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e-Printed Reasons
Journal of Theoretical & Experimental Pharmacology
2008; Volume 1: Number 1 (January – April)

CONTENTS

e-Printed Reasons
Journal of Theoretical & Experimental Pharmacology
2008; Volume 1 (Number 1): Pages 1-2
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Editorial

Good news: the Journal of Theoretical and Experimental Pharmacology was born

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The story on how this journal was planned is full of anecdotes, friendship, and passion for work. However, I am sure that none of members of the small group of colleagues who conceived the original idea of the journal realized all the difficulties that would need to be sorted out to see the first issue of the journal published.

The ***Journal of Theoretical and Experimental Pharmacology*** welcomes any pharmacological research performed *in silico*, *in vitro*, and in experimental animal models including computer simulations with potential pharmacological interest; SAR and QSAR analyses; dose-response evaluations; drug-drug interactions; drug effects at cellular and sub-cellular levels; *in vitro* and *in vivo* metabolic studies; and pharmacokinetic, pharmacodynamic, and teratogenic studies. The journal is also accepting for consideration for publication studies in the field of pharmacogenetics, pharmacogenomics, and preclinical toxicology.

Studies evaluating the effects of vitamins, hormones, immunological mediators, or chemicals are also welcomed if any of the topics mentioned above are the focus of the study. At this time, only invited review articles are being accepted. However, systematic reviews and meta-analysis of preclinical pharmacological studies are also welcomed through the standard submission process.

The journal will also consider for publication manuscripts devoted to the analysis of philosophical and ethical aspects of biomedical sciences. These latter topics are intended to enrich the content of the journal and to make it an open space where ethical and philosophical dilemmas can be analyzed and debated.

I am excited by the response of colleagues from around the world to my invitation to join us in this adventure. Currently, 24 scientists from 13 different countries around the world are members of the Editorial Board. In this very first issue the journal publishes two review papers and two original studies. All of them are of such a high quality that I should thank the authors, including my Mexican colleagues, for considering this journal as their option for publishing these papers instead of preferring well-established journals.

SERAFIM et al. [1] reviewed the potential role of several phenolic acids and derivatives as anti-cancer agents, highlighting the role of mitochondria as a primary subcellular target for this class of compounds. Plant-derived phenolic compounds are widely consumed in a normal diet, especially in fruits and vegetables. Interestingly, phenolic acids have been reported to display antiproliferative activity by promoting selective induction of tumor cell apoptosis and by triggering the mitochondrial pathway for apoptosis. CANAPARO & SERPE [2] reviewed single nucleotide polymorphisms as a source of human genetic and phenotypic variation and their contribution to the variability of drug action. However, as the authors duly noted, any time genetics plays a role modifying drug effects, there likely is a complex interplay of several genes rather than the action of a single one.

GONZÁLEZ-LOZANO et al. [3] evaluated the obstetric and fetal outcomes in dystocic sows receiving udder massage stimulation alone or in combination with

oxytocin. The authors observed that dystocic sows receiving udder massage had better obstetric and fetal outcomes than control sows. However, co-administration of oxytocin might reverse the advantages of udder massage. Finally, KIM et al. [4] examined the molecular mechanism of how Histone deacetylase (HDAC) inhibitors modulate the cell cycle regulators and tumor suppressor genes in prostate cancer cells. The study found that HDAC inhibitors may prevent the proliferation of prostate cancer cells by altering the expression of the cell cycle regulators and tumor suppressor genes, which might be associated with epigenetic regulation.

The journal is published electronically in the English language only. However, the abstracts are available in three different languages. Translation of the abstracts to French and Spanish was made possible by colleagues who volunteered for this task.

This issue was supported by submission fees for original papers as well as by generous support from PharmaReasons, Toronto, Canada. However, the final acceptance of the manuscripts was independent of the journal's supporter. All the manuscripts can be downloaded at no cost.

As Editor, I express gratitude to my friends and colleagues who have worked alongside me since the beginning of this project. The authors and the readers of this journal deserve a special note of gratitude for trusting us.

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e-Printed Reasons
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MITOCHONDRIA AS A TARGET FOR NOVEL CHEMOTHERAPEUTIC AGENTS BASED ON PHENOLIC ACIDS

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ABSTRACT

Every day, new molecules with antineoplastic potential are discovered. Unfortunately, most of these molecules lack cell-type specificity, and are unable to kill tumor cells any more efficiently than normal cells. Also, a high number of those molecules are very toxic. In order to design more effective chemotherapeutic drugs, it is important to understand the interaction between novel molecules and biological systems. Certain cellular components are particularly relevant in the context of specific targeting and mechanisms of action. Mitochondria are not only the major source of cell energy but are also important in the control of processes that culminate in apoptotic cell death. Particular aspects of mitochondrial physiology (e.g. the negative transmembrane electric potential) facilitate selective targeting by anti-cancer molecules. Such mitochondria-specific drugs are referred to as 'mitocans'. Among potential mitocans, phenolic acids are attractive candidates. Plant-derived phenolic compounds are widely consumed in a normal diet, especially in fruits and vegetables. Besides their antioxidant properties, phenolic acids have been reported to display antiproliferative activity by promoting selective induction of tumor cell apoptosis. In some cases, the molecule acts by triggering the mitochondrial pathway for apoptosis. Here we review the potential role of several phenolic acids and derivatives as anti-cancer agents, highlighting the role of mitochondria as a primary subcellular target for this class of compounds. The present review intends to raise awareness for this promising direction of research.

Key words

Antineoplastic drugs; Antioxidants; Hydroxybenzoic acids; Mitochondria

RÉSUMÉ

Des nouvelles molécules anticancéreuses potentielles sont découvertes quasi journellement. Malheureusement la plupart de ces molécules n'exercent pas de spécificité cellulaire et ne tuent pas plus les cellules tumorales que les cellules normales. Un grand nombre de ces molécules sont par ailleurs toxiques. Dans le but de développer des molécules anticancéreuses plus efficaces il est dès lors important de comprendre l'interaction entre de nouvelles molécules et les systèmes biologiques. Certains composants cellulaires sont particulièrement appropriés comme cibles spécifiques pour leurs mécanismes d'action. Les mitochondries sont la source principale d'énergie cellulaire, mais sont aussi importantes dans le contrôle des processus qui aboutissent à la mort cellulaire par apoptose. Certaines caractéristiques physiologiques des mitochondries, comme leur potentiel de membrane, permettent une attaque spécifique des mitochondries par des molécules anticancéreuses appelées «mitocans». Les acides phénoliques sont candidats mitocans très intéressants. Les composés phénoliques naturels sont abondamment présents dans l'alimentation, en particulier dans les fruits et les légumes. Outre leurs propriétés antioxydantes il a été dans certains cas démontré qu'ils possédaient des propriétés antiprolifératives par promotion d'une apoptose sélective par mécanisme mitochondrial. Dans cet article nous revoyons le rôle potentiel de certains acides phénoliques et de leurs dérivés comme agents anticancéreux, en insistant sur le rôle des mitochondries comme cible subcellulaire pour cette classe de composés. Cette revue de la littérature souligne l'importance de cette nouvelle voie d'investigation.

Mots clés

Médicaments anticancéreuses; Antioxydants ; Acides hydroxybenzoïques; Mitochondries

RESUMEN

Todos los días se descubren nuevas moléculas con actividad antineoplásica. Sin embargo, la mayoría de ellas carecen de especificad celular, lo que se traduce en una incapacidad para eliminar a las células tumorales en forma más eficiente que a las células normales. Un número importante de estas moléculas también son altamente tóxicas. En el diseño de fármacos quimioterapéuticos más efectivos es importante entender la interacción entre las nuevas moléculas y los sistemas biológicos. Algunos componentes celulares son especialmente relevantes en el contexto de su especificidad y mecanismo de acción. Las mitocondrias, además de ser la principal fuente de energía celular, también son importantes en el control de la apoptosis. Algunos aspectos fisiológicos de las mitocondrias, p.ej. el potencial eléctrico de membrana negativo, podrían ser un blanco selectivo molecular de algunos anticancerígenos. Estos fármacos antimitocondriales específicos son citados en la literatura como 'mitocanos'. Los ácidos fenólicos son candidatos atractivos para actuar como mitocanos. Los compuestos fenólicos derivados de plantas son ampliamente consumidos en la dieta diaria, especialmente en las frutas y los vegetales. Además de su actividad antioxidante, los ácidos fenólicos han mostrado actividad antiproliferativa promoviendo la inducción selectiva de la apoptosis de las células tumorales; en algunos casos, activando la vía mitocondrial de la apoptosis. En este manuscrito hemos revisamos el papel potencial de varios ácidos fenólicos y sus derivados como agentes anticancerígenos, enfatizando el papel de las mitocondrias como el principal blanco subcelular de esta clase de compuestos. Esta revisión intenta dirigir la atención a esta prometedora línea de investigación.

Palabras clave

Medicamentos antineoplásicos; Antioxidantes; Ácidos hidroxibenzoicos; Mitocondrias

INTRODUCTION

Significant progress has been made in cancer research in recent decades. Profound changes are occurring in several new experimental approaches currently under pursuit [1], including genetic strategies, recombinant biology, irradiation, and anticancer chemotherapy. Despite improved pre-clinical data, some cancers are not easy to control while others display a high rate of remission.

Chemotherapy has been one of the strategies with a higher success rate, particularly when based on natural compounds. The use of phytochemical-type molecules has emerged as a promising and pragmatic clinical approach: besides reducing cancer risk, these molecules constitute a wide family of natural compounds with a considerable range of important properties such as low toxicity, low cost, and effectiveness when orally administrated [2].

Among phytochemicals, phenolic compounds [such as phenolic acids, stilbenes, curcuminoids, vanilloids, chalcones, and flavonoids (Figure 1)], are one of the most numerous and ubiquitous groups of plant metabolites, and an integral part of the human diet. Phenolic acids and derivatives are a particular phenolic class known to display a wide variety of biological functions: besides their high antioxidant capacity, phenolic acids have shown remarkable modulating properties in the carcinogenic process. In this context, phenolic derivatives have been under intense scrutiny, in some cases having a higher biological activity than their parent compounds [2].

The determination of cellular target(s) for these natural compounds, as well as the characterization of the corresponding molecular mechanisms of action, can lead to the design of new and more effective agents based on the chemical structure of the parent (lead) compound. One possible experimental strategy is the development of molecules capable of triggering the mitochondrial pathway for apoptosis

Targeting particular aspects of mitochondrial physiology may provide an effective strategy to selectively trigger apoptosis in cancer cells. In fact, there are several differences between normal and transformed cells concerning the main routes for ATP production: although neoplastic cells are mostly dependent on glycolysis for ATP generation, mitochondria are usually functional, with other roles on cellular physiology besides ATP production [3].

Mitochondria also participate in the regulation of intracellular calcium and iron concentrations, as well as in several biosynthetic pathways. Also, production of reactive oxygen species (ROS), which might be harmful if excessively produced, and more importantly, the regulation of cell death pathways, are major roles of mitochondria in cancer cells. Finding a strategy to destabilize mitochondrial homeostasis can lead to compromised integrity and to the elimination of malignant cells [4].

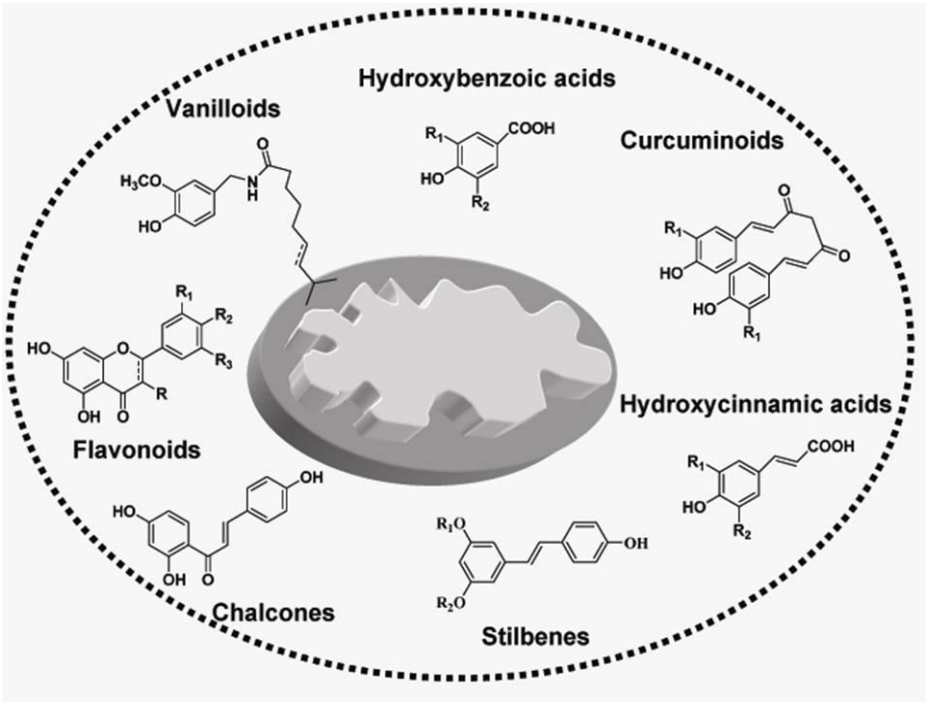

Figure 1 Examples of phenolic systems that can be used to investigate potential chemotherapeutic properties by inducing the mitochondrial pathway for apoptosis.

CHEMISTRY OF PHENOLIC ACIDS AND DERIVATIVES

Structural diversity is one of the main characteristics of phenolic systems, which constitute one of the most numerous and ubiquitous families of plant metabolites. Phenolic molecules display an impressive array of structures (more than 8,000 compounds have been described to date) classified into 10 different groups according to their chemical characteristics [5].

Apart from variations in the carbon skeleton, these compounds differ in the hydroxylation pattern of the phenolic ring, in the presence of alkyl ester or amide side chains, in the occurrence of stereoisomers, in the tendency for oligomerization in condensed phases, or in the conjugation to amines and sugars [6]. The biological activity of polyphenols is known to be largely by this structural wealth as much as by their antioxidant potency. In fact, their biochemical properties have been shown to be highly structure-dependent [7]: bioactivity varies considerably upon minor structural modifications, since these often induce significant conformational changes [8]. The bioavailability of phenolic compounds is directly related to their lipophilicity, which, in turn, is strongly influenced by chemical and structural preferences.

Several structure-activity relationship (SAR's) studies carried out on promising anticancer phenols [9] have shown that geometrical preferences are mainly determined by electrostatic factors, as well as by the formation of both intra- and intermolecular hydrogen bonds. Consequently, the most stable geometries display a clear preference for planarity. In the case of hydroxycinnamates, the presence of an ethylenic spacer in the carbon chain allows the formation of a conjugated system, strongly stabilized through π-electron delocalization.

An E-orientation of the aromatic ring and the C=O group relative to the carbon chain double bond was verified to be essential for the cytotoxic activity of caffeates and their derivatives [10]. The well known ability of phenolic compounds to form dimeric structures through OH•••O and/or CH•••O close contacts [11] should be taken into account, since their conformation and, consequently, their chemical properties vary considerably upon dimerization.

Furthermore, the biological activity of these systems has been reported to be affected by the introduction of alkyl groups in the carboxylic moiety of the molecule

(ester or amide formation) [12]. Several reports describe a higher growth-inhibition and cytotoxic activity of phenolic esters relative to their acid counterparts [13].

Regarding the phenolic aromatic ring substitution pattern, trihydroxylated phenolic acids and esters were found to display a greater cytotoxicity, as well as antiproliferative properties, in comparison with their dihydroxylated analogues [14].

Preliminary studies on the antioxidant activity of trihydroxy- and dihydroxyphenolic acids showed that the former reveal a significantly higher activity [15]. Concerning the relevance of the degree of saturation of the carbon chain, it has been described that the presence of a double bond in cinnamates is associated with increased cell viability as opposed to the saturated compounds [16], which may be partially related to the increased antioxidant ability of the unsaturated phenols [17].

The biological behavior of phenolic systems may also be affected by their conjugation with other biomolecules (e.g. polyamines and glycosides), either by intracellular metabolic processes, or even by in vivo absorption and transport mechanisms. Since in vivo biological efficacy of phenolic compounds is influenced by bioavailability, knowledge of these types of structural factors is considered crucial for predicting the absorption versus concentration and metabolic profile.

On the other hand, a thorough understanding at the molecular level of the physicochemical properties (such as partition coefficients or redox behavior) and structural preferences including hydrogen-bonding motif of phenolic systems is essential for establishing the structure-property-activity relationships, allowing for optimization as antineoplastic agents with selective targeting.

Phenolic derivatives aimed for chemopreventive or chemotherapeutic applications can thus be rationally designed, through tailored chemical changes, in light of the structure-activity relationships or structure-property-activity relationships. Benzoic and cinnamic acids, as well as the phenolic flavonoids, have been extensively used as templates for this purpose, as the chemical modification of the lead phenolic compound is an important research area in medicinal chemistry.

APOPTOSIS

Transformed cells in cancer tissue are normally characterized by excessive proliferation, accompanied by the loss of control of cell processes. Activation of cell death by chemotherapy is beneficial if occurring in preneoplastic or tumor cells, but it may result in toxicity when taking place in normal cells. Of obvious clinical interest, some phenolic compounds were found to selectively induce time- and dose-dependent apoptosis only in immortalized (malignant) cells [18].

Apoptosis is a programmed cell death mechanism, finely regulated and genetically encoded. A typical phenotype consists in a progressive shrinkage of cells and formation of apoptotic bodies, as well as nuclear (chromatin condensation and nuclear fragmentation) and plasma membrane (e.g. phosphatidylserine exposure) alterations. Apoptosis can be monitored through the evaluation of several markers such as decreased mitochondrial membrane potential (mitochondrial depolarization), enhanced mitochondrial release of cytochrome c, increased caspase activation, chromatin condensation, or cleavage of Poli-ADP-ribose polymerase [19], among others. Apoptosis develops in different stages: 1) an initiation phase, which depends on the nature of the lethal signal; 2) a decision phase, characterized by equilibrium between pro- and anti-apoptotic molecules levels and 3) a common degradation phase, when the cell ceases its function [20]. However, cells that are deficient in cell death processes may not exactly follow this sequence.

Programmed cell death can follow two major and well characterized routes, the extrinsic and the intrinsic pathway. The former is a death receptor-dependent pathway, consisting in the regulation of death receptors located on the cell surface. The intrinsic route is mitochondrial-dependent and includes the release of apoptotic signals from the mitochondrial intermembrane space [21].

In the extrinsic pathway, a cell death signal is transduced within cells, either by the activation of the receptor-mediated process (absence of the corresponding ligands), or by the ligand-induced activation of receptors such as TNFR1, Fas, CD95/Fas (the receptor of CD95L/FasL) and of the tumor necrosis factor-α (TNF-α) receptor-1 [22]. In some cases, activated TNF receptors have been demonstrated to promote a different signalosome through NF-kB activation, leading to a release of inflammatory cytokines and to anti-apoptosis responses. TNF can also target angiogenesis, inducing tumor necrosis, overall having an antiproliferative activity [23]. If coupled to pro-apoptotic processes, death-inducible signaling complexes (DISC) are formed and cause activation of pro-caspase-8.

It has been demonstrated that caspase-8 can activate pro-caspase-3, which then cleaves target proteins [21]. Also, in several cell types, caspase-8 first cleaves Bid

(a Bcl-2 family protein) which, in turn, is directed to mitochondria or induces translocation, oligomerization and insertion of Bax and/or Bak, another Bcl-2 family member. Mitochondrial permeabilization follows, which leads to the release of several proteins from the mitochondrial inter-membrane space, including cytochrome c. A cytosolic complex is formed in the presence of dATP, comprising cytochrome c, the apoptosis activating factor-1 (Apaf-1) and pro-caspase-9, resulting in caspase-9 activation, which triggers caspase-3 activity and signal amplification [22].

The intrinsic pathway is also characterized by the initial role of subcellular compartments such as the nucleus, lysosomes, the endoplasmic reticulum, or the cytosol itself, originating death-promoting stimuli that will act (directly or indirectly) on mitochondria. Permeabilization of the mitochondrial outer membrane (MOM) can occur, leading, as described above, to the release of intermembrane space proteins, including caspase activators such as cytochrome c, Omi/HtrA2 (Omi stress-regulated endoprotease/High temperature requirement protein A 2) and Smac/Diablo (second mitochondria-derived activator), as well as caspase-independent death effectors such as the apoptosis-inducing factor (AIF) and endonuclease G. AIF, endonuclease G and HtrA2/Omi are then directly translocated to the nucleus, triggering chromatin condensation and the appearance of high-molecular weight chromatin fragments [19].

Also, as previously referred, cytochrome c promotes activation of caspase-9, while Omi/HtrA2 and Smac/Diablo indirectly favor the caspase cascade by antagonizing the activity of the inhibitor of apoptosis proteins (IAPs), an endogenous caspase inhibitor. Besides IAP activation, another mechanism capable of rescuing cells from death involves Bcl-2 and Bcl-xL, which act by preventing the permeabilization of MOM and the release of proteins from the inter-membrane space [24]. Interestingly, studies have demonstrated that Bcl-2 proteins are strongly expressed in human breast cancer cells; in particular, Bcl-xL expression confers resistance to chemotherapy-induced apoptosis [25].

It has also been described that the intrinsic pathway for apoptosis can be triggered by the induction of the mitochondrial permeability transition (MPT), which involves the formation of protein pores that span the inner and outer mitochondrial membranes, resulting in mitochondrial depolarization, oxidative stress, and in some cases in outer membrane rupture due to matrix expansion, allowing pro-apoptotic proteins to escape to the cytosol [26]. The degree of MPT induction among the mitochondrial network, and the consequent decrease of mitochondrial ATP production, determines if apoptosis can be redirected to necrosis [27].

MITOCHONDRIA AS CHEMOTHERAPEUTIC TARGET

The discovery that mitochondria play a critical role in the process of cell death promoted a radical change in cell death research. Several oxidative stress-related diseases, in particular cancer, involve the understanding of mitochondrial-mediated apoptosis [3].

Mitochondria are thus potential targets for anti-cancer therapy. Different processes are suggested to interfere with vital mitochondrial functions in cancer cells. The vast majority of conventional anti-cancer drugs indirectly exploit the activation of intrinsic pathways in order to exert their cytotoxic action by using multiple activation routes (e.g. p53 or death receptors) [3]. Conventional anticancer agents, as well as numerous dietary components and micronutrients, have considerable potential for hindering in vivo deleterious oxidative processes and for inhibiting carcinogenesis through induction of apoptosis in neoplastic cells, in some cases via mitochondria-mediated pathways [28].

As discussed in the previous section, MOM permeabilization is generally regarded as a crucial control point in the commitment of a cell to apoptosis. During normal physiology, the MOM is considered freely permeable to solutes and small metabolites (approximately 5 kDa) due to the presence of the abundant protein voltage-dependent anion channel (VDAC), which allows the diffusion of such molecules through the MOM [29]. MOM rupture due to excessive osmotic influx can be lethal, not only by releasing caspase-activating molecules and caspase-independent death effectors, but also by initiating metabolic failure of mitochondria. For example, phenols such as gallic acid down-regulate Bcl-xL protein and activate Bak, Bax and Bad proteins, causing the release of mitochondrial cytochrome c to the cytosol, through MOM permeabilization [30].

Furthermore, oncogene products modulate MOM permeability by regulating Bcl-2 family proteins, which modulate the bioenergetic metabolite flux and putative components of the MPT pore. In this regard, phenolic acids may act through similar mechanisms. Several authors have reported that CAPE (caffeic acid phenyl ester) is cytotoxic against tumors, but not against normal cells, the mechanism involving mitochondria through altered Bcl-2 protein expression [31]. A number of other experimental chemotherapeutic agents have the opposite effect, acting on mitochon-

drial lipids and proteins and inhibiting MPT pores opening through direct binding to certain protein components [32].

A general rule is that antioxidants act as MPT inhibitors, decreasing or hindering the oxidative stress responsible for oxidation of proteins of the pore complex, and is a prime cause for the consequent inner membrane permeabilization [33].

In contrast to the MOM, the inner membrane is impermeable to the majority of ions, including protons. Membrane selectively is important for maintaining the proton gradient that is required for oxidative phosphorylation [21]. It is at the inner mitochondrial membrane that ATP synthesis occurs, through electron transfer coupled to proton ejection. Some potential anticancer molecules can also inhibit the respiratory chain, promoting mitochondrial failure and the release of proteins from the inter-membrane space, increasing the generation of reactive oxygen species (ROS) and decreasing ATP generation. Ultimately, cell death occurs through mitochondrial failure [3].

The selective accumulation of promising anticancer molecules inside the mitochondria of tumor cells, rather than normal cells, is a key point in the design of novel molecules tested in new pharmacological strategies [34]. Several reports indicate that mitochondria have a higher transmembrane electric potential in tumor cells than in normal, non-neoplastic cells [35]. This difference has been used to induce a selective accumulation of specific molecules in the mitochondria of tumor cells, explaining its selective toxicity. We have reported a similar mechanism for the alkaloid berberine, which was found to inhibit proliferation in a murine metastatic melanoma cell line through a mitochondrial mechanism, mostly due to enhanced mitochondrial accumulation [36].

BIOLOGICAL ACTIVITY OF PHENOLIC ACIDS AND DERIVATIVES

Although naturally occurring phenolic acids have been the subject of numerous studies in cells, information on the molecular basis of their mechanisms of action at the sub-cellular level (e.g. mitochondria) is still somewhat scarce [37]. Phenolic compounds are usually associated with a beneficial effect on carcinogenesis principally due to their antioxidant activity, which mainly occurs by a free radical scavenging process [38].

Other pathways have been suggested, including the modulation of specific enzymes and signal transduction processes leading to cell proliferation arrest, cell cycle regulation, inhibition of DNA synthesis, or inhibition of angiogenesis and metastasis [39].

Phenolic acids can be subdivided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acid derivatives include p-hydroxybenzoic, protocatechuic, vannilic, syringic, and gallic acid. These derivatives can be found in plants in free or bound forms, either as structural components of lignins and hydrolyzable tannins, or as a sugar derivatives and organic acids in plant foods. Hydroxycinnamic acids include p-coumaric, caffeic, ferulic, and sinapic acids. Like hydroxybenzoic acids, these molecules are present in the bound form, linked by ester bonds to components of the plant cell-wall such as cellulose, lignin and proteins [40].

Gallic acid and its esters are presently used as antioxidant additives in both food and pharmaceutical industry – propyl gallate (E-310) and octyl gallate (E-311) – since they are known to protect against oxidative damage induced by reactive oxygen, nitrogen and sulphur species [41]. Gallic acid derivatives have been described to cause apoptosis in tumor cell lines, to inhibit lymphocyte proliferation and to inhibit protein tyrosinase kinase (PTK's) activity [42].

Among hydroxycinnamic acids, caffeic acid is one of the most relevant and potent antioxidants found in the human diet, causing a significantly decrease in in vitro oxidative damage to DNA [43]. It has been demonstrated that the antioxidative capacity of caffeic acid attenuates the proliferation of Vascular Smooth Muscle Cells (VSMC) induced by angiotensin II stimulation, through a mechanism that involves inhibiting generation of reactive oxygen species generation and partially blockade of the JAK/STAT and the Ras/Raf-1/ERK1/2 cascades [44].

As potential anti-carcinogenic agent, caffeic acid inhibits cell growth during S-phase, causing further apoptosis in MCF-7 and T47D human breast cancer cells by activating pro-apoptotic proteins such as Bax and caspases [43] [45]. Alternatively, caffeic acid can also promote apoptosis in a number of cancer cell lines by inhibiting the NF-kB pathway [45]. Interestingly, caffeic acid is mentioned in some studies as a chemosensitizer, having a synergistic effect with doxorubicin treatment on MCF-7 human breast cancer cells [43].

Caffeic acid phenyl ester (CAPE) is a well-known phenolic acid derivative with promising potential, possessing antioxidant, anti-inflammatory, anti-viral, immunostimulatory, anti-angiogenic, anti-invasive, and anti-metastatic activities [46]. CAPE, together with other phenolic acid phenethyl esters, also possesses anti-

cancer activity against some human cancer cells [47]. In human ME180 cervical cancer cells, CAPE promotes apoptosis mediated by down-regulation of Mcl-1 gene expression and activation of caspase-8 [46]. Moreover, CAPE was also reported to decrease cell viability in human U937 myeloid leukemic cells, in a dose and time-dependent manner. The apoptotic action of CAPE is mitochondrial-mediated and is accompanied by a decrease in Bcl-2 expression, increase of Bax expression, release of cytochrome c, activation/cleavage of caspase-3, and PARP cleavage [31].

Ferulic acid (4-hydroxy-3-methoxycinnamic acid), is a cinnamic acid structurally related to caffeic acid, which is present in rice bran and other plants. Food processing, such as thermal processing, pasteurization, fermentation, and freezing, contributes to the release of ferulic acid [40], which is rapidly absorbed and excreted as glucuronides or sulphates in men. Radical scavenging activity experiments showed that ferulic acid has antioxidant activity and that its esterification leads to a modification of its efficacy [48]. Ferulic acid derivatives present in the diet possess interesting biological effects including chemoprevention of colon and tongue cancers. One particular example is 3-(4'-geranyloxy-3'-methoxyphenyl)-2-trans-propenoic acid and its derivatives [49].

Curcumin is a phenolic derivative present in plants and is formed by two ferulic acids linked by a methylene group, in a diketone structure. The compound is the active ingredient of turmeric (Curcuma longa) and displays anti-inflammatory, antioxidative and anticarcinogenic properties, with an immense potential in cancer chemotherapy because of its effect in cell growth regulatory mechanisms. Analysis of its structure revealed that the presence of the beta-diketone moiety and phenolic groups in the structure have a direct contribution to its antioxidant behavior [50]. Curcumin exhibits growth inhibitory activity against prostate, colon and breast cancer [51]. Some studies evidence a selective growth-inhibiting effect of curcumin on transformed cell lines, as compared to non-transformed cell lines [52].

It was also previously demonstrated that the anticarcinogenic action of curcumin in colon have the involvement of the phenolic groups and the conjugated bonds in the central seven carbon chain [53]. Curcumin also affects various cell cycle proteins and checkpoints, leading to the down-regulation of some cyclins and cyclin-dependent kinases, as well as to up-regulation of cdk inhibitors, and to inhibition of DNA synthesis [54]. Of special relevance is the fact that curcumin can also induce apoptosis by targeting mitochondria, affecting p53-related signaling and blocking NF-kB activation, in a similar fashion to caffeic acid [50]. Prostate cancer cells can be sensitized with curcumin to TRAIL treatment, inducing both the extrinsic and the intrinsic pathways for apoptosis, which suggests that curcumin is a beneficial adjunct against chemoresistance to standard therapeutic drugs [54].

Phenolic compounds in general have been reported to affect nitric oxide (NO) production and its activity on physiologic functions. The phenolic acid 3,4-dihydroxyphenylacetic acid (PAA) decreases the activity of NO synthase and consequently the possible production of endogenous reactive nitrogen species, besides arresting cells in the S-phase and inducing apoptosis of T47D breast cancer cells. The induction of apoptosis in this cell line occurs via FKHRL1 (FOXO3a) kinase pathway, while in MCF-7, a different breast cancer cell line, apoptosis is activated by NO via p53-associated pathways [45].

A second molecule, either a classic anti-cancer molecule (doxorubicin, taxol, cis-platin, etc) or an alternative molecule (a second phenolic acid or another natural phenolic compound), will be able to trigger signaling pathways that will ultimately end up affecting the already destabilized mitochondrial network. The outcome will be the release of mitochondrial proteins including cytochrome c or the apoptosis-inducing factor (AIF). These proteins are involved in the intrinsic pathway of apoptosis and the cascade of events will cause tumor cell death. It is noteworthy to point out that one advantageous aspect of mitochondrial physiology is that the transmembrane electric gradient (negative inside) can be used to drive the accumulation of lipophilic cations. Also, it is possible that at least part of the mitochondrial transmembrane electric gradient is maintained during the process of drug-induced cell killing, thus allowing mitochondrial ATP to be used during apoptosis. A complete collapse of the cell energy processes (mitochondrial oxidative phosphorylation and glycolysis) can drive the process of cell death from apoptosis to necrosis

FUTURE PERSPECTIVES

The efficacy of many clinically used chemotherapeutic drugs is often compromised by acquired chemoresistance by cancer cells. In fact, many cancer cells undergo mutations or loss of proteins that are intimately involved in cell death (e.g. p53 and Bcl-2 proteins). Phenolic derivatives present in fruits and vegetables, have long been recognized to display antioxidant and antiproliferative activities, thus being promising chemopreventive/chemotherapeutic compounds.

Exploiting the effect of these bio-antioxidants on cellular processes will lead to new chemopreventive and chemotherapeutic phenolic-based strategies that will hopefully be non-toxic to healthy cells. In order to overcome chemoresistance, mitochondria are an attractive biological target due to their major role in bioenergetics and vital signaling in mammalian cell. One new approach suggested is the use of compounds that specifically interact with the MOM, contributing to the release of pro-apoptotic proteins contained in mitochondria. Secondly, since the electrochemical gradient is apparently higher in mitochondria from cancer cells, lipophilic cations can be preferentially accumulated in the mitochondrial matrix [20].

A third method is based on the synergistic action of two or more compounds, the first of which is used to sensitize mitochondria to undergo failure and trigger apoptosis, if and when a second or a third molecule is added to the cell (Figure 2). These strategies rely on the condition that derivatives from phenolic acids are small and positively charged, although neutral molecules can also, in theory, accumulate in mitochondrial membranes. Although the presence of a positive moiety does not guarantee that the molecules will be accumulated by mitochondria, it is likely that once inside the cell lipophilic cations will tend to accumulate within organelles with a negatively-charged environment.

Thus, the secret for success is to rationally design and synthesize phenolic acid derivatives that can be selectively accumulated by mitochondria in tumor cells, in such a way that the threshold for cytotoxicity exerted by a second molecule is significantly lowered (Figure 2). The mechanism of action results in a novel pharmacological strategy, coupling high efficacy against neoplastic cells to a low toxicity towards non-tumor cells. The advantage of using phenolic acids which are normally present in human diet is that the toxicity threshold is already understood.

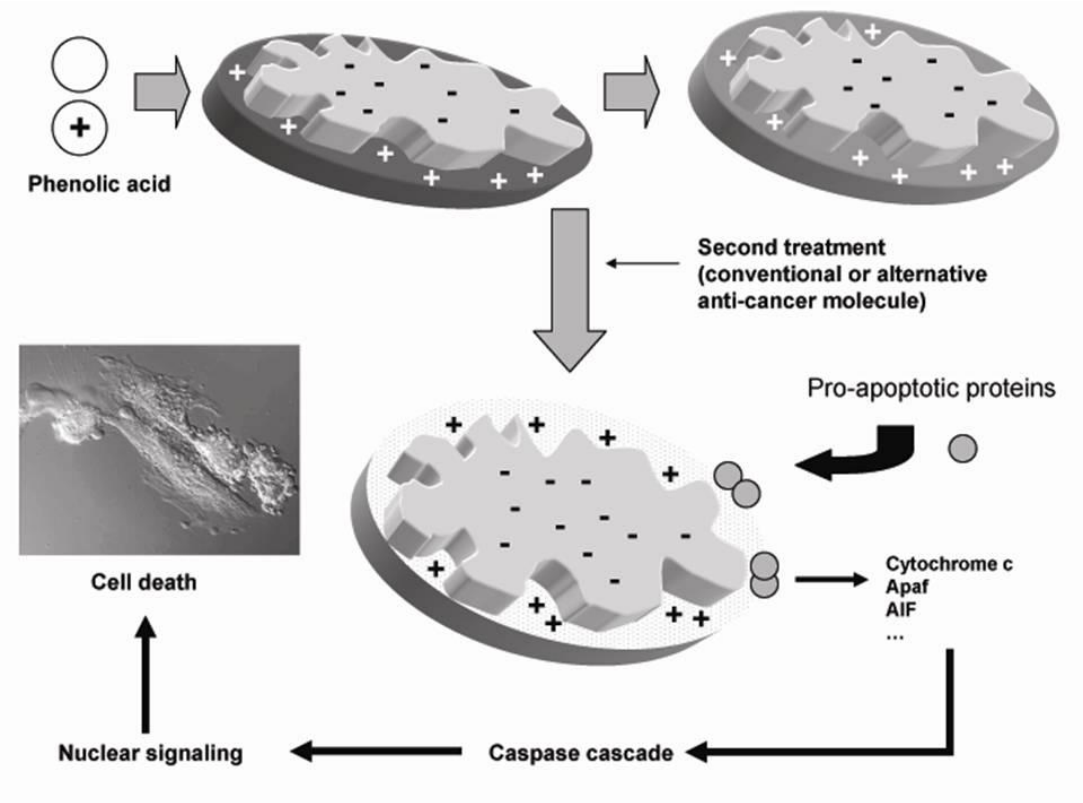

Figure 2 A proposed strategy in which the co-treatment of tumor cells with both a phenolic acid or derivative and a second molecule results in toxicity for the cancer cell. By altering the structure of the molecule (including modifying the polarization/charges), it is possible to direct the phenolic acid or derivative to mitochondria in order to create an initial destabilization in the mitochondrial membranes.

AUTHORS' PARTICIPATION

All authors participated in the composition of the text, figures and review of the manuscript.

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CONFLICT OF INTERESTS/DISCLAIMERS

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BEYOND SINGLE NUCLEOTIDE POLYMORPHISMS

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ABSTRACT

Similar medications have been known to cause considerable heterogeneity in efficacy and toxicity across human populations. Therefore, individualized, or personalized, therapy has been highlighted as a declared goal of modern medicine. In this paper, we briefly describe the main strategies for dose individualization and then focus our attention on Single Nucleotide Polymorphisms (SNPs), the main source of human genetic and phenotypic variation. This genetic variation was long recognized as the principal genetic contribution to the variability of drug action, but the advent of more powerful molecular technologies has uncovered other abundant DNA variations and changed this perception. It should also be taken in consideration that most drug effects are determined by the interplay of several genes (the genomic approach), rather than candidate gene approaches. Although pharmacogenetics and pharmacogenomics mainly focus on human genetic variations linked to SNPs, we believe that this approach is only a starting point, from which it will be necessary to proceed to a more complex stage of research to better individualize drug therapy.

Key words

Pharmacogenetics; Pharmacogenomics; Pharmacokinetics; Single nucleotide polymorphism

RÉSUMÉ

Il est bien connu qu'un même médicament peut montrer une hétérogénéité dans son efficacité et sa toxicité au sein de l'espèce humaine. Pour cette raison la médecine moderne met de plus en plus l'accent sur une médecine individualisée ou personnalisée. Cet article décrit brièvement les stratégies principales pour une posologie individualisée et focalise ensuite l'attention sur les SNPs (polymorphismes d'une seule paire de base du génome), la source principale de variation génétique et phénotypique humaine. Cette variation génétique a été longtemps reconnue comme la contribution génétique principale à la variabilité de l'action des médicaments. L'arrivée de technologies moléculaires plus performantes a découvert de nombreuses autres variations de l'ADN et a ainsi changé cette perception. Il faut en effet prendre en considération que la plupart des effets des médicaments sont déterminés par l'interaction de plusieurs gènes (l'approche génomique) plutôt que d'un gène unique. Quoique la pharmacogénétique et la pharmacogénomique se focalisent actuellement surtout sur les variations génétiques humaines en relation avec les SNPs, nous croyons que cette approche n'est qu'un point de départ à partir duquel il sera nécessaire d'instaurer des étapes plus complexes de recherche pour aboutir à une médecine mieux individualisée.

Mots clés:

Pharmacogénétique; Pharmacogénomique; Pharmacocinétique; SNP

RESUMEN

Medicamentos similares puede producir una respuesta heterogénea en cuanto a su eficacia y toxicidad. Por lo que una terapéutica individualizada, o personalizada, es considerada como un objetivo específico de la medicina moderna. En este artículo, describimos brevemente las estrategias principales para la individualización de la dosis y posteriormente enfocamos la atención sobre el polimorfismo de un solo nucleótido (PSN), la principal fuente de variación genética y fenotípica humana. Esta variación genética fue extensamente reconocida como la principal contribución genética a la variabilidad de los efectos farmacológicos. Sin embargo, el desarrollo de técnicas moleculares más avanzadas ha descubierto otras variaciones de ADN abundantes y ha cambiado esta perspectiva. También debe de considerarse que la mayoría de los efectos farmacológicos están determinados por la interacción de varios genes por lo que también el trabajo hace un análisis no solo bajo la óptica de algunos genes específicos sino de de una perspectiva genómica. Resumiendo, aunque la farmacogenética y la farmacogenómica enfocan su atención en las variaciones genéticas humanas, principalmente ligadas al PSN, nosotros consideramos que esta perspectiva es sólo el principio desde el cual tendremos necesariamente que movernos a un nivel más complejo de investigación para individualizar la farmacoterapia.

Palabras clave

Farmacogenética; Farmacogenómica; Farmacocinética; Polimorfismo de un solo nucleótido

INTRODUCTION

The rule "the right drug at the right dose at the appropriate time in the right patient" may appear to be a very ambitious goal in drug therapy, but an overview of scientific pharmacological literature over the last 30 years indicates it as one of the main targets in third-millennium medicine.

There are many reasons for the move from the "one drug fits all" approach to personalized medicine: 1) the enormous increase, during the 20th century, in the range of therapies against all major diseases, 2) the increasing life expectancy, 3) drug therapy sometimes fails to be curative, 4) adverse drug reactions are the fifth leading cause of death in the United States [1] [2], 5) powerful new technologies have produced advances in biomedical research.

DOSE INDIVIDUALIZATION

To date, the main strategy for dose individualization is to determine dosage from the drug's pharmacokinetic properties; the classic example of this approach is carboplatin, an analogue of cisplatin, which is used to treat lung and ovarian cancer [3] [4]. Alternatively, initial treatment doses can be used to establish the individual's pharmacokinetic (PK) response to the drug, with subsequent doses being based on this information; this strategy has been used for oral busulfan, an alkylating agent that is used to treat leukemia, which has linear kinetics but high interindividual variability [5] [6] [7] [8] [9].

Another PK-based method for dose individualization is the population pharmacokinetic model. This approach studies the variability of plasma drug concentrations among individuals who receive standard regimens determined from PK data relating to the target patient population. Population pharmacokinetic models aim to account for observed interindividual variation in terms of patient variables called covariates; these include all sources of variability. Population modeling enables the relative importance of covariates to be quantified; Bayesian estimation, which evaluates an individual's data relative to the population pharmacokinetic model, can then be used to estimate patient-specific pharmacokinetic parameters from which to calculate the optimal dose for an individual [10] [11] [12] [13] [14].

Epirubicin, an anthracycline used in breast cancer therapy, and digoxin, used in the treatment of various heart conditions, exemplify how population modeling can lead to dose individualization [13] [15].

However, each of these strategies suffers from some disadvantages, such as subtherapeutic or supratherapeutic dosing during the initial stages and difficulty of adaptation to clinical practice. It has therefore been suggested, following the complete mapping and understanding of all human genes through the Human Genome Project, that pharmacogenetics and pharmacogenomics might have the potential to overcome these drawbacks and to facilitate reaching the goal of optimizing drug therapy [16] [17] [18] [19] [20] [21] [22].

PHARMACOGENETICS AND PHARMACOGENOMICS

The British physician Archibald Garrod was probably the first to realize that certain individuals inherited a predisposition to alcaptonuria or other conditions [23]. In particular, he observed that parental consanguinity was more common than usual among parents of children with alcaptonuria.

However, it was probably William Bateson [24], a biologist who was ahead of his time, who interpreted Garrod's reports as recessive inheritance when he popularized Mendelian genetics in Britain. Bateson discovered genetic linkage and introduced the term "genetics" at some time between 1902 and 1913. With particular foresight, Garrod went on to develop the concept known as the "Chemical Individuality in Man" [24]. He proposed that drugs undergo biotransformation by specific pathways, similar to endogenous substrates. As occurs with inborn errors of metabolism, defects in such pathways could alter drug concentrations and therefore their effects [24].

The concept of familial clustering of unusual xenobiotic responses was reinforced during the 1940s with the observation of a high incidence of hemolysis, on exposure to antimalarial drugs, among individuals with glucose-6-phosphate dehydrogenase deficiency [25]. In the 1950s, Evans et al. identified N-acetylation as a major route of isoniazid elimination [26]. Although individuals varied substantially in terms of the extent to which a single dose of the drug was acetylated, variability between monozygotic twins was found to be small compared with that between dizygotic twins.

This observation laid the groundwork for later studies that have defined the clinical consequences and genetic basis underlying the fast and slow acetylator phenotypes. More generally, the past half century has seen developments towards understanding the molecular basis of drug disposition and action, and of the mechanisms that determine the observed variability in drug action.

The concept of a familial component in drug action thus initiated the field of 'pharmacogenetics' even before the discovery of DNA as the repository of genetic information. An increased understanding of the molecular, cellular, and genetic determinants of drug action elicited an appreciation that variants in many genes might contribute to variability in drug action. The concept of using whole-genome information to predict drug action is one definition of the more recent term, 'pharmacogenomics' [27] [28] [29].

SNP

Whether we are discussing pharmacogenetics - the study of the relationship between individual gene variants and variable drug effects [30] - or pharmacogenomics - the study of the relationship between variants in a large collection of genes, up to the whole genome, and variable drug effects [30] - we are chiefly talking about Single Nucleotide Polymorphisms (SNPs).

An SNP is a change in one nucleotide (base pair) in a DNA sequence. SNPs can be in coding regions (where they may be either synonymous or non-synonymous) or, more commonly, in non-coding regions; they frequently vary with ethnicity [30]. It has been estimated that there are at least 10 million SNPs within the human population [31], averaging one every 300 nucleotides, of the approximately 3 billion nucleotide base pairs that constitute the genome of an individual. It is precisely in these heritable variations among individuals on which the principles of pharmacogenetics and pharmacogenomics are based.

There are many cases in which SNPs have been correlated with significant changes in drug effects [17] [32]. One of the best examples of SNPs relating to the outcome of therapy is the polymorphism of the gene thiopourine S-methyl transferase (TPMT). TPMT is a cytosolic drug-metabolizing enzyme that catalyzes the S-methylation of 6-MP and azathioprine. In their original study, Weinshilboum et al. demonstrated a very clear tri-modal frequency of TPMT activity in red blood cells from 298 unrelated control adults [33]. One in 300 subjects lacked TPMT activity and 11% had intermediate levels. Family studies showed that the frequency distribution was due to inheritance. While phenotypic studies have shown a clear tri-modal distribution, the genetic basis of phenotypic variation has proved more complex.

Seventeen variant TPMT alleles have been identified to date, although 3 variant alleles account for the majority (>95%) of persons with intermediate (1 variant allele) or low (2 variant alleles) TPMT activity [34] [35]. Subsequent clinical studies have demonstrated very clearly that TPMT polymorphism can predict toxicity of 6-MP and consequences of therapy. Children with acute lymphocytic leukemia (ALL) with intermediate or no TPMT activity are at higher risk of myelosuppression when prescribed standard doses of 6-MP [36]. Moreover, a number of studies have shown that TPMT phenotype or genotype influences the effectiveness of therapy, with low TPMT activity being associated with higher levels of cytotoxic 6-thioguanine nucleotides (6-TGN) and reduced relapse [37].

Another example of SNPs influencing therapeutic efficacy involves polymorphism of genes belonging to the superfamily of cytochrome P450 enzymes (CYP) [38]. For example, patients carrying some of the 78 variants of CYP2D6 currently identified (http://www.imm.ki.se/cypalleles) have a greater risk of adverse effects from metoprol, venlafaxine or tricyclic antidepressants [39] [40] [41].
CYP2C19 is important in the metabolism of proton-pump inhibitors (omeprazole, lansoprazole, rabeprazole, and pantoprazole), fluoxetine, sertaline and nelfinavir. Several inactive genetic variants exist, although two (CYP2C19*2 and CYP2C19*3) account for more than 95 percent of cases involving poor metabolism of these drugs [42]. Marked differences in the plasma levels of protein-pump inhibitors occur between genotypes and phenotypes and are reflected in drug-induced changes in gastric pH [43].

CYP2C9 is involved in the hydroxylation of the S-form of the anti-epileptic agent phenytoin and the anticoagulant warfarin. Many CYP2C9 variant alleles have now been reported (http://www.imm.ki.se/cypalleles). Of these, decreased activity has been confirmed in cases with CYP2C9*3, by means of an expression system using COS cells and yeast, and an *in vivo* test on healthy volunteers and patients whose genetic polymorphism was known [44] [45].

For example, oral clearance of (S)-warfarin decreased to below half in subjects with heterozygous polymorphism for CYP2C9*3 (CYP2C9*1/*3) and to below 10% in patients who were homozygous for CYP2C9*3 [46].

BEYOND SNPs

Several pharmacogenetic research studies published in recent decades have demonstrated gene-drug interactions in cases where a SNP has been identified in one or more genes with functional consequences [47]. Nevertheless, many other studies show a poor correlation between SNPs in candidate genes and phenotypes. For instance, Irinotecan is a prodrug that has been widely used to treat advanced cancers and is activated by human carboxylesterase 2. Although the

carboxylesterase 2 gene exhibits several instances of polymorphism, and although an intronic SNP has been found to be associated with reduced carboxylesterse mRNA expression in colorectal tumors, none of the variations in this gene have been found to be associated with protein activity [48] [49]. Furthermore, CYP3A4 is the human enzyme known to be involved in the metabolism of numerous medications. Thus far, no completely inactivating mutations have been discovered in the human CYP3A4 gene, although a common polymorphism in the CYP3A4 promoter has been described [50] [51] [52].

This method is beginning to change as haplotype studies and other approaches appear in the literature [46][30] [53]. An important manuscript in this context evaluated whether the response to inhaled β_2-agonist therapy for asthma was best predicted by individual SNPs or by gene haplotype in the β_2-adrenoceptor gene (ADRB2) [54]. None of the 13 SNPs were significant predictors of response to β_2-agonist therapy. Of the 8,192 haplotypes theoretically possible for (ADRB2), only 5 were commonly observed. Importantly, haplotype analyses did define a patient group with a significantly superior response to β_2-agonist therapy.

A study correlating thiopurine-related adverse drug reactions with TPMT gene polymorphism, one of the best pharmacogenetic examples, noted that 78% of adverse drug reactions were not associated with the TPMT genotype. Researchers are thus moving from SNPs to other human genetic variations, such as small insertions or deletions of repetitive DNA sequences in the promoter region of the TPMT gene, in an attempt to elucidate this association [55] [56].

The idea to move onwards from SNPs to other human genetic variations arises from the advent of genome-scanning technologies that have uncovered an unexpectedly-large number of structural variations in the human genome. These consist of microscopic and submicroscopic variants, including deletions, duplications, insertions, inversions and translocations, which involve segments of DNA that are larger than 1 kb [57].

For example, gene deletions and duplications have been discovered in debrisoquin hydroxylase (CYP2D6), probably the best-characterized genetic polymorphism among the cytochrome P450 enzymes, and concordance between genotype and phenotype has been well established for many drug substrates [58]. However, this monogenic approach, in which a single DNA variant site is associated with a specific alteration, has been criticized in that it fails to consider potential polygenic contributions. For instance, the field of systems biology reflects gene–gene interactions resulting from a particular stimulus that affects a complex circuitry of pathways, ending in a response by the cell or organism [59]. Gene–gene sensing, or gene–gene warfare within the genome, has been called molecular drive or meiotic drive [60]. Gene conversion can lead to one gene repairing, or altering the expression of, its neighbor gene [47]. Gene silencing can occur through several different mechanisms, including DNA hypermethylation and RNA interference [61].

Genomic imprinting also results from DNA methylation [47]. Nutrition and dietary supplementation have been shown to affect epigenetic gene regulation in man [62]. Extensive transmission distortion can lead to unequal genetic sharing among relatives [47]. Several studies have suggested that more than 70% of all human multi-exon genes are alternatively spliced [47]. Exonic splicing enhancers can be disabled by a synonymous single-nucleotide polymorphism, regarded by many as unimportant compared to a non-synonymous single-nucleotide polymorphism [47]. Stochastic events (random noise during transcription and other cellular processes) can also markedly affect gene expression [47]. Another area of active inquiry is the transcriptional regulation of normal proteins, which can be highly variable because of allelic variants in regions of DNA that regulate expression [63] [64].

Variation in the function or expression of genes encoding various factors, such as AhR (arylhydrocarbon receptor), PPAR (peroxisome proliferator activated receptor), PXR (pregnane X receptor) and CAR (constitutive androstane receptor), that control the transcription of genes encoding drug-metobolizing enzymes and transporters, could also contribute to variable drug action [65] [66] [67] [68] [69]. Finally, transcriptional mutagenesis results in mutated mRNA and, therefore, in mutated protein, although the mutation does not exist in the DNA [47].

CONCLUSIONS

Although pharmacogenetics and pharmacogenomics focus attention on human genetic variation mainly linked to SNPs, we have shown in outline that this approach is only a starting point, from which it will be necessary to proceed to a more complex stage of research. We believe, however, that SNPs comprise a useful step in individualizing therapy, although we are less optimistic than some [70]. The chief role of SNPs, in our view, is as one covariate in population pharmacokinetic models [71] [72], with the goal of preventing subtherapeutic or supratherapeutic dosage (during the initial stages) in particular therapeutic groups [73] [74]. They may also help to avoid high-risk subjects developing severe adverse drug reactions [35]. In

summary, it is clear that no single approach is likely to identify, in all individuals, the contribution made by all genes and gene products responsible for a particular drug response.

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CONFLICT OF INTERESTS/DISCLAIMERS

RC is member of the Editorial Board of the journal.

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PERINATAL OUTCOMES TO UTERINE STIMULATION WITH BREAST MASSAGE ALONE OR IN COMBINATION WITH OXYTOCIN IN DYSTOCIC SOWS

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ABSTRACT

Objective: Our goal was to evaluate the obstetric and fetal outcomes in dystocic sows receiving udder massage stimulation alone or in combination with oxytocin. **Methods:** 120 sows with maternal or fetal dystocia were randomly allocated to receive either intravulvar (I-Vu) saline (control group), udder massage followed by I-Vu saline, or udder massage followed by I-Vu oxytocin 10 IU (groups 1, 2 and 3, respectively). Udder massage consisted in the application of natural oil while rubbing the udder in a cephalic-to-caudal direction. Obstetric and fetal outcomes were obtained and compared among the three groups. The statistically significant limit was fixed to a two-tailed $P < 0.05$ in every test. **Results:** Intensity of uterine contractions decreased from 18.1 ± 3.6 mmHg in the control group to 14.1 ± 3.8 mmHg ($P < 0.001$) in group 2. Compared to the control group, there was an approximately 3.3-fold reduction in the number of moderate-to-severe meconium stained live-born piglets and 50% reduction of intrapartum deaths in group 2. In group 3, the reduction in these two outcomes was less evident but remained statistically significant. **Conclusions:** Dystocic sows that received udder massage had better obstetric and fetal outcomes than control sows. However, co-administration of oxytocin might reverse the advantages of udder massage.

Key words

Dystocia; Experimental animal models; Neuroendocrine system; Uterine stimulants

RÉSUMÉ

Objectif: Les effets obstétricaux et fœtaux de la stimulation mammaire seule ou associée à l'ocytocine ont été évalués chez des truies avec mise-bas dystocique. **Méthodes:** 120 truies avec dystocie maternelle ou fœtale ont été randomisées à un des trois groupes suivants: administration de sérum physiologique intravulvaire (I-Vu) (groupe témoin), stimulation mammaire suivie d'une administration de sérum physiologique I-Vu, ou stimulation mammaire suivie d'une administration de 10 UI d'ocytocine I-Vu (groupes 1, 2 et 3). La stimulation mammaire a consisté en l'application d'huile naturelle avec massage des mamelles en direction céphalo-caudale. Les effets obstétricaux et fœtaux ont été enregistrés et comparés entre les trois groupes. Une valeur de $P < 0,05$ en bidirectionnel a été considérée comme statistiquement significative. **Résultats:** Une diminution de l'intensité des contractions utérines de 18,1 ± 3,6 mmHg dans le groupe témoin à 14,1 ± 3,8 mmHg est observée dans le groupe 2 ($P < 0,001$). De même, le nombre de porcelets nés vivants avec liquide amniotique modérément à sévèrement méconial est réduit de 3,3 fois et la mortalité périnatale est réduite de 50 % dans le groupe 2. Dans le groupe 3, la réduction de ces paramètres est moins marquée, la différence vis-à-vis du groupe témoin restant cependant statistiquement significative. **Conclusions**: La stimulation mammaire procure des avantages obstétricaux et fœtaux chez les truies dystociques. L'administration concomitante d'ocytocine pourrait en diminuer les avantages.

Mots clés

Dystocie; Modèles expérimentaux animaux; Système neuroendocrinien; Stimulants utérins

RESUMEN

Objetivo: Evaluar los efectos obstétricos y fetales en cerdas distócicas que recibieron estimulación con masaje mamario solo o en combinación con oxitocina. **Métodos:** 120 cerdas con distocia materna y fetal fueron asignadas en forma aleatoria a cualquiera de los siguientes tres grupos: solución salina intravulvar (I-Vu) (grupo control); masaje mamario y solución salina I-Vu; o masaje mamario y oxitocina 10 UI (grupos 1, 2 y 3, respectivamente). El masaje mamario consistió en la aplicación de aceite natural mientras se masajeaba a la ubre en dirección cefálico-caudal. Los resultados obstétricos y fetales observados se compararon entre los tres grupos. El límite de significancia estadística fue de $P < 0.05$ de dos colas en todos los análisis realizados. **Resultados:** La intensidad de las contracciones uterinas disminuyó de 18.1 ± 3.6 mmHg en el grupo control a 14.1 ± 3.8 mmHg ($P < 0.001$) en el grupo 2. En comparación con el grupo control, en el grupo 2, el número de lechones nacidos vivos y con tinción de meconio de moderada a severa se redujo 3.3 veces mientras que el número de lechones muertos intraparto se redujo un 50%. La reducción de estos dos parámetros fue menos evidente en el grupo 3, aunque la diferencia resultó estadísticamente significativa. **Conclusiones:** Las cerdas distócicas sometidas a masaje mamario tuvieron mejores resultados obstétricos y fetales que las cerdas controles. Sin embargo, la coadministración de oxitocina revertió parcialmente las ventajas del masaje mamario.

Palabras clave

Distocia; Modelos animales experimentales; Sistema neuroendócrino; Estimulantes uterinos

INTRODUCTION

Dystocia is a common obstetric and public health problem because of the increased risk of intrauterine asphyxia, operative delivery, and maternal morbidity [1]. In pregnant women, breast stimulation may result in labor induction and reduction of rates of postpartum hemorrhage [2] [3]. However, breast stimulation may fail to enhance uterine response in 50% of parturient women [4], requiring the subsequent administration of oxytocin. Despite its technical simplicity, a number of problems have been recognized for developing appropriate trials to evaluate labor augmentation by breast stimulation in parturient women [5] [6]. This is probably the reason why insufficient information is available in the literature on this topic [3].

Pregnant sows appear to be a relevant animal model for studying uterine stimulation. In sows, low plasma levels of oxytocin are associated with prolonged parturition [7]. Conversely, udder stimulation has been associated with increased plasma levels of oxytocin in ewes [8]. However, few studies have focused on the uterotonic activity of teat massage during parturition, as breast massage had shown very strong effects, similar to the use of oxytocin.

The objective of the present study was to evaluate the obstetric and fetal outcomes in dystocic sows receiving udder massage stimulation alone or in combination with oxytocin.

METHODS

Animals

Previous approval by the Institutional Review Board of the Universidad Autónoma Metropolitana-Xochimilco, México DF, Mexico, and in accordance with the guidelines of the ethical use of animals in applied ethologic studies [9], the study was performed at a commercial farm of Yorkshire-Landrace sows located in the State of Hidalgo, Mexico. Approximately 120 sows deliver per month at this farm, and 2% to 4% have dystocia. The animals were artificially inseminated and received prenatal care throughout pregnancy including a diagnostic ultrasound (Renco Pregnant-Alert, Minneapolis MN, USA) at 5 weeks of pregnancy. Parturition was induced with an intramuscular prostaglandin injection (dinoprost tromethamine 10 mg; Lutalyse, Pharmacia & Upjohn, Mexico DF, Mexico) 24 hours prior to the expected delivery date, at 114 days of gestation, according to the farm's breeding-farrowing records. Animals were housed in individual crates of 1.76 m × 1.0 m for 5 days prior to the expected delivery date and for 21 days after delivery until complete weaning occurred in all the litters. The sows were in their first to fifth pregnancy.

Treatments

By means of a predesigned table of random numbers, at the time of delivery, 120 sows with maternal or fetal dystocia were allocated to one of the following 3 groups. In group 1 (controls), saline was injected by intravulvar route. In group 2, animals received an udder massage for 5 min starting after maternal or fetal dystocia was identified, followed by an intravulvar injection of saline. In group 3, sows received udder massage for 5 min starting after maternal or fetal dystocia was identified, followed by intravulvar administration of 10 IU of oxytocin. Udder massage involved the application of natural oil while rubbing the udder in a cephalic-to-caudal direction; this procedure was performed by the same investigator in all occasions. For purpose of the study, fetal dystocia was considered as present when a sow delivered one or two consecutive intrapartum stillbirths prior to the inclusion of the animal into the study. Maternal dystocia was defined as a 40-min period of uterine quiescence that occurred in the parturient sow after the delivery of any of the piglets. Parturition was manually assisted by two of

the investigators when the interval of time between piglets was longer than 1 hour.

Study outcomes

As obstetric outcomes, the number, intensity (mmHg) and duration (seconds) of uterine contractions were monitored by means of an electronic digital cardiotocograph (Fetal Monitor Coriometric, Medical Systems Inc. Co., Connecticut, CN, USA). Duration of parturition (min) was registered starting from the first tocographic evidence of consistent uterine activity until the delivery of the placenta. The time to deliver each piglet (time interval between piglets; min) was measured and averaged for each sow. The number of manual obstetric interventions per sow, performed by one of the investigators, was also registered.

As fetal outcomes, the number of live-born and stillborn piglets was registered. Stillbirths were classified into type I or type II, according to the classification criteria previously described in detail elsewhere [10] [11]. Type I or ante-partum stillbirths had a characteristic edematous and hemorrhagic appearance, a grayish-brown discoloration because of beginning mummification; if the process was advanced then the fetuses were dehydrated and started to lose hair. Type II or intra-partum stillbirths had the exact appearance of their normal littermates with the exception that they did not breathe. Finally, based on the degree of meconium staining at birth, life-born piglets were classified as unstained-to-mildly and moderate-to-severely stained [11].

Statistical analysis

The number of piglets born per sow was compared among groups by means of the Kruskal-Wallis test followed by the Mann-Whitney test in order to distinguish the differences between group 1 (controls) and either group 2 (udder massage) or 3 (udder massage and oxytocin). The other continuous variables were compared among groups by an Analysis of Variance (ANOVA) followed by a Dunnett test to distinguish the differences between group 1 and either group 2 or 3. Categorical variables were compared among the three groups by a "2 by k" X^2 test followed by a comparison between group 1 and either group 2 or 3 by a "2 by 2" X^2 test. The statistically significant limit was fixed to a two-tailed $P < 0.05$ in every test.

RESULTS

Udder massage alone or in combination with oxytocin did not increase the number of uterine contractions in comparison to the control group (Table 1). However, the duration of uterine contractions and the overall duration of parturition were decreased by approximately 9 s and 20 min by udder massage, respectively; as expected, the reduction was more evident in group 3 (approximately 12 s and 50 min, respectively). Paradoxically, the intensity of uterine contractions decreased from 18.1 ± 3.6 mmHg in group 1 to 14.1 ± 3.8 mmHg in group 2 ($P < 0.001$) (Table 1). In group 3, however, the intensity of uterine contractions increased approximately 3 mmHg above that observed in the control group ($P \leq 0.0025$). Piglets were delivered at intervals of approximately 16 min in the control group and 15 min in the group treated with udder massage; time interval between piglets was reduced by 3 min ($P < 0.001$) in group 3 compared to the control. The number of manual obstetric interventions significantly decreased in sows in group 2 compared to the control group; this reduction was more evident in group 3.

Table 1 Obstetric outcomes of dystocic sows treated with udder massage alone or in combination with oxytocin

	Group 1 (controls) n = 40	Group 2 (udder massage) n = 40	Group 3 (udder massage + oxytocin) n = 40	P value
Uterine contractions per piglet (n)	3.8 ± 1.2	3.7 ± 0.7	4.3 ± 1.8	0.08
Intensity of uterine contraction (mmHg)	18.1 ± 3.6	14.1 ± 3.8*	21.1 ± 4.1*	< 0.01
Duration of uterine contraction (s)	25.6 ± 5.7	16.7 ± 4.5*	13.1 ± 2.8*	< 0.001
Duration of parturition (min)	205.2 ± 33.1	185.2 ± 36.6*	156.3 ± 37.3*	< 0.001
Time interval between piglets (min)	16.9 ± 3.3	15.4 ± 3.3	13.1 ± 3.6*	< 0.001
Manual obstetrics interventions [n (% of piglets)]	76 (14.9)	49 (10.1)*	30 (6.2)*	< 0.001

*P < 0.05, control group versus either udder massage or udder massage followed by oxytocin administration.
Data are mean ± SD or where specified n (%).

Table 2 Fetal outcomes of dystocic sows treated with udder massage alone or in combination with oxytocin

	Group 1 (controls) n = 40	Group 2 (udder massage) n = 40	Group 3 (udder massage + oxytocin) n = 40	P value
Piglets per sow [median (range)]	13 (11 – 15)	12 (10 -16)*	12 (8 -15)	0.048
Live born piglets [n (%)]	407 (79.6)	433 (89.1)*	398 (82.2)	< 0.001
Antepartum deaths [n (%)]	6 (1.2)	4 (0.8)	10 (2.0)	0.2
Intrapartum deaths [n (%)]	98 (19.2)	49 (10.1)*	76 (15.7)	< 0.001
Moderate-to-severe meconium stained piglets [n (% of live-born piglets)]	117 (28.7)	36 (8.8)*	58 (17.2)*	< 0.001

*P < 0.05, control group versus either udder massage or udder massage followed by oxytocin administration

The number of piglets born per sow was similar between groups 1 and 3; it was slightly, but significantly, lower in group 2 (Table 2). The number of live born piglets was significantly higher in group 2 than in the control group, whereas no differences were observed between groups 1 and 3. There was an approximately 3.3-fold reduction in the number of moderate-to-severe meconium stained piglets born to dystocic sows treated with udder massage (P < 0.001). In group 3, there was a 1.7-fold reduction (P < 0.001). Although the number of antepartum-death piglets was similar among the three groups, there was a reduction of approximately 50% in the number of intrapartum deaths in group 2 whereas the difference was not significant between groups 1 and 3 (Table 2).

DISCUSSION

The current study showed that udder massage applied to dystocic sows resulted in better obstetric and fetal outcomes than sows without any intervention. The demonstration that breast stimulation can successfully be used for ripening the cervix and labor induction was described as early as in the 18th and 19th centuries [5]. However, few advances to study the uterotonic effects, produced in response to breast stimulation, have been performed.

The uterotonic effect of udder massage in parturient sows can be enhanced by the subsequent administration of oxytocin. However, the rate of adverse obstetric and fetal outcomes also increased with the co-administration of oxytocin. Gestation-related changes in uterine activity include alterations in hormonal, metabolic, and neural inputs to the uterus, changes in the responsiveness of the myometrium to bioactive substances through alterations in receptors, and coupled signal transduction mechanisms resulting in myometrial layer-dependent differences in responsiveness to bioactive substances less marked than that observed in non-pregnant sows [12].

Uterine response to breast stimulation has not shown any correlation with oxytocin levels [13], and the effects observed in the present study further support that breast stimulation may act through a different mechanism to oxytocin. For example, udder massage in dystocic sows significantly decreased uterine activity whereas oxytocin administration evidently enhanced it. In contrast, either udder massage alone or followed by oxytocin administration resulted in shorter duration of uterine contractions and parturition. In pregnant mammals, including humans, an abrupt increase in oxytocin binding sites in the uterus may occur at approximately 24 h before the onset of labor, reaching the greatest levels during labor and sharply decreasing after parturition to reach baseline levels 2-5 days postpartum [14] [15] [16]. These physiological changes explain the participation of endogenous oxytocin during parturition as well as the efficacy of this hormone when exogenously administered. Breast stimulation may facilitate the oxytocin-induced uterine stimulation by other pathways involved in the parturition process.

In our study, a 50% reduction in the rates of meconium-stained live-born piglets was observed in the group of dystocic sows that received udder massage in comparison to the control group. Similar significant, though less evident, results were previously reported in studies performed in parturient women [3]. In this study, however, the positive effect of udder massage was partially reversed by the administration of oxytocin. The adverse fetal and obstetric outcomes to oxytocin could be related to uterine hyperstimulation [17]. However, dystocia may not only result from uterine

hyperstimulation but also from the lack of uterine response to oxytocin administration [18]. In a study on breast stimulation in parturient women, this maneuver failed to produce adequate uterine stimulation in 50% of cases, but there was no case of uterine hyperstimulation in those patients who subsequently received oxytocin [4]. The dose and the rate at which oxytocin is administered might define whether it may or may not produce uterine hyperstimulation. However, the current evidence points to a mechanism of action different to that produced by breast stimulation in mammals.

In conclusion, udder massage applied to dystocic sows resulted in better obstetric and fetal outcome than sows without any intervention. The mechanism of action of udder massage appears to be different to oxytocin. However, the subsequent administration of oxytocin partially reversed the benefits of udder massage.

AUTHORS' PARTICIPATION

D MR, M AS, R RN, M GL, and ME TO conceived the idea, drafted the protocol and proposed the study design. D MR, MGL, and M Z were responsible for the experimental part of the study. AA NO, and EY VA performed the data analysis. D MR, M AS, R RN, and ME TO drafted the manuscript. All participated in the discussion of the results and contributed to the final version of the manuscript.

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CONFLICT OF INTERESTS/DISCLAIMERS

M GL, D MR, AA NO, M AS, EY VA, and R RN are members of the Editorial Board of the journal.

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DIFFERENTIAL EFFECTS OF HISTONE DEACETYLASE INHIBITORS ON CELL CYCLE REGULATION AND APOPTOSIS IN HUMAN PROSTATE CANCER CELLS

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ABSTRACT

Objectives: Histone deacetylase (HDAC) inhibitors may suppress the proliferation of cancer cells and induce apoptosis. This study examined the molecular mechanism of how HDAC inhibitors modulate the cell cycle regulators and tumor suppressor genes in prostate cancer cells. **Methods**: HDAC inhibitors were evaluated according to the following parameters and techniques: (1) cell viability determined by a microculture tetrazolium assay (MTA); (2) induction of apoptosis according to 4'-6-diamidine-2'-phenylindole dihydrochloride (DAPI) staining; (3) the expression of cell cycle related proteins such as CDK2, CDK4, cyclin D1, and cyclin E by Western blot analysis; (4) p16 mRNA expression by RT-PCR; (5) the acetylation of the histone protein by nuclear protein extraction; and (6) cell cycle analysis by flow cytometry. **Results**: The HDAC inhibitors decreased the level of cell proliferation and induced apoptosis in human prostate cancer cell lines. Trichostatin A (TSA) caused a significant decrease in cyclin D1/CDK4 expression in LNCaP cells. Cell cycle analysis showed that the TSA treatment increased the proportion of LNCaP cells in the G1 phase and decreased the proportion of cells in the S phase. The TSA treatment resulted in the induction of apoptotic cell death which was confirmed by DAPI staining. TSA increased the level of p16 and WT1 expression in the androgen-dependent LNCaP or androgen-independent PC3 and DU145 cells. **Conclusions**: HDAC inhibitors may prevent the proliferation of prostate cancer cells by altering the expression of the cell cycle regulators and tumor suppressor genes, which might be associated with epigenetic regulation.

Key words

Apicidin; Apoptosis; Cell cycle regulator; Histone deacetylase; Trichostatin A

RÉSUMÉ

Objectif: Les inhibiteurs de l'histone-désacétylase (HDCA) peuvent réduire la prolifération de cellules cancéreuses et induire une apoptose. Nous avons examiné dans cette étude les mécanismes moléculaires impliqués par les inhibiteurs de l'HDAC dans la modulation du cycle cellulaire et des gènes répresseurs des tumeurs dans les cellules du cancer de la prostate. **Méthodes:** Les effets des inhibiteurs de l'HDAC ont été étudiés par les techniques suivantes: (1) la viabilité cellulaire par le TTM (test de tétrazolium sur microculture), (2) l'induction de l'apoptose cellulaire par marquage avec le DAPI (4'-6-diamidine-2'-phénylindole dihydrochlorure), (3) l'expression des protéines liées au cycle cellulaire telles que les CDK2, CDK4, cycline D1 et cycline E par immunotransfert, (4) l'expression de l'ARN messager p16 par RT-PCR, (5) l'acétylation des histones par extraction des protéines nucléaires, et (6) l'analyse du cycle cellulaire par cytométrie en flux. **Résultats:** Les inhibiteurs de l'HDAC réduisent la prolifération et induisent l'apoptose des cellules du cancer de la prostate. La trichostatine A (TSA) produit une réduction significative de l'expression de cycline D1/CDK4 dans les cellules LNCaP. L'analyse du cycle cellulaire montre une proportion augmentée de cellules LNCaP en phase G1 et une diminution des cellules en phase S suite an traitement par la TSA. Ce traitement induit la mort cellulaire par apoptose confirmée par marquage par le DAPI. La TSA augmente le taux de p16 et l'expression de WT1 tant dans les cellules androgéno-dépendantes LNCaP que dans les cellules androgéno-indépendantes PC3 et DU145. **Conclusions:** Les inhibiteurs de l'HDAC peuvent prévenir la prolifération des cellules du cancer de la prostate par la modulation du cycle cellulaire et des gènes répresseurs des tumeurs vraisemblablement par régulation épigénétique.

Mots clés

Apicidine; Apoptose; Régulateur du cycle cellulaire; Histone-déacétylase; Trichostatine A

RESUMEN

Objetivos: Los inhibidores de la histona desacetilasa (HDAC) suprimen la proliferación de las células cancerígenas e inducen la apoptosis. Este estudio examinó los mecanismos moleculares de los reguladores del ciclo celular y los genes supresores tumorales por los inhibidores de la HDAC, en las células prostáticas cancerígenas. **Métodos:** Para evaluar el

efecto de los inhibidores de HDCA, nosotros estudiamos (1) la viabilidad celular determinada mediante la prueba de microcultivo con tetrazolium; (2) la inducción de la apoptosis de acuerdo a la tinción de cloruro de 4'-6-diamino-2'-fenilindol (DAFI); (3) la expresión de CDK2, CDK4, ciclina D1, y ciclina E, mediante análisis de Western blot; (4) la expresión de p16 RNA mediante RT-PCR; (5) la acetilación de la histona mediante extracción proteica nuclear; y (6) el ciclo celular mediante citometría de flujo. **Resultados:** Los inhibidores de la HDAC disminuyeron los niveles de proliferación celular e indujeron la apoptosis en las células de cáncer de próstata. La tricostatina A (TSA) disminuyó significativamente la expresión de ciclina D1/CDK4 en las células LNCaP. El tratamiento con TSA incrementó la proporción de células LNCaP en la fase G1, disminuyó la proporción de células en la fase S del ciclo celular, y resultó en la inducción de la muerte celular por apoptosis confirmada mediante la tinción de DAFI. La TSA incrementó los niveles de p16 y la expresión de WT1 en las células dependientes de andrógenos LNCaP así como en las células independientes de andrógenos PC3 y DU145. **Conclusiones:** Los inhibidores de la HDAC pueden prevenir la proliferación de las células del cáncer de próstata mediante la alteración de la expresión de los reguladores del ciclo celular y de los genes supresores tumorales, los cuales podrían estar asociados con regulaciones epigenéticas.

Palabras clave

Apicidina; Apoptosis; Reguladores del ciclo celular; Histona deacetilasa; Trichostatina A

INTRODUCTION

Prostate cancer is one of the most common malignancies and the second leading cause of death among men in the United States [1] [2]. Although the incidence of prostate cancer in Korea is still much lower than in Western populations, it has increased rapidly over the past 10 years [3]. Epidemiological studies have shown that a high incidence of prostate cancer may be related to dietary factors. Excessive consumption of polyunsaturated fats has been associated with prostate cancer in humans [4]. Recent studies suggest that additional factors, including environmental agents (endocrine disrupting chemicals), aging, and oxidative stress are causally related to the development of prostate cancer [5].

In the initial stages, prostate cancer cells depend on androgens for their survival. Therefore, the main form of systemic treatment is androgen-ablation [6]. However, within a few years, most patients progress to an androgen-refractory state, which is capable of growing without androgens [7]. Hence, there is a need for alternative therapeutic strategies for the treatment of hormone-refractory prostate cancer with several chemotherapy trials being carried out for this purpose [8].

DNA methyltransferases (DNMTs) and HDACs are potential targets for regulating these epigenetic changes. DNA methylation and histone modification, which are regulated by several classes of enzymes, play an important role in the epigenetic mechanisms of gene regulation in various tumor initiation and progression stages. Epigenetic changes are defined as heritable changes in gene expression that occur without any changes in DNA sequence. The importance of an epigenetic alteration to cancer has probably been understated, since there has been more than ten years of remarkable advances in the knowledge of the mutational events that lead to the activation of protooncogenes or the inactivation of tumor suppressor genes [9]. Therefore, DNMT and HDAC inhibitors are believed to be one of the most promising classes of new anticancer agents in current clinical trials.

Recently, either naturally derived or synthetic compounds exhibiting HDAC inhibitory activity were shown to have anti-proliferative, pro-differentiating, and pro-apoptotic properties in a variety of cancer cells [10] [11] [12] [13]. TSA, which was originally developed as an antifungal agent, is a potent and reversible HDAC inhibitor. TSA has HDAC inhibitory effect at low concentrations; it targets the cell cycle progression of several cell types, induces cell growth arrest at both the G1 and G2/M phases, and in some cases, induces apoptosis [14] [15]. Apicidin [cyclo(N-O-methyl-L-tryptophanyl-L-iso leucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)] is a fungal metabolite shown to inhibit both mammalian and protozoan HDACs. It has also been reported to inhibit the proliferation of variety of tumor cells and selectively up-regulate the expression of p21WAF/Cip1, which allows tumor cells to undergo cell cycle arrest at the G0/G1 phase and cause morphological changes [16] [17]. However, the precise mode of actions of these agents in prostate cancer cells is unclear.

Therefore, this study examined the molecular mechanism for the epigenetic alterations of the cell cycle regulator proteins and tumor suppressor genes in prostate cancer cells by DNMT and HDAC inhibitors.

METHODS

Materials

The TSA and apicidin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The medium and its supplements containing antibiotics, fetal bovine serum was obtained from Gibco Invitrogen Corporation (CA, USA). Western blot detection reagents were obtained from Amersham Bioscience Corporation (Bucks, United Kingdom). The polyvinylidene difluoride (PVDF) membranes were obtained from Bio-Rad (CA, USA).

The RT-PCR reagents were supplied by Invitrogen Corporation. All other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell lines and culture media

The LNCaP, PC3 and DU145 cells, which are human prostate cancer cells, were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). The cells were grown in Minimum Essential Medium (MEM), pH 7.2, containing 2.2 g L^{-1} $NaHCO_3$, 1 mM sodium pyruvate, 100 U mL^{-1} antibiotics, and 10% heat-inactivated fetal bovine serum (FBS). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The cells were plated on plastic dishes. The culture medium was replaced every 2 days. Apicidin and TSA were dissolved in dimethyl sulfoxide (DMSO) and absolute ethanol, respectively. These agents were diluted to the appropriate concentrations with the culture medium containing 5% charcoal-dextran treated FBS (CD-FBS). The final concentration of DMSO or ethanol was < 0.1% (vol vol^{-1}).

Microculture tetrazolium assay

The cell viability was determined using an MTA. The cultures were initiated in 96 well plates at a density of 2500 cells per well. Cells were allowed to reattach for 48 hr and then exposed to the test compounds. At the end of the treatment period, 15 μL of a 5 mg mL^{-1} MTT reagent [3-(4,5-dimethyl- thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide in PBS] was added to each well. The plates were incubated again for 4 h at 37°C in the dark. The supernatant was aspirated, and formazan crystals were dissolved in 100 μL DMSO at 37°C for 10 min with gentle agitation. The absorbance per well was measured at 540 nm using a VersaMax Microplate Reader (Molecular Devices Corp, CA, USA).

Nuclear protein preparation

Nuclear protein from the cells was prepared using the following procedure. All solutions, tubes, and centrifuges were maintained at 4°C. The cells were harvested by tripsinization, washed with cold PBS, centrifuged at 3,000 rpm for 5 min, and incubated in 50 μL of lysis buffer I (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.5 mM PMSF) for 20 min. After centrifugation at 12,000 rpm for 10 min, supernatant was removed and the pellet was re-suspended in 30 μL of a lysis buffer II (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF and 0.05% NP-40) for 20 min. After centrifugation at 12,000 rpm for 10 min, the pellet was re-suspended in 40 μl of lysis buffer III (5 mM HEPES, pH 7.9, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 26% glycerol) for 30 min. After the final centrifugation at 12,000 rpm for 30 min, the protein concentration was quantified using a Bio-Rad protein assay reagent.

Western blot analysis

The cells were harvested and washed twice with PBS at 4°C. The total proteins were prepared using a PRO-PREP protein extract solution (Intron, Seongnam, Korea), and quantified using the protein assay reagent (Bio-Rad). Fifty microgram of the proteins were denatured by boiling at 96°C for 5 min in a sample buffer (0.5 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.1% bromphenol blue, 10% β-mercaptoethanol) at 1:1 ratio. Each sample was separated using a 10-15% polyacrylamide gel (SDS-PAGE), and transferred to a PVDF membrane at 100 V for 1 h in a transfer buffer. The membrane was blocked using a blocking buffer containing 5% non-fat dry milk in a TNT buffer (10 mM Tris-Cl, pH 7.6, 100 mM NaCl and 0.5% Tween 20) at room temperature for 1 h. The membrane was then incubated overnight with the primary antibodies specific to CDK4, cyclin D1, CDK2, cyclin E, Rb, p-Rb, WT1 (Santa Cruz Biotechnology Inc., CA, USA), and Ac-H3 (Upstate Biotechnology) at 4°C. After washing for 1 hr with TNT buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Santa Cruz Biotechnology Inc., 1:10,000) at room temperature for 30 min, and then washed for 60 min with the TNT buffer. Antibody labeling was detected using ECL plus Western blotting detection reagents (Amersham Bioscience Corporation).

Assay of RT-PCR

The total RNA was extracted using Trizol Reagent (Gibco). cDNA was generated using the Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using a reverse transcription method. Briefly, 2 μg of the total RNA was reverse transcribed for 50 min at 42°C in a 20 μL reaction mixture (containing 1 μL of oligo primer (0.5 g), 2 μL of 10 mM dNTP mix, 2 μL of 10x RT buffer, 4 μL of 25 mM $MgCl_2$, 2 μL of 0.1 M DTT, 40 U of RNase Out inhibitor, and 100 U of Superscript II), followed by denaturation at 70°C for 15 min. RNase inhibitor (2 U) was then added and incubated at 37°C for 20 min. cDNA was used for the subsequent PCR reaction, 2 μL of cDNA was amplified in a 25 μL reaction mixture containing 2.5 μL of 10x PCR buffer, 0.75 μL of 50 mM $MgCl_2$, 0.5 μL of 10 mM dNTP mix, 1 U Taq polymerase, and 0.25 μL of sense and anti-sense primer (20 μM).

The primers were p16 (F; 5'-CAACGCACCGAATAGT TACG-3', R; 5'-ACCAGCGTGTC CAGGAA-3') and b-actin (F; 5'-CCTCGCCTTTGCCG ATCC-3', R; 5'-GGATCTTCATGA

GGTAG TCAGTC-3'). The reaction was initiated at 94°C for 5 min and PCR was performed using a variable number of cycles of amplification defined as follows: denaturation at 94°C for 45 sec, annealing at 55°C (p16, 177 bp) or 60°C (b-actin, 626 bp) for 45 sec and extension at 72°C for 45 sec. Electrophoresis was performed in 2% agarose gel containing 1 μg of ethidium bromide (EtBr). The molecular sizes of the amplified products were determined by comparison with the molecular weight markers, a 100 bp DNA ladder (iNtRON) run in parallel with the RT-PCR products.

DAPI staining

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA binding fluorochrome DAPI. The cells were grown in 6 well plate at a density of 4×10^4 cells per well. After treatment, cells were fixed in absolute methanol, and stained with 300 μL of DAPI solution (1 g mL^{-1}) at 37°C for 15 min. After removing the staining solution, the cells were examined by fluorescence microscopy (Axiovert 200, ZEISS Inc., Germany).

Flow cytometry analysis

Cells were harvested and washed in 1% BSA before being fixed in 75% ethanol containing 0.5% Tween 20 for at least 1 h at 4°C. The cells were washed in 1% BSA and re-suspended in a cold propidium iodine (PI) staining solution (100 g mL^{-1} RNase and 10 g mL^{-1} PI in PBS) 1 mL for 40 min at 4°C. Data acquisition and analysis was carried out using a flow cytometry system (Becton Dickinson, CA, USA).

Statistical analysis

The results are reported as the means ± S.E. of three independent triplicate measurements. Statistically significant differences between untreated control and treated groups were determined using one-way analysis of variance (ANOVA).

RESULTS

The antiproliferative effect of TSA and apicidin on human prostate cancer cells was determined using a MTT assay and the results are shown in Figure 1. The prostate cancer cell lines (LNCaP, PC3, and DU145 cells) were treated with either TSA or apicidin at the consecutive serial concentrations. TSA and apicidin inhibited the proliferation of LNCaP and DU145 cells in a concentration-dependent manner and significantly decreased the level of cell proliferation after a 48 h treatment at the lowest concentration of 50 nM. In contrast, the PC3 cells were resistant under these conditions (Figure 1, plots A and B).

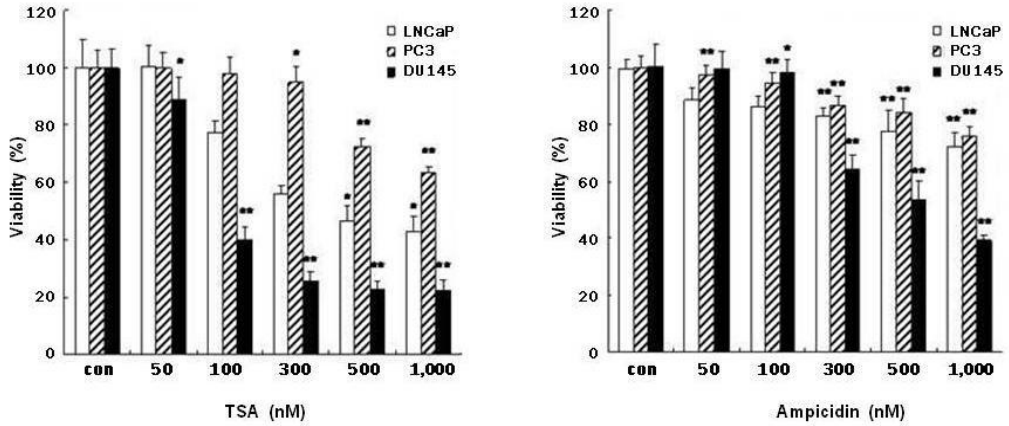

Figure 1 **Growth inhibition of human prostate cancer cell lines by trichostatin A and apicidin.**
The cells were treated with different concentrations of trichostatin A (TSA) and apicidin for 48 h and the cell viability was determined by a MTT assay.
The error bars show a S.D.
*P < 0.005, **P < 0.001 as determined by a Student's t test, difference between the treated and the control group.

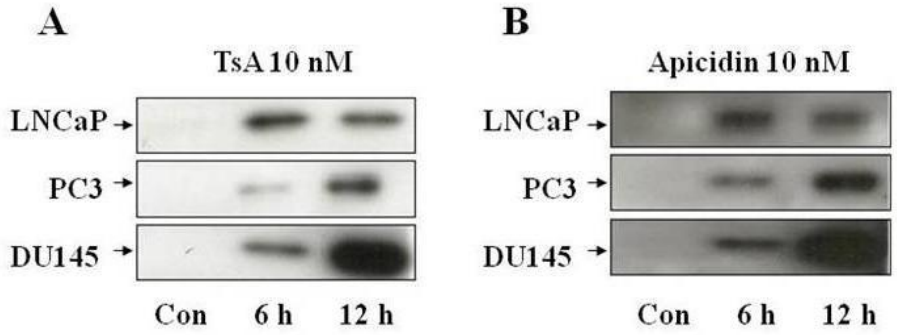

Figure 2 **Accumulation of acetylated histone proteins by treatment with the HDAC inhibitors.**
The cells were treated with 10 nM trichostatin A (TSA) and apicidin for 12 h. The acetylated histone tails were determined by western blot analysis using anti-acetylated H3 antibodies.

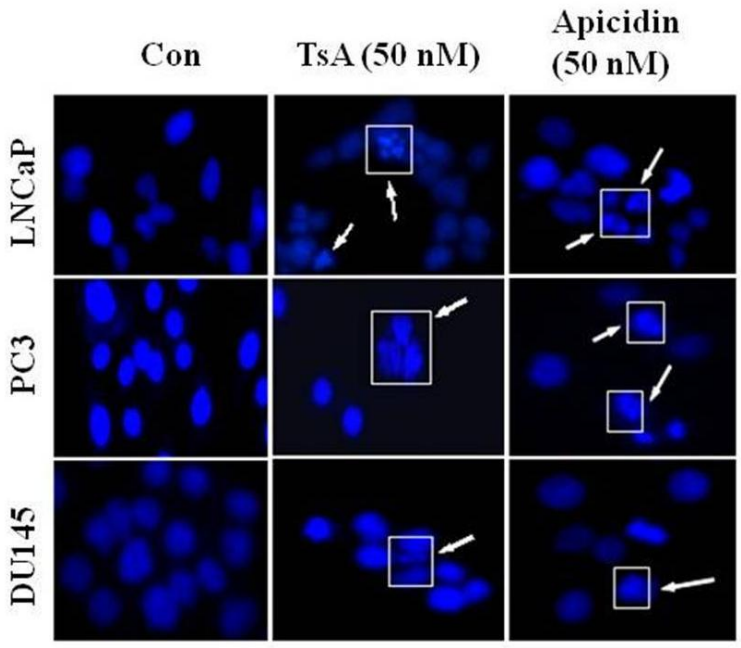

Figure 3 **Apoptosis induction in prostate cancer cell lines by trichostatin A and apicidin treatment.**
The induction of apoptosis by trichostatin A (TSA) and apicidin in LNCaP, PC-3, and DU-145 was analyzed by fluorescence microscopy after nuclear staining with DAPI. The cells were incubated with or without TSA (50 nM) and apicidin (50 nM) for 24 hr. The cells undergoing apoptosis and nuclear fragmentation are identified with arrows.
The magnification is 400X.

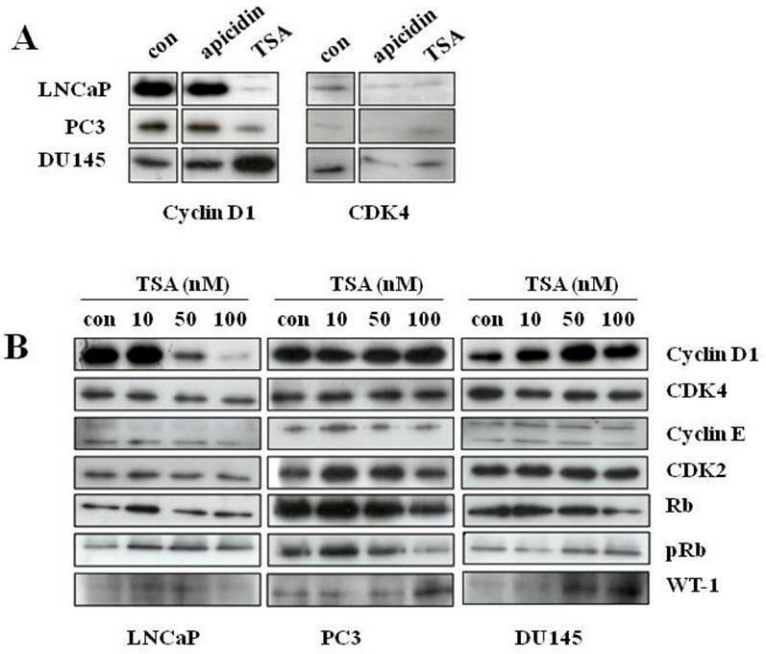

Figure 4 **Effect of the HDAC inhibitors on cell cycle regulators.**
The human prostate cancer cells were treated with 10, 50 and 100 nM trichostatin A (TSA) and apicidin (100 nM) for 24 hr. The proteins were isolated from LNCaP, PC-3, and DU145 cells and lysates (50 μg) of the cells were examined by 10% SDS-PAGE and analyzed by immunoblotting using specific antibodies.

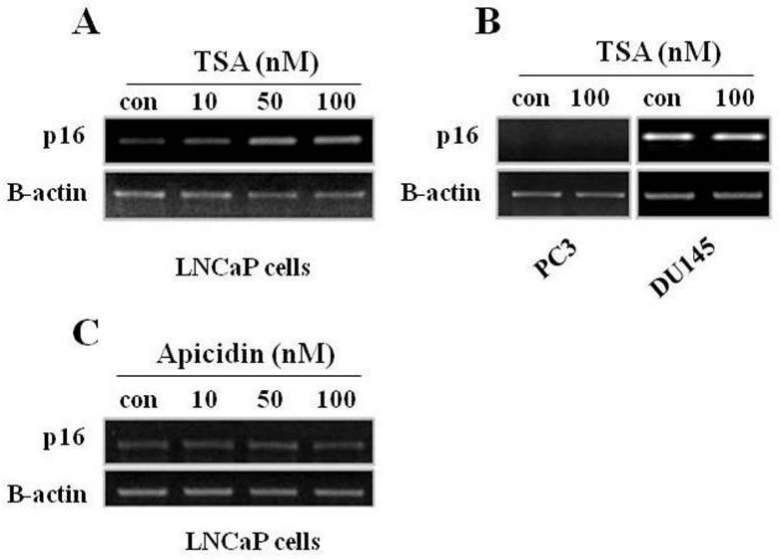

Figure 5 **Effect of HDAC inhibitors on tumor suppressor gene p16 expression in prostate cancer cells.**
The cells were treated with the indicated concentrations of trichostatin A (TSA) and apicidin for 48 h. Total RNA was isolated, and RT-PCR was performed using the specific primers described in the Materials and Methods section. Actin was used as a housekeeping control gene.

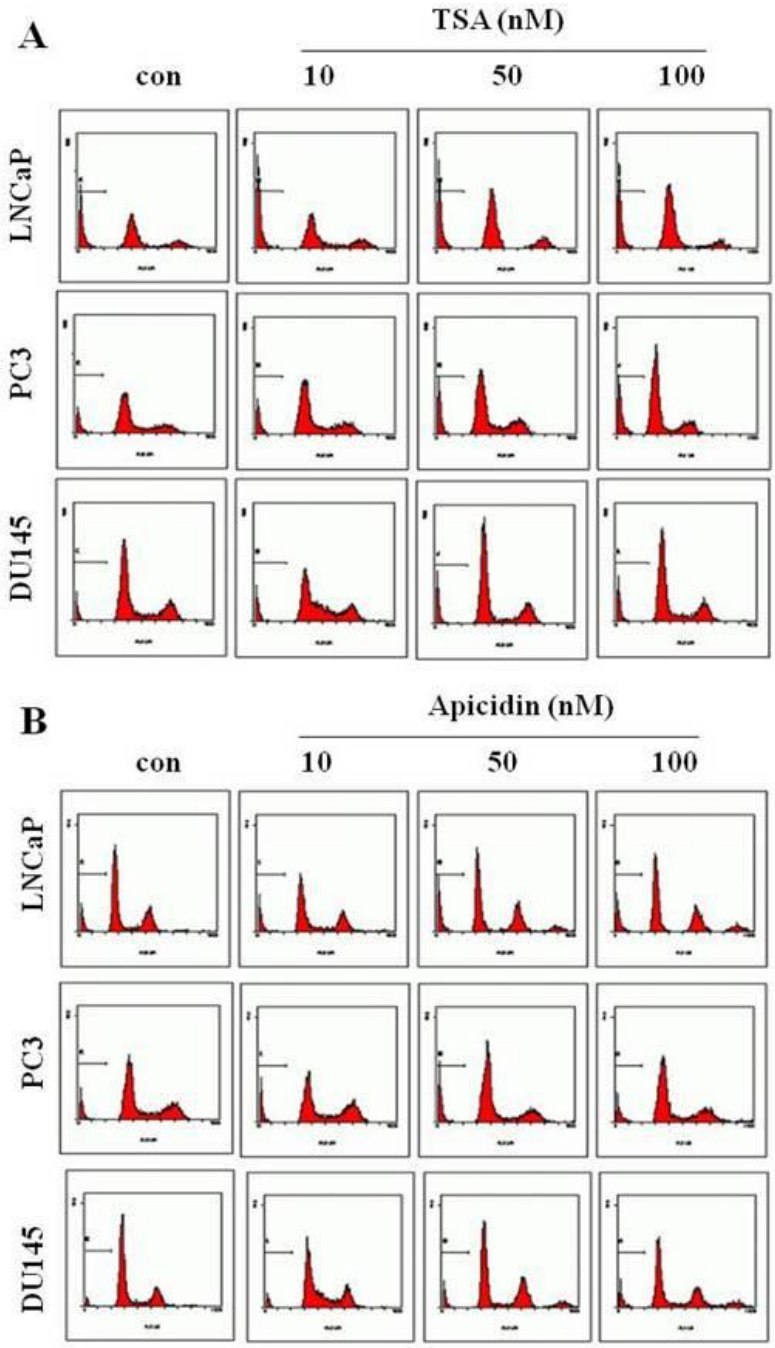

Figure 6 **Effect of apicidin on cell cycle distribution.**
Human prostate cancer cells were treated with trichostatin A (TSA) (A) and apicidin (B) at the indicated concentrations for 24 h. The cells were fixed, permeabilized and stained with propidium iodide to measure DNA content by fluorescence-activated cell sorting (FACS) analysis. The percentages of cells in each phase of the cell cycle (G1, S and G2) are indicated.

The effect of apicidin on the level of histone acetylation was then analyzed by Western blotting using the specific antibody to acetylated histone H3. Treatment of the prostate cancer cells with TSA (10 nM) and apicidin (10 nM) for 12 h resulted in a significant increase in acetylated H3 levels (Figure 2).

In order to determine if the decrease in cell viability was related to apoptosis, the cells were treated with 50 nM TSA and 50 nM apicidin for different times. The cells were then stained with DAPI, which enters the cells and binds to the fragmented DNA. The nuclear changes were observed by fluorescence microscopy. TSA and apicidin markedly increased the number of apoptotic bodies after a 24 h treatment (Figure 3).

Whole cell protein extracts were made from each cell line after apicidin and TSA treatment for 24 h in order to determine if the cell death observed in prostate cancer cell lines related to cell cycle regulation. In the LNCaP cells treated with TSA at 100 nM, there was a significant decrease in cyclin D1 and CDK4 expression (Figure 4A). When treated with 10, 50, and 100 nM TSA for 24 h, there was a significant decrease in cyclin D1, CDK4, cyclin E, and CDK2 expression in the LNCaP cells but not in the PC3 and DU145 cells (Figure 4B). However, the apicidin treatment had no effect on cyclin E and CDK2 expression (which act in the G1 late stage) in the LNCaP, PC3 and DU145 cells (Figure 4A).

The tumor suppressor, Wilm's tumor suppressor gene (WT1), is essential for normal kidney and gonadal development and regulates cell differentiation and apoptosis *in vitro*. The expression of WT1 was examined to determine the effect of TSA on prostate cancer cells. The results showed that the expression of WT1 was induced dose-dependently in androgen-independent PC3 and DU145 cells. In contrast, the androgen-dependent LNCaP cells did not express a detectable level of WT1 (Figure 4B). In general, the p16 gene regulates progression through the G1/S phase of the cell cycle by binding CDK 4/6, which limits the formation of the CDK 4/6-cyclin D complex. The mRNA levels of the tumor suppressor gene, p16, were measured by RT-PCR to determine if the prostate cancer cell death, when treated with TSA, is related to p16 expression. The levels of p16 mRNA in LNCaP cells increased after TSA treatment in a dose-dependent manner, whereas there was no effect observed in the androgen-independent PC3 and DU145 cells (Figure 5, plots A and B). However, apicidin did not affect the levels of p16 expression in the LNCaP cells (Figure 5C).

Finally, flow cytometry analysis was used to examine the cell cycle progression of prostate cancer cells treated with TSA or apicidin for 24 h. TSA induced cell cycle arrest in the G1 phase in the LNCaP and DU145 cells. However, this was not observed in PC3 cells. In PC3 cells, the cell cycle arrest in the G1 phase was induced only after 24 h of treatment (Figure 6A). On the other hand, 100 nM of apicidin for 24 h induced cell cycle arrest in the G1 phase in PC3 cells only. In contrast, apicidin decreased the proportion of LNCaP and DU145 cells in the S phase and increased the proportion of cells in the G2 phase (Figure 6B).

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DISCUSSION

This study examined whether or not HDAC inhibitors induced apoptotic cell death and cell cycle arrest in various human prostate cancer cell lines. It was demonstrated that the inhibition of histone deacetylation leads to cell death, which is associated with nuclear chromatin condensation and cell cycle arrest in the G1 or G2 phase [18] [19] [20]. In this study, TSA suppressed the growth of prostate cancer cells in a dose-dependent manner. These effects were related to epigenetic regulation of the specific genes associated with cell cycle regulation. In particular, TSA exhibited strong anticancer efficacy against cultured human androgen-dependent and -independent prostate cancer cells. Additionally, TSA inhibited cell growth and induced apoptosis and cell cycle arrest, induced a decrease in cyclin D1 and CDK4 expression in LNCaP cells, and restored the levels of p16 expression in prostate cancer cells. The p16 gene is localized to chromosome 9p21 and encodes the CDK inhibitor. This protein regulates cell cycle progression through the G1/S by binding CDK 4/6, which limits the formation of the CDK 4/6-cyclin D complex and the phosphorylation of Rb [21]. Recently, aberrant DNA methylation of the 5' promoter region, an epigenetic event, was associated with the transcriptional silencing of p16 expression in several cancer cell lines including lung, bladder, colon, and prostate cancers [22] [23] [24].

Based on these results, the level of p16 expression was measured to determine if its expression is related

to the cell cycle regulation of prostate cancer cells treated with TSA. The results showed that p16 expression was induced in androgen-dependent LNCaP cells in a dose-dependent manner, whereas there was no expression observed in the androgen-independent PC3 and DU145 cells.

However, apicidin did not affect the expression of p16 in LNCaP cells. Therefore, TSA can restore the expression of p16 by inducing the relaxed chromatin conformation in androgen-dependent prostate cancer cells. This restored p16 expression induces prostate cancer cell death by inhibiting the cell cycle regulators, cyclin D1 and CDK4 in G1 phase of the cell cycle.

Another tumor suppressor gene, WT1 is essential for normal kidney and gonadal development and regulates cell differentiation and apoptosis in vitro [25] [26]. WT1 represses the androgen-signaling pathway. Moreover, WT1 expression in prostate cancer cell lines is limited to those cell lines lacking functional AR [27] [28]. PC3 and DU145 cells express high levels of WT1 mRNA and protein, whereas the androgen-dependent LNCaP cell did not express WT1 [29]. Furthermore, WT1 modulates the expression or activity of apoptotic genes, such as the prostate apoptosis response gene, par-4 [30] [31] or bcl-2 [32] [33].

In this study, the WT1 protein level was measured to determine the anticancer effect of TSA on human prostate cancer cells. The results showed that expression of WT1 was induced in androgen-independent PC3 and DU145 cells in a dose-dependent manner, whereas androgen-dependent LNCaP cells did not express detectable levels of WT1. Therefore, the HDAC inhibitor TSA would restore WT1 expression by inducing a relaxed chromatin conformation in androgen-independent prostate cancer cells. In addition, the intracellular level of acetylated histone H3 in prostate cancer cell lines induced by HDAC inhibitors was measured. Low levels of histone acetylation were observed in the absence of TSA, whereas hyperacetylated H3 was observed in all prostate cancer cells tested. This suggests that the induction of histone hyperacetylation by TSA due to the inhibition of HDAC and the activation of the transcriptional factors for WT1 gene expression.

TSA was found to dramatically induce cell cycle arrest in the G1 phase and increase the level of apoptosis in LNCaP cells. The transition from the G1 to S phase is believed to be triggered mainly by CDK4 after binding to its regulatory subunit, cyclin D1 [27] [28]. In general, G1 arrest depends on the ability of p16 to activate the transcription of specific genes, while G2/M arrest involves the repression of the transcription of CDK1/cyclin B1 complex [30]. This shows that TSA decreases the expression of cyclin B1 and CDK1 at both the mRNA and protein levels, and apicidin also increased the expression p21 and p27, which play a key role in the cell cycle arrest at the G2/M phase and apoptosis.

In conclusion, HDAC inhibitors suppress the proliferation of prostate cancer cells by altering the expression of cell cycle regulators and inducing apoptosis. In particular, TSA may have different mechanism of action in either androgen-dependent or androgen-independent prostate cancer cells by altering the tumor suppressor genes, which might be associated with the epigenetic regulations.

AUTHORS' PARTICIPATION

HS Kim and HJ Kim conceived of and designed the study. WS Choi and JY Han analyzed and interpreted the data. H.J. Kim drafted the paper and J Lee and JY Han revised it for important intellectual content. All authors gave final approval of the version to be published.

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CONFLICT OF INTERESTS/DISCLAIMERS

HS Kim is member of the Editorial Board of the journal.

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