (2018) 'Resibufogenin suppresses colorectal cancer growth and metastasis through RIP3-mediated necroptosis', *Journal of Translational Medicine*, 16(1), pp. 1–13.

Hanahan D, Weinberg RA (2000) 'Hallmarks of Cancer', *Cell*, 100, pp. 57–70.

Hanahan D, Weinberg RA (2011) 'Hallmarks of Cancer: The Next Generation', *Cell*. Elsevier Inc., 144(5), pp. 646–674.

Hartmann RW, Hector M, Haidar S, Ehmer PB, Reichert W, Jose J (2000) 'Synthesis and evaluation of novel steroidal oxime inhibitors of P450 17 (17α-hydroxylase/c17-20-lyase) and 5α-reductase types 1 and 2', *Journal of Medicinal Chemistry*, 43(22), pp. 4266–4277.

Heidenreich A, Bastian PJ, Bellmunt J, Bolla M, Joniau S, Kwast TVD, Mason M, Matveev V, Weigel T, Zattoni F, Mottet N (2014) 'EAU Guidelines on Prostate Cancer. Part II: Treatment of Advanced, Relapsing, and Castration-Resistant Prostate Cancer', *European Urology*. European Association of Urology, 65(2), pp. 467–479.

Hejaz HAM, Purohit A, Mahon MF, Reed MJ, Potter BVL (1999) 'Synthesis and biological activity of the superestrogen (E)-17-oximino- 3-O-sulfamoyl-1,3,5(10)-estratriene: X-ray crystal structure of (E)-17- oximino-3-hydroxy-1,3,5(10)-estratriene', *Journal of Medicinal Chemistry*, 42(16), pp. 3188–3192.

Herbst RS, Heymach JV, Lippman SM (2008) 'Molecular origins of cancer - Lung Cancer', *The New England journal of medicine*, 359, pp. 1367–1380.

Holland HL, Kumaresan S, Tan L, Njar VCO (1992) 'Synthesis of 6-Hydroximino-3-oxo Steroids, a New Class of Aromatase Inhibitor', *Journal of the Chemical Society, Perkin Transactions 1*, (5), pp. 585–587.

Honisch S, Alkahtani S, Kounenidakis M, Liu G, Alarifi S, Al-Yahya H, Dimas K, AlKahtane AA, Prousis KC, Al-Dahmash B, Calogeropoulou T, Alevizopoulos K, Lang F, Stournanras C (2014) 'A Steroidal Na^+/K^+ ATPase Inhibitor Triggers Pro-apoptotic Signaling and Induces Apoptosis in Prostate and Lung Tumor Cells', *Anti-Cancer Agents in Medicinal Chemistry*, 14(8), pp. 1161–1168.

Hou Q, He C, Lao K, Luo G, You Q, Xiang H (2019) 'Design and synthesis of novel steroidal

imidazoles as dual inhibitors of AR/CYP17 for the treatment of prostate cancer', *Steroids*. Elsevier, (February), pp. 0–1.

Huang Y, Cui J, Zheng Q, Zeng C, Chen Q, Zhou A (2012) '6-Hydroximino-4-aza-A-homo-cholest-3-one and related analogue as a potent inducer of apoptosis in cancer cells', *Steroids*. Elsevier Inc., 77(8–9), pp. 829–834.

Huggins DJ, Sherman W, Tidor B (2012) 'Rational Approaches to Improving Selectivity in Drug Design', *Journal of Medicinal Chemistry*, 55(4), pp. 1424–1444.

Hughes D, Mehmet H *Cell Proliferation & Apoptosis*. 1st Edition (2003) Oxford: Bios Scientific Publishers Limited.

Hughes TB, Miller GP, Swamidass SJ (2015) 'Modeling epoxidation of drug-like molecules with a deep machine learning network', *ACS Central Science*, 1(4), pp. 168–180.

Hussein S, Satturwar S, Van Der Kwast T (2015) 'Young-age prostate cancer', *Journal of Clinical Pathology*, 68(7), pp. 511–515.

Joshua AM, Evans A, Kwast TVD, Zielenska M, Meeker AK, Chinnaiyan A, Squire JÁ (2008) 'Prostatic preneoplasia and beyond', *Biochimica et Biophysica Acta - Reviews on Cancer*, 1785(2), pp. 156–181.

Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, Schellhammer PF (2010) 'Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer Philip', *The New England Journal of Medicine*, 363(5), pp. 1543–1554.

Knowles M, Selby P '*Introduction to the cellular and molecular biology of cancer*'. 4th Edition (2005) Oxford: Oxford University Press.

Koskenkorva-Frank TS, Weiss G, Koppenol WH, Burckhardt (2013) 'The complex interplay of iron metabolism, reactive oxygen species, and reactive nitrogen species: Insights into the potential of various iron therapies to induce oxidative and nitrosative stress', *Free Radical Biology and Medicine*. Elsevier, 65, pp. 1174–1194.

Labianca R, Nordlinger B, Beretta GD, Mosconi S, Mandalà M, Cervantes A, Arnold D (2010) 'Primary colon cancer: ESMO clinical practice guidelines for diagnosis, adjuvant treatment and follow-up', *Annals of Oncology*, 21(SUPPL. 5).

Larsen JE, Minna JD (2011) 'Molecular Biology of Lung Cancer: Clinical Implications', *Clinics in Chest Medicine*. Elsevier Inc, 32(4), pp. 703–740.

Leist M, Single B, Castoldi AF, Kühnle S, Nicotera P (1997) 'Intracellular Adenosine Trisphosphate (ATP) Concentration: A Switch in the Decision Between Apoptosis and

Necrosis', *Journal of Experimental Medicine*, 185(8), pp. 1481–1486.

Leist M, Jäättelä M (2001) 'Four deaths and a funeral: From caspases to alternative mechanisms', *Nature Reviews Molecular Cell Biology*, 2(8), pp. 589–598.

Li L, Wang H (2016) 'Heterogeneity of liver cancer and personalized therapy', *Cancer Letters*. Elsevier Ireland Ltd, 379(2), pp. 191–197.

Lopez J, Tait SWG (2015) 'Mitochondrial apoptosis: Killing cancer using the enemy within', *British Journal of Cancer*. Nature Publishing Group, 112(November 2014), pp. 957–962.

Lu X, Qiao YB, Li Y, Yang B, Chen MB, Xing CG (2017) 'Preclinical study of cinobufagin as a promising anti-colorectal cancer agent', *Oncotarget*, 8(35), pp. 988–998.

Ly JD, Grubb DR, Lawen A (2003) 'The mitochondrial membrane potential ($\Delta\psi_m$) in apoptosis; an update', *Apoptosis*, 8(2), pp. 115–128.

Mamede AC, Abrantes AM, Pedrosa L, Casalta-Lopes JE, Pires AS, Teixo RJ, Gonçalves AC, Sarmento-Ribeiro AB, Maia CJ, Botelho MF (2013) 'Beyond the Limits of Oxygen: Effects of Hypoxia in a Hormone-Independent Prostate Cancer Cell Line', *ISRN Oncology*, 2013, pp. 1–8.

Mandal P, Berger SB, Pillay S, Moriwaki K, Huang C, Guo H, Lich JD, Finger J, Kasparcova V, Votta B, Ouellete M, King BW, Wisnoski D, Lakdawala AS, DeMartino MP, Casillas LN, Haile PA, Sehon CA, Marquis RW, Upton J, Daley-Bauer LP, Roback L, Ramia N, Dovey CM, Carette JE, Chan FKM, Bertin J, Gough PJ, Mocarski ES, Kaiser WJ (2014) 'RIP3 Induces Apoptosis Independent of Pronecrotic Kinase Activity', *Molecular Cell*. Elsevier Inc., 56(4), pp. 481–495.

Mansinhos IFP '*Detection of New Actionable Mutations in Lung Cancer Precision Therapy*'. Master Thesis, University of Coimbra (2017).

Manson MM (1980) 'Epoxides – Is There a Human Health Problem?', *British Journal of Industrial Medicine*, 37, pp. 317–336.

Marco-Contelles J, Molina MT, Anjum S (2004) 'Naturally occurring cyclohexane epoxides: Sources, biological activities and synthesis', *Chemical Reviews*, 104(6), pp. 2857–2899.

Marques, IAF '*Rádio-223 no tratamento do carcinoma da próstata metastático: Estudo dos mecanismos de atuação'*. Master Thesis, Universidade de Coimbra (2016).

Masayuki N, Morikawa A, Kwasaki M, Matsuno Y, Yamada T, Hirohashi S, Kondo H, Shimosato Y (1995) 'Small adenocarcinoma of the lung. Histologic characteristics and prognosis', *Cancer*, 75(12), pp. 2844–2852.

McDonald ER, El-Deiry WS (2000) 'Cell cycle control as a basis for cancer drug

development', *International journal of oncology*, 16(5), pp. 871–886.

McLaughlin F, Finn P, La Thangue NB (2003) 'The cell cycle, chromatin and cancer: Mechanism-based therapeutics come of age', *Drug Discovery Today*, 8(17), pp. 793–802.

Merlani MI, Amiranashvili LS, Mulkidzhanyan KG (2006) 'Synthesis and biological activity of certain amino-derivatives of 5α-steroids', *Chemistry of Natural Compounds*, 42(3), pp. 263–265.

Micheletti R, Mattera GG, Rocchetti M, Schiavone A, Loi MF, Zaza A, Gagnol RJP, Munari SD, Melloni P, Carminati P, Bianchi G, Ferrari P (2002) 'Pharmacological Profile of the Novel Inotropic Agent (E,Z)-3-((2-Aminoethoxy)imino)androstane-6,17-dione Hydrochloride (PST2744)', *The Journal of pharmacology and experimental therapeutics*, 303(2), pp. 592–600.

Mijatovic T, Roland I, Quaquebeke EV, Nilsson B, Mathieu A, Vynckt FV, Darro F, Blanco G, Facchini V, Kiss R (2007) 'The α1 subunit of the sodium pump could represent a novel target to combat non-small cell lung cancers', *Journal of Pathology, The*, 212, pp. 170–179.

Miller K, Siegel R, Jemal A (2016) 'Cancer Treatment & Survivorship - Facts & Figures', *American Cancer Society*.

Miller WL, Auchus RJ (2011) 'The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders.', *Endocrine reviews*, 32(1), pp. 81–151.

Moldogazieva NT, Lutsenko SV, Terentiev AA (2018) 'Reactive Oxygen and Nitrogen Species-Induced Protein Modifications: Implication in Carcinogenesis and Anticancer Therapy.', *Cancer Research*, pp. 1–9.

Mosmann T (1983) 'Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays', *Journal of Immunological Methods*, 65(1–2), pp. 55–63.

Naahidi S, Jafari M, Logan M, Edalat F, Khademhosseini A, Dixon B, Chen P (2014) 'Immuno-and hemocompatibility of amino acid pairing peptides for potential use in anticancer drug delivery', *Journal of Bioactive and Compatible Polymers*, 29(3), pp. 254–269.

Nechifor M, Neagu TM, Manda G (2012) 'Reactive Oxygen Species, Cancer and Anti-Cancer Therapies', *Current Chemical Biology*, 3(1), pp. 22–46.

Newman RA, Yang P, Pawlus AD, Block KI (2008) 'Cardiac Glycosides as Novel Cancer Therapeutic Agents', *Molecular Interventions*, 8(1), pp. 36–49.

De Oliveira AC, Hillard EA, Pigeon P, Rocha DD, Rodrigues FAR, Montenegro RC, Costa-

Lotufo LV, Goulart MOF, Jaouen G (2011) 'Biological evaluation of twenty-eight ferrocenyl tetrasubstituted olefins: Cancer cell growth inhibition, ROS production and hemolytic activity', *European Journal of Medicinal Chemistry*. Elsevier Masson SAS, 46(9), pp. 3778–3787.

Peng P, Lv J, Cai C, Lin S, Zhou E, Wang S (2017) 'Cinobufagin, a bufadienolide, activates ROS-mediated pathways to trigger human lung cancer cell apoptosis in vivo', *RSC Advances*. Royal Society of Chemistry, 7(40), pp. 25175–25181.

Petros AM, Olejniczak ET, Fesik SW (2004) 'Structural biology of the Bcl-2 family of proteins', *Biochimica et Biophysica Acta - Molecular Cell Research*, 1644(2–3), pp. 83–94.

Pires AS '*Vitamina C e cancro*'. Doctoral Thesis, University of Coimbra (2016).

Pires AS, Marques CR, Encarnação JC, Abrantes AM, Marques IA, Laranjo M, Oliveira R, Casalta-Lopes JE, Gonçalves AC, Sarmento-Ribeiro AB, Botelho MF (2018) 'Ascorbic acid chemosensitizes colorectal cancer cells and synergistically inhibits tumor growth', *Frontiers in Physiology*, 9(JUL), pp. 1–14.

Qi F, Inagaki Y, Gao B, Cui X, Xu H, Kokudo N, Li A, Tang W (2011) 'Bufalin and cinobufagin induce apoptosis of human hepatocellular carcinoma cells via Fas- and mitochondria-mediated pathways', *Cancer Science*, 102(5), pp. 951–958.

Rah B, Amin H, Yousuf K, Khan S, Jamwal G, Mukherjee D, Goswami A (2012) 'A Novel MMP-2 Inhibitor 3-azidowithaferin A (3-azidoWA) Abrogates Cancer Cell Invasion and Angiogenesis by Modulating Extracellular Par-4', *PLoS ONE*, 7(9), pp. 1-14.

Rasheed A, Qasim M (2013) 'A Review of natural steroids and their applications', *Internation Journal of Pharmaceutical Sciences and Reserach*, 4(2), pp. 520–531.

Reed JC (2006) 'Proapoptotic multidomain Bcl-2/Bax-family proteins: Mechanisms, physiological roles, and therapeutic opportunities', *Cell Death and Differentiation*, 13(8), pp. 1378–1386.

Reed JC (2008) 'BCL-2-family proteins and hematologic malignancies: history and future prospects', *Blood*, 111(7), pp. 3322–3330.

Rhee SG, Chang TS, Jeong W, Kang D (2010) 'Methods for detection and measurement of hydrogen peroxide inside and outside of cells', *Molecules and Cells*, 29(6), pp. 539–549.

Ribeiro da Silva RC '*Efeitos da radiação em carcinoma do pulmão*'. Master Thesis, University of Coimbra (2012).

Rodriguez J, Nuñez L, Peixinho S, Jiménez C (1997) 'Isolation and Synthesis of the First Natural 6-Hydroximino 4-en-3-one- Steroids from the Sponges Cinachyrella spp.',

Tetrahedron, 38(10), pp. 1833–1836.

Roleira FMF, Varela C, Amaral C, Costa SC, Correia-da-Silva G, Moraca F, Costa G, Alcaro S, Teixeira N, Tavares da Silva, E (2019) 'C-6α- vs C-7α-Substituted Steroidal Aromatase Inhibitors: Which Is Better? Synthesis, Biochemical Evaluation, Docking Studies, and Structure-Activity Relationships', *Journal of Medicinal Chemistry*, 62(7), pp. 3636–3657.

Rytting M, Worth L, Jaffe N (1996) 'Hemolytic Disorders Associated With Cancer', *Hematologic Complications of Cancer*, 10(2), pp. 492–495.

Sakai H, Suzuki T, Maeda M, Takahashi Y, Horikawa N, Minamimura T, Tsukada K, Takeguchi N (2004) 'Up-regulation of Na+,K+-ATPase α3-isoform and down-regulation of the α1-isoform in human colorectal cancer', *FEBS Letters*, 563(1–3), pp. 151–154.

Sánchez-Sánchez L, Hernández-Linares MG, Escobar ML, López-Muñoz H, Zenteno E, Fernández-Herrera MA, Guerrero-Luna G, Carrasco-Carballo A, Sandoval-Ramírez J (2016) 'Antiproliferative, cytotoxic, and apoptotic activity of steroidal oximes in cervicouterine cell lines', *Molecules*, 21(11), pp. 1–17.

Santafé G, Paz V, Rodríguez J, Jiménez C (2002) 'Novel cytotoxic oxygenated C29 sterols from the Colombian marine sponge Polymastia tenax', *Journal of Natural Products*, 65(8), pp. 1161–1164.

Schottenfeld D, Fraumeni JF '*Cancer Epidemiology and Prevention*'. 3rd edition (2006), *Oxford University Press, Inc.*

Sciarra A, Mariotti G, Salciccia S, Gomez AA, Monti S, Toscano V, Silverio FD (2008) 'Prostate growth and inflammation', *Journal of Steroid Biochemistry and Molecular Biology*, 108(3–5), pp. 254–260.

Shah SJ, Blair JEA, Filippatos GS, Macarie C, Ruzyllo W, Korewicki J, Bubenek-Turconi SI, Ceracchi M, Bianchetti M, Carminati P, Kresmastinos D, Grzybowski J, Valentini G, Sabbah HN, Gheorghiade M (2009) 'Effects of istaroxime on diastolic stiffness in acute heart failure syndromes: Results from the Hemodynamic, Echocardiographic, and Neurohormonal Effects of Istaroxime, a Novel Intravenous Inotropic and Lusitropic Agent: A Randomized Controlled Trial in P', *American Heart Journal*. Mosby, Inc., 157(6), pp. 1035–1041.

Shaikh S, Verma H, Yadav N, Jauhari M, Bullangowda (2012) 'Applications of Steroid in Clinical Practice: A Review', *ISRN Anesthesiology*, 2012, pp. 1–11.

Shen M, Abate-Shen C (2010) 'Molecular genetics of prostate cancer: new prospects for old challenges', *Genes & development*, (212), pp. 1967–2000.

Siegel R, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, Cooper D, Gansler T, Lerro C,

Fedewa S, Lin C, Leach C, Cannady RS, Cho H, Scoppa S, Hachey M, Kirch R, Jemal A, ward E (2012) 'Cancer Treatment and Survivorship Statistics , 2012', *CA Cancer J Clin*, 00(0), pp. 1–22.

Siegel R, Jemal A (2016) 'Colorectal Cancer Facts & Figures', *American cancer society*.

Stagno MJ, Zacharopoulou N, Bochem J, Tsapara A, Pelzl L, al-Maghout T, Kallergi G, Alkahtani S, Alevizopoulos K, Dimas K, Calogeropoulou T, Warmann SW, Lang F, Schmid E, Stournaras C (2017) 'Istaroxime Inhibits Motility and Down-Regulates Orai1 Expression, SOCE and FAK Phosphorylation in Prostate Cancer Cells', *Cellular Physiology and Biochemistry*, 42(4), pp. 1366–1376.

Stark GR, Taylor WR (2004) 'Analyzing the G2 / M Checkpoint', *Methods in Molecular Biology*, 280(1), pp. 51–82.

Syntichaki P, Tavernarakis N (2002) 'Death by necrosis Uncontrollable catastrophe, or is there order behind the chaos?', *EMBO Reports*, 3(7), pp. 604–609.

Teh E, Belche E (2014) 'Lung cancer: Diagnosis, staging and treatment', *Surgery (United Kingdom)*. Elsevier Ltd, 32(5), pp. 242–248.

Thibodeaux CJ, Chang WC, Liu HW (2012) 'Enzymatic chemistry of cyclopropane, epoxide, and aziridine biosynthesis', *Chemical Reviews*, 112(3), pp. 1681–1709.

Toksöz H, Ünal S, Kirazli Ş, Gürgey A (2004) 'The effect of short course high dose methylprednisolon therapy on fibrinogen level in children with acute lymphoblastic leukemia: A preliminary study', *Leukemia Research*, 28(5), pp. 533–535.

Varela C, Tavares da Silva EJ, Amaral C, Correia-da-Silva G, Baptista T, Alcaro S, Costa G, Carvalho RA, Teixeira NAA, Roleira FMF (2012) 'New structure-activity relationships of A-and D-ring modified steroidal aromatase inhibitors: Design, synthesis, and biochemical evaluation', *Journal of Medicinal Chemistry*, 55(8), pp. 3992–4002.

Varela CL, Amaral C, Tavares da Silva E, Lopes A, Correia-da-Silva G, Carvalho RA, Costa SCP, Roleira FMF (2014) 'Exemestane metabolites: Synthesis, stereochemical elucidation, biochemical activity and anti-proliferative effects in a hormone-dependent breast cancer cell line', *European Journal of Medicinal Chemistry*, 87, pp. 336–345.

Vermes I, Haanen C, Steffens-nakken H, Reutelingsperger C (1995) 'A novel assay for apoptosis Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V', 184(95), pp. 39–51.

Vermes I, Haanen C, Reutelingsperger C (2000) 'Flow cytometry of apoptotic cell death', *Journal of Immunological Methods*, 243(1–2), pp. 167–190.

Vieira RA, Tramonte MS, Lopes LF (2015) 'Colorectal carcinoma in the first decade of life: a systematic review', *International Journal of Colorectal Disease*, 30(8), pp. 1001–1006.

Vineis P (2005) 'Environmental tobacco smoke and risk of respiratory cancer and chronic obstructive pulmonary disease in former smokers and never smokers in the EPIC prospective study', *British Medical Journal*, 330(7486), pp. 277–280.

Vollhardt P, Schore N *'Organic Chemistry Structure and Function'*. 6th Edition (2011). W. H. Freeman and Company.

Waring MJ (2010) 'Lipophilicity in drug discovery', *Expert Opinion on Drug Discovery*, 5(3), pp. 235–248.

World Health Organization (2019) 'Portugal', *International Agency for Research on Cancer*.

Yeh JY, Huang WJ, Kan SF, Wang PS (2003) 'Effects of bufalin and cinobufagin on the proliferation of androgen dependent and independent prostate cancer cells', *Prostate*, 54(2), pp. 112–124.

Yiu AJ, Yiu CY (2016) 'Biomarkers in Colorectal Cancer', *Anticancer Research*, 36, pp. 1093–1102.

Young A, Hobbs R, Kerr D *'ABC of colorectal cancer'*. 2nd edition (2011). John Wiley & Sons, Ltd.

Zappa C, Mousa SA (2016) 'Non-small cell lung cancer: current treatment and future advances', *Translational Lung Cancer Research*, 5(3), pp. 288–300.

Zhang L, Wang H, Ding K, Xu J (2015) 'FTY720 induces autophagy-related apoptosis and necroptosis in human glioblastoma cells', *Toxicology Letters*. Elsevier Ireland Ltd, 236(1), pp. 43–59.