Cytotoxic and COX-2 Inhibition Properties of Hydroxycinnamic Derivatives

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Abstract: The anticancer (cytotoxicity against human mammary gland adenocarcinoma cells) and anti-inflammatory properties (COX-2 inhibition) of the hydroxycinnamic derivatives *trans*-3-(3,4,5-trihydroxyphenyl)-2-propenoic acid, *trans*-ethyl(3,4,5-trihydroxyphenyl)-2-propenoate and diethyl 2-(3,4,5-trihydroxy-phenylmethylene)malonate were screened. Data point out a putative correlation between anti-inflammatory and anticancer properties and suggest hydroxycinnamic derivatives as promising lead compounds for the development of anti-inflammatory/chemopreventive agents.

Keywords: Hydroxycinnamic derivatives, anti-inflammatory, COX-2, anticancer, mammary gland adenocarcinoma cells.

INTRODUCTION

Polyphenols are bioactive compounds believed to be involved in the defence process against deleterious oxidative damage, at least in part, due to their antioxidant properties [1-5]. Among polyphenolic compounds, hydroxycinnamic acids (*e.g.* ferulic and caffeic acids) are a well-known group of phytochemicals, which are present in the human diet in representative amounts. Many epidemiologic studies and preclinical experiments have suggested that this type of dietary antioxidants play important roles in the prevention of cancer through their antioxidant and/or anti-inflammatory activities [6-13]. In fact, cinnamic acids and its derivatives, such as caffeic acid phenylethyl ester (CAPE), were reported to act as efficient chemopreventive agents, displaying remarkable growth-inhibition properties in several human cancer cell lines [14-19].

One relevant aspect of carcinogenesis is recognised to be the inflammatory response, which may be prevented by hindering oxidative stress conditions, namely by phenolic antioxidants. Furthermore, anti-inflammatory agents are currently used in some cancer chemopreventive strategies. One of the chemoprevention mechanisms associated to phenolic compounds is related to their scavenging capacity towards deleterious reactive oxygen and/or nitrogen species (ROS/RNS) [20,21]. In addition, polyphenols can inhibit ROS generating transcription factors closely linked to inflammation (*e.g.* NF-κB), as well as enzymes that mediate the inflammatory process [22], such as xanthine oxidase [23], lipoxygenase (LOX) [24] and cycloxygenase-2 (COX-2) [25].

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It is well established the occurrence of two distinct isofoms of cyclooxygenase (COX): COX-1 and COX-2 [26]. COX-1 is constitutively expressed in most tissues and is involved in normal cellular homeostasis whereas COX-2 expression is induced by a variety of mitogenic stimuli such as phorbol esters, LPS, and cytokines [27] leading to the production of prostaglandins. Inhibition of COX-2 decreases the conversion of arachidonic acid to prostaglandins (PGs), which are important biological mediators of inflammation and have been implicated in the initiation/promotion of some types of cancer, namely, colon cancer [28] and renal, prostate, bladder and testicular tumors [29]. In addition, COX-2 has been considered an angiogenic factor in malignancies such as ovarian cancer [30]. Chemoprevention by non-steroidal anti-inflammatory drugs (NSAIDs) is thought to reflect inhibition of COX-2, although non-COX-mediated mechanisms have also been implicated [31]. Similarly, phenolic antioxidant compounds with proven chemopreventive actions were found to inhibit both COX-2 transcription and activity in cancer cell lines: resveratrol [32] and caffeic acid phenylethyl ester (CAPE: [19]).

However, the mechanisms underlying the protective action of phenolic compounds towards degenerative pathologies are not yet completely understood, although numerous evidences indicate that they are strongly dependent on their structural characteristics. Accordingly, an interactive project is currently being developed focusing on the antioxidant, anticancer, and anti-inflammatory activities of several phenolic systems of both natural and synthetic origin, aiming at attaining a more reliable understanding of the structure-activity relationships (SAR´s) underlying their biological role [15,16]. The results obtained will hopefully lead to a better insight into the mechanisms of action of this kind of systems.

In the present study, the anticancer and anti-inflammatory properties of the synthetic hydroxycinnamic derivatives

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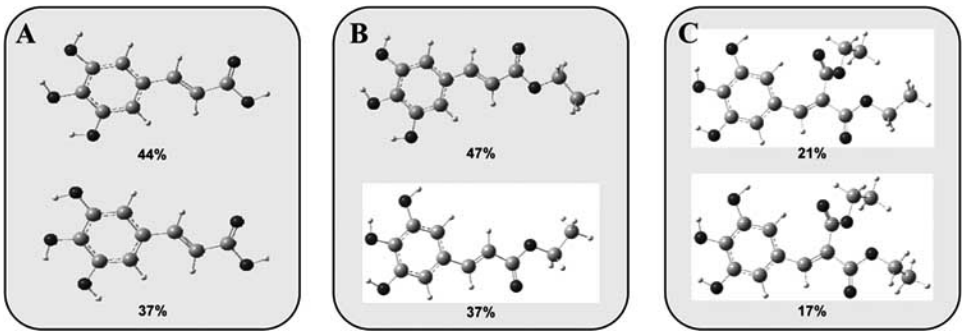


Fig. (1). Calculated (B3LYP/6-31G**) structures for compounds A (*trans*-3-(3,4,5-trihydroxyphenyl)-2-propenoic acid), B (*trans*-ethyl(3,4,5-trihydroxyphenyl)-2-propenoate) and C (diethyl 2-(3,4,5-trihydroxyphenylmethylene)malonate). For each compound, the two most stable conformers are represented, as well as their populations at room temperature.

trans-3-(3,4,5-trihydroxyphenyl)-2-propenoic acid (A), *trans*-ethyl(3,4,5-trihydroxyphenyl)-2-propenoate (B) and diethyl 2-(3,4,5-trihydroxy-phenylmethylene)malonate (C) (Fig. (**1**)) were screened. Anticancer properties were evaluated by measuring their cytotoxicity against cancer (mammary gland adenocarcinoma, MDA-MB-231) and non-neoplastic (skin fibroblasts, BJ) human cell lines. Furthermore their effect on COX-2 activity was assessed, using human recombinant COX-2, as an indicator of their anti-inflammatory properties. These studies were also complemented by the determination of their conformational preferences using theoretical methods (*ab initio* MO calculations).

MATERIALS AND METHODS

Reagents and other Conditions

All chemical reagents (*pro analysis*) were purchased from Merck (Lisbon, Portugal) and Aldrich (Sintra, Portugal). The MDA-MB-231 and BJ cell lines were obtained from the American Type Culture Collection (ATCC, USA) and from the European Collection of Cell Cultures (ECACC, United Kingdom), respectively.

Synthesis

The synthetic procedure followed for the preparation of the phenolic compounds presently investigated was an adaptation of the process reported by Hubner *et al.* [33].

Cytotoxicity Evaluation

Cytotoxicity evaluation after drug exposure – for drug concentrations between 50 and 100 μM – was performed using the Alamar blue colorimetric test [34,35]. This assay is based on the oxidation-reduction potential of the cells affected by the dye indicator in the medium, resulting in fluorescence and color changes.

The cells were exposure to the drugs after 24 hours of incubation (37°C; 5% CO_2). Then were then treated with Alamar Blue (10% v/v) and incubated for 4 hours, this procedure having been repeated every 24 hours (for different plates). The absorbance was measured at 570 nm ($A\lambda_1$, $A'\lambda_1$) and 600 nm ($A\lambda_2$, $A'\lambda_2$). Cell viability (Alamar Blue reduction) is expressed as a percentage and calculated according to the following equation:

$$\%\ \text{Reduced} = \frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2}{(\varepsilon_{RED})\lambda_1 A'\lambda_2 - (\varepsilon_{RED})\lambda_2 A'\lambda_1} \times 100 \quad (1)$$

ε_{ox} and ε_{RED} representing the molar absorptivity coeficients of the oxidised and reduced forms of Alamar blue, respectively, at 570 nm and 600 nm: $(\varepsilon_{ox})_{\lambda 1}$=80.856, $(\varepsilon_{RED})_{\lambda 1}$=155.677, $(\varepsilon_{ox})_{\lambda 2}$=117.216 and $(\varepsilon_{RED})_{\lambda 2}$=14.652.

In order to check whether the effect of the drug was reversible, this was removed and fresh culture medium was added to the cell culture after 3 days of drug exposure. Cell viability was evaluated after 4 days of incubation in the absence of drug.

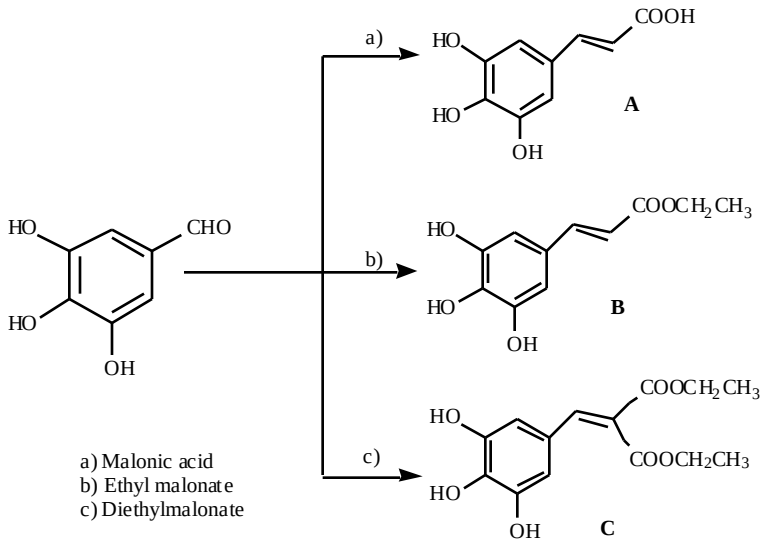
All experiments were performed in triplicate. The results are expressed as mean ± SD. Statistical analysis was performed using ANOVA, followed by *post hoc* test of Fisher´s Protected Least Significant Difference. Statistical comparison between the data was based on Pearson´s correlation coefficients and values lower than 0.05 were considered statistically significant.

Anti-Inflammatory Activity Determination

The human COX inhibitor screening assay (Cayman Chemical Company, Ann Harbor, MI, USA) was used to test the ability of the drugs to inhibit COX-2, in which PGF2α is directly measured after $SnCl_2$ reduction of COX-derived PGH2. The prostanoid product is quantified *via* enzyme immunoassay (EIA) using a broadly specific antibody that binds to all the major prostaglandin compounds [36].

Results are presented as the COX-2 inhibitory potential (% COX-2 inhibition) of the compounds in study (tested at the concentration of 100 μM, the highest concentration used in the cytotoxicity studies) in comparison to the standard COX inhibitor used (indomethacin [37]) at the same concentration. The amounts of prostaglandin PGF2α pg/ml) produced (in the presence and the absence of drugs) were determined by plotting the standard curve: ratios of the absorbance of a particular sample well to that of the maximum binding well (%B/B_0 : %Bound/Maximum Bound) against concentration of prostaglandin standards. A different standard curve was used for independent experiments.

To obtain the percentage (%) of COX-2 inhibition, each concentration of PGF2α obtained in the presence of drugs



Scheme 1.

(values obtained from the standard curve) was subtracted from the amount of PGF2α produced by COX-2 100% active (in the absence of drugs). This value was then divided by the amount of PGF2α obtained in the absence of drugs (COX-2 100% active) and multiplied by 100 to give the % of COX-2 inhibition.

Results are presented as mean ± SD of 2-3 independent experiments and were compared by one-way ANOVA followed by Dunnet´s test. P values lower than 0.05 were considered to indicate statistically significant differences.

RESULTS AND DISCUSSION

Chemistry

The phenolic compounds under study were obtained by a Knoevenagel type reaction ocurring between trihydroxy-benzaldehyde and malonic acid or its ester derivatives (Scheme **1**). The purification and characterisation of the synthesised compounds was carried out as previously described [38,39]. The geometries and relative energies of the distinct possible conformers of the phenolic compounds

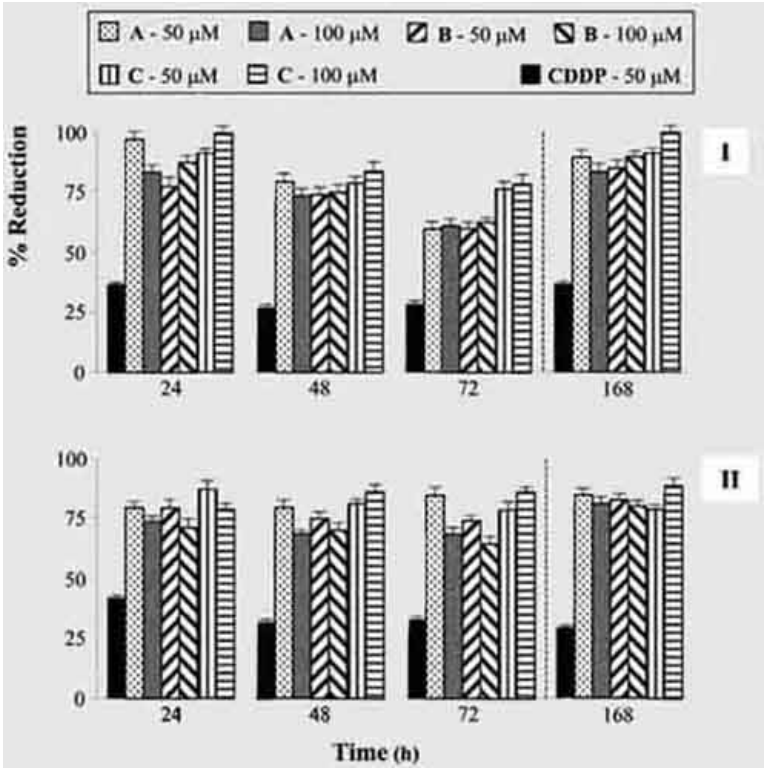


Fig. (2). Time and dose-dependence plots of the cytotoxic effect of compounds A, B and C against: I – mammary adenocarcinoma (MDA-MB-231); II – human skin fibroblasts (BJ). Cells (2,84 x 10^4 cells/ml and 1,25 x 10^4 cells/ml, respectively) were incubated with the drugs for periods of 24 to 72 h, and reversibility was assessed at 168 h. Data is expressed as percentage of the control Alamar reduction (100%) and represent mean ± SD from two independent experiments carried out in triplicate. P<0.05. (Cisplatin (CDDP) is included for comparison purposes).

were obtained, through *ab initio* MO calculations (Fig. (**1**)). The most stable conformers are completely planar and display an *S-cis* orientation of the terminal carbonyl group. The conformational preferences of these systems were found to be mainly ruled by the stabilising effect of π-electron delocalisation [40].

Cytotoxicity Evaluation

The cytotoxic effect of the phenolic compounds presently investigated towards the MDA-MB-231 and BJ human cell lines was evaluated – variation of cell viability as a function of the incubation time and drug concentration (between 50 and 100 μM). From these time- and dose-response plots (Fig. (**2**)) it is possible to relate the particular structural differences of these compounds to their antineoplastic activity, thus learning on factors such as specificity of action and reversibility of the drug effect. The hydroxycinnamic acid (A) and its linear ester (B) showed the highest cytotoxic effect against the cancer MDA-MB-231 line (down to a cell viability of *ca.* 60 %, Fig. (**2**) - I), followed by the diester counterpart (C). This viability decrease was determined to be significant only after 72 hours of incubation in the presence of these drugs, and it was found to recover back to 85 to 100% cell viability, upon removal of the phenolic agents (after 4 days in the absence of drug), thus evidencing high reversibility of their effect. In fibroblasts (non-neoplastic cells) however, none of the compounds tested displayed significant toxicity (Fig. (**2**) - II). Moreover, the cell viability was found to reach *ca.* 100% after removal of the compounds. As to the effect of drug concentration, the results clearly evidence that doses above 50 μM should be used in order to achieve a significant cytotoxic effect.

Anti-Inflammatory Activity Determination

The human COX inhibitor screening assay was used to test the ability of the synthesised phenolic compounds to inhibit COX-2. The assay is based on the fact that COX (also called prostaglandin H synthase or PGHS) catalyses the first step in the biosynthesis of PGs, tromboxans and prostacyclins; the conversion of arachidonic acid to PGH2 [25]. This assay directly measures PGF2α produced by $SnCl_2$ reduction of COX-derived PGH2. At the concentration studied (100 μM) two of the hydroxycinnamic derivatives (A and B) presented significant COX-2 inhibitory activity. Furthermore, at this concentration the compound A inhibited COX-2 similarly to the potent anti-inflammatory drug currently used as standard in numerous works (indomethacin [37]). The ester derivative (B), at this same concentration, inhibited COX-2 to a much lower extent and the diester (C) failed to inhibit COX-2 (see Table **1**).

Table 1. Effect of Indomethacin and Phenolic Compounds on Inhibition of COX-2

Drugs	COX-2 inhibition (%)
Indomethacin	95.7 ± 1.8* (8)
A	94.0 ± 2.3* (8)
B	76.6 ± 10.8*# (6)
C	10.8 ± 13.3# (4)

Results are mean ± SD (n); * P<0.01, from values obtained in the absence of drugs; # P < 0.01, from values obtained in the presence of indomethacin.

These are very promising results and compounds A and B seem to be potent COX-2 inhibitors, which can be related to their anti-inflammatory activities.

CONCLUDING REMARKS

Taken together, the preliminary results obtained in the present work showed that the free cinnamic acid (A) and its linear monoester (B), in opposition to the diester (C) counterpart, possess higher anticancer and anti-inflammatory activities. Compound A even seems to be equipotent to indomethacin, used normally as the standard drug at the same molar concentration. Interestingly, compounds A and B, that possess a pyrogallol moiety, also display remarkable antioxidant activity as previously shown throughout TRAP assays [38,41]. This observed antioxidant activity may be related to their anti-inflammatory capacity and to the ability to reduce carcinogenesis *via* cell protection against deleterious oxidative damage. The difference in the activity between the compounds A e B, could be related, at least in part, to lipophilicity variation as previously reported [38].

To note that none of the three compounds displayed a significant toxic effect against healthy cells, which can be of interesting if these compounds, should be used in chemopreventive strategies.

The results gathered along this and related works [15,16,41,42] clearly evidence that the antioxidant, anti-inflammatory and antineoplastic properties of this type of phenolic compounds seem to correlate to their structural characteristics, such as the number of phenolic hydroxyl groups and the presence of alkyl ester side chains (linear *vs* branched, saturated *vs* unsaturated). Furthermore, these properties do not seem to be independent: a plausible mechanism for the neoplastic properties of these compounds, observed in tumor cell lines, can be related to COX-2 inhibition as described to occur with non-steroidal anti-inflammatory drugs [31].

Theoretical (*ab initio*) calculations has also been found, throughout this type of studies, to be a valuable tool for an accurate interpretation of the experimental results as well as for achieving reliable SAR´s, capable of explaining the biological role of this kind of systems.

In conclusion, these preliminary results confirmed the importance of exploring the phenolic systems as safer templates to build new drug candidates, using rational design approaches, for anti-inflammatory/chemopreventive therapy.

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