In vitro Antitumour Activity of Orsellinates

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Lichen phenolic compounds exhibit antioxidant, antimicrobial, antiproliferative, and cytotoxic activities. The purpose of this study was to evaluate the anticancer activity of lecanoric acid, a secondary metabolite of the lichen *Parmotrema tinctorum*, and its derivatives, orsellinates, obtained by structural modification. A cytotoxicity assay was carried out *in vitro* with sulforhodamine B (SRB) using HEp-2 larynx carcinoma, MCF7 breast carcinoma, 786-0 kidney carcinoma, and B16-F10 murine melanoma cell lines, in addition to a normal (Vero) cell line in order to calculate the selectivity index of the compounds.

n-Butyl orsellinate was the most active compound, with IC_{50} values (the concentration that inhibits 50% of growth) ranging from 7.2 to 14.0 μg/mL, against all the cell lines tested. The compound was more active (IC_{50} = 11.4 μg/mL) against B16-F10 cells than was cisplatin (12.5 μg/mL). Conversely, lecanoric acid and methyl orsellinate were less active against all cell lines, having an IC_{50} value higher than 50 μg/mL. Ethyl orsellinate was more active against HEp-2 than against MCF7, 786-0, or B16-F10 cells. The same pattern was observed for *n*-propyl and *n*-butyl orsellinates. *n*-Pentyl orsellinate was less active than *n*-propyl or *n*-butyl orsellinates against HEp-2 cells. The orsellinate activity increased with chain elongation (from methyl to *n*-butyl), a likely consequence of an increase in lipophilicity. The results revealed that the structural modification of lecanoric acid increases the cytotoxic activity of the derivatives tested.

Key words: Orsellinates, Lecanoric Acid, Cytotoxic Activity

Introduction

Plants synthesize numerous secondary metabolites, including alkaloids, steroids, terpenoids, flavonoids, quinones, lignans, and anthocyanins, many of which have biological and/or pharmacological properties. Among these metabolites, phenolic compounds constitute a group with important biological and pharmacological activities, acting, for instance, as antimutagens, anticarcinogens, antipromoters, radical scavengers, antioxidants, antivirals, and antibacterials (Nandi *et al*., 2007; Stich, 1991; Selassie *et al*., 2005). On the other hand, phenolic compounds can also be mutagenic, carcinogenic, and promotory agents (Stich, 1991).

Lichens – symbiotic associations between a fungus and one or more algae – produce several classes of phenolic compounds, many of which are unique to these associations, although some are also found in nonlichenized fungi and higher plants (Nash, 1996; Hale, 1983). Phenolic compounds extracted from lichens exhibit a wide variety of biological actions, including antibiotic, antimycobacterial, antiviral, antiproliferative, and cytotoxic activities (Bézivin *et al*., 2003; Cohen *et al*., 1996; Ingólfsdóttir *et al*., 1985; Kumar and

Müller, 1999; Neamati *et al.*, 1997; Ögmundsdóttir *et al.*, 1998).

Given the growing number of cases of cancer, the investigation of potentially active compounds for the treatment of these patients is currently the focus of many research groups. Substances extracted from plants, lichens, and fungi have been evaluated for their antitumour activity. Although many compounds have shown activity against several cell lines, they are potentially toxic and of low solubility in biological media. Structural modifications of these compounds have been carried out with the purpose of improving their solubility and increasing their activity.

Our group has investigated lichens from the Cerrado biome of the state of Mato Grosso do Sul in Brazil, involving the isolation, structural elucidation, structural modification, and evaluation of biological activities of phenolic substances. Lecanoric acid (**1**), isolated from the lichen *Parmotrema tinctorum*, and orsellinates, obtained by reacting this acid with alcohols, were tested against *Artemia salina* and microorganisms and evaluated for their activity as free-radical scavengers (Gomes *et al.*, 2006; Lopes *et al.*, 2008).

The present paper reports the results of our investigation of the cytotoxic activity of lecanoric acid (**1**), methyl (**2**), ethyl (**3**), *n*-propyl (**4**), *n*-butyl (**5**), *n*-pentyl (**6**), *iso*-propyl (**7**), *sec*-butyl (**8**), and *tert*-butyl (**9**) orsellinates (Fig. 1) against HEp-2 larynx carcinoma, MCF7 breast carcinoma, 786-0 kidney carcinoma, and B16-F10 murine melanoma cell lines.

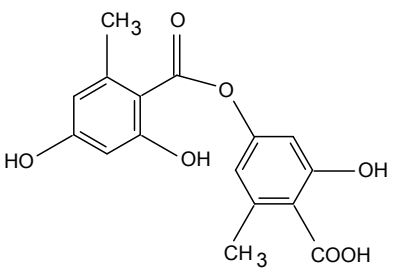
Experimental

Preparation of derivatives

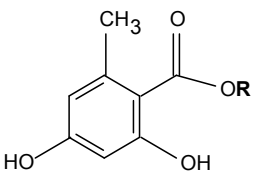
Lecanoric acid (**1**) was isolated and puri fied from *Parmotrema tinctorum* (Nyl.) Hale as proposed by Ahmann and Mathey (1967). The orsellinates **2**–**9** were prepared by reacting **1** (200 mg) with 50 mL of the corresponding alcohol at 40 °C in a steam bath. Once the reaction was completed, the mixture was concentrated and the compounds were separated by silica column chromatography using chloroform and chloroform/acetone gradients (Gomes *et al.*, 2002). All the reactions yielded the corresponding esters – 2,4-dihydroxy-6-methylbenzoates (orsellinates, **2**–**9**) – whose structures were confirmed by ^{1}H NMR, ^{13}C NMR, DEPT 135, and EI-MS analyses (Lopes *et al.*, 2008).

Cell lines

The cell lines utilized were: HEp-2 (ATCC CCL-23, larynx carcinoma) and Vero (ATCC CCL-81, African green monkey kidney), both purchased from Instituto Adolpho Lutz, São Paulo, Brazil; B16-F10 (ATCC CRL-6322, murine melanoma), donated by Dr. Auro Nomizo of the School of Pharmaceutical Sciences, Universidade de São Paulo, Ribeirão Preto, Brazil; and MCF7 (ATCC



Lecanoric acid (**1**)



Orsellinates

R = -CH_3 (**2**), -CH_2CH_3 (**3**),

-$CH_2CH_2CH_3$ (**4**), -$CH_2(CH_2)_2CH_3$ (**5**),

-$CH_2(CH_2)_3CH_3$ (**6**), -$CH(CH_3)_2$ (**7**),

-$CH(CH_3)CH_2CH_3$ (**8**), -$C(CH_3)_3$ (**9**)

Fig. 1. Compounds extracted, **1**, and derived, **2**–**9**, from the lichen *Parmotrema tinctorum*, collected in Brazil.

HTB-22, breast carcinoma) and 786-0 (ATCC CRL-1932, kidney carcinoma), both donated by Dr. João Ernesto de Carvalho of the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA), Universidade Estadual de Campinas (Unicamp), Campinas, Brazil.

Cell culture

HEp-2 and Vero cells were cultured in DMEM medium (Dulbecco's modified Eagle's medium), while the other cell lines were grown in RPMI-1640 medium (Roswell Park Memorial Institute) (purchased from Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% v/v fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. All reagents were obtained from Sigma. The cells were maintained at 37 °C with 5% CO_2. The medium was changed every 2 d until the cells reached 80% confluence.

Adherent cell lines were detached from the culture flasks by adding 0.5 mL 0.25% trypsin solution. Trypsin was inactivated by adding 9 mL of 10% FBS in RPMI-1640 medium. Single-cell suspensions were obtained by a gentle pipetting action. After counting, the cells were plated in 96-well microtiter plates (10,000–15,000 cells per well) with a fixed volume of 100 μL per well. The cell-containing microtiter plates were pre-incubated for 24 h at 37 °C to allow for stabilization, after which the test substances (100 μL) were added and the plates incubated for 48 h at 37 °C with 5% CO_2.

Solubilization and dilution of test substances

All compounds were solubilized in DMSO and tested at four dilutions (5 to 50 μg/mL). The final DMSO content was lower than 0.5% of the total volume. Each compound was tested in quadruplicate wells. Cisplatin, an anticancer drug, was used as positive control for all the cell lines tested.

Sulforhodamine B (SRB) assay

This assay relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue-culture plates by trichloroacetic acid (TCA). SRB is a bright-pink aminoxanthene dye with two sulfonic groups that bind to basic amino acid residues under acidic conditions and dissociate under basic conditions. The SRB assay was performed as described by Skehan *et al.* (1990). Briefly, the cells were fixed with 100 μL of ice-cold 20% TCA (Sigma) and incubated at 4 °C for 30 min. The supernatant was then discarded and the plates were washed five times with cold water. The cells were stained for 30 min with 0.1% SRB in 1% acetic acid (50 μL/well) (Sigma) and subsequently washed four times with 1% acetic acid to remove the unbound dye. The plates were air-dried and the protein-bound dye was solubilized with 100 μL 10 mM Trizma buffer (Sigma). The plates were shaken for 10 min on a shaker and the resulting optical density was read in a multiwell plate reader at 540 nm.

Data calculations

IC_{50} (the concentration that inhibits 50% of growth) values were calculated from the difference in concentrations between negative control and cells receiving the test compounds using a program for nonlinear regression. The data were subjected to two-way ANOVA, with a 95% confidence interval.

Results and Discussion

As shown in Table I, of all the compounds evaluated, *n*-butyl orsellinate (**5**) was the most active against HEp-2, MCF7, 786-0, and B16-F10 cells, with IC_{50} values of 7.2 μg/mL, 14.0 μg/mL, 12.6 μg/mL, and 11.4 μg/mL, respectively. Also, *n*-butyl orsellinate was more active than cisplatin against B16-F10 cells (IC_{50} = 12.5 μg/mL).

Ethyl orsellinate (**3**) was more active against HEp-2 than against MCF7, 786-0, or B16-F10 cells. The same pattern was observed for *n*-propyl (**4**) and *n*-butyl (**5**) orsellinates. *n*-Pentyl orsellinate (**6**) was less active against HEp-2 cells than were *n*-propyl (**4**) and *n*-butyl (**5**) orsellinates.

Lecanoric acid (**1**) and methyl orsellinate (**2**) were less active against any of the cell lines used, with IC_{50} values higher than 50 μg/mL.

In linear-chain orsellinates (methyl to *n*-pentyl), the activity increased from methyl to *n*-butyl against all cell lines. *n*-Pentyl orsellinate (**6**) was less active than *n*-butyl orsellinate (**5**). Of the ramified-chain esters, *sec*-butyl (**8**) and *tert*-butyl (**9**) orsellinates showed similar activity against HEp-2, MCF7, and B16-F10 cells and were more active than *iso*-propyl orsellinate (**7**). *tert*-Butyl orsellinate was more active than *iso*-propyl or

Table I. Antitumour activity (IC_{50} in μg/mL) and log *P* values of lecanoric acid and its structurally modified derivatives on four tumour cell lines.

Compound	HEp-2	MCF7	786-0	B16-F10	Vero	log *P**
Lecanoric acid (**1**)	>50	>50	>50	>50	>50	
Methyl orsellinate (**2**)	>50	>50	>50	>50	>50	2.38 ± 0.33
Ethyl orsellinate (**3**)	31.2 ± 1.4	70.3	47.5 ± 0.62	64.8 ± 4.0	28.1 ± 1.76	2.91 ± 0.33
n-Propyl orsellinate (**4**)	13.5 ± 0.9	23.5 ± 4.18	18.8 ± 1.68	33.4 ± 0.65	12.1 ± 0.14	3.44 ± 0.33
n-Butyl orsellinate (**5**)	7.2 ± 0.2	14.0 ± 0.035	12.6 ± 1.49	11.4 ± 3.89	18.9 ± 0.84	3.97 ± 0.33
n-Pentyl orsellinate (**6**)	17.3 ± 0.6	18.5 ± 0.58	14.3 ± 4.9	17.98 ± 0.05	47.3 ± 2.05	4.50 ± 0.33
iso-Propyl orsellinate (**7**)	34.9 ± 0.3	30.2 ± 4.73	22.0 ± 7.03	26.5 ± 2.86	15.0 ± 1.48	3.26 ± 0.33
sec-Butyl orsellinate (**8**)	8.9 ± 1.1	16.2 ± 0.098	20.6 ± 1.06	17.3 ± 0.11	26.3 ± 1.55	3.79 ± 0.33
tert-Butyl orsellinate (**9**)	10.2 ± 0.6	17.8 ± 2.33	14.9 ± 6.01	15.4 ± 1.94	2.84 ± 0.219	3.31 ± 0.33
Cisplatin (positive control)	1.8 ± 0.3	3.22 ± 1.33	1.4 ± 0.46	12.5 ± 2.79	2.84 ± 0.21	

* Values calculated with the ACD-Log*P* software (95% confidence interval).

sec-butyl orsellinates against 786-0 and B16-F10 cells.

The mechanism underlying the toxicity of most phenols is related to their lipophilicity, expressed by log *P* (Hansch *et al.*, 2000). When tested against HEp-2, the compounds with stronger activity, **5**, **8**, and **9**, showed higher log *P* values than less active substances like **2**, **3**, and **4**. The orsellinate activity increased with chain elongation (from methyl to *n*-butyl) and therefore with the increase in lipophilicity.

Toxicity, however, is not only related to lipophilicity, but also to electronic and steric parameters and pK_a values (Thakur *et al.*, 2004). Compounds **1**–**9** have pK_a values (C4-OH) of around 8.0 and are nearly 10% ionized at pH 7.0 (Gomes *et al.*, 2006). Higher pK_a values facilitate anion stabilization in the membrane phase, since the negative charge is more delocalized (Miyoshi *et al.*, 1990). According to Benz and McLaughlin (1983), anion mobility is critical, being much lower than that of neutral molecules. The mode of action of the phenolic OH moiety in chemical-biological interactions depends on the ultimate receptor where the reaction takes place. This may be promoted by either decreasing or increasing electron density on the phenolic ring system and/or simply by varying the hydrophobicity of the phenolic entity (Selassie *et al.*, 1998).

The selectivity index (SI, the ratio between the IC_{50} value of a compound tested against normal cells and its IC_{50} value against a given neoplastic cell line) was also calculated (data not shown), since it expresses the selectivity of a compound for cancer cell lines as opposed to normal cells (Houghton *et al.*, 2007). Three compounds – ethyl orsellinate (**3**), *n*-pentyl orsellinate (**6**), and *tert*-butyl orsellinate (**9**) – were most selective against HEp-2 cells. Cisplatin and *iso*-propyl orsellinate (**7**) were more selective against 786-0 cells (SI ≥ 2.0). No significant selectivity was found for the compounds tested against other cell lines.

Phenols may be classified into two main groups – namely electron-releasing phenols, which inhibit cell growth by electron-releasing substituents, and electron-attracting phenols, which inhibit cell growth by electron-attracting substituents. Biological activity data may vary, depending on the cell lines against which phenols are tested (Nandi *et al.*, 2007).

Since differences are found even in cells of the same histological type – as is the case with the high metastatic B16-F10 and low metastatic B16-F1 melanoma cell lines, which have markedly different membrane structures (Schroeder, 1984) – the features, and therefore the behaviour, of different cell lines can vary even more widely. This might partly explain the small differences observed in the action of the compounds tested in our experiments. On the other hand, membrane lipid bilayer fluidity plays a crucial role in signal transduction for a variety of biologically active molecules that activate the cellular function. For instance, membrane fluidity decreases in hepatomas, but increases in lymphomas and leukemias. Cancer cell plasma possibly contains antigens that become exposed when fluidity is changed – a feature that may affect the behaviour of compounds against cells and therefore interfere with the treatment of neoplastic diseases (Deliconstantinos, 1987).

Furthermore, genetic and morphophysiological differences in tumour cells hamper identi-

fication of the pharmacological action of new drugs against neoplastic tissues. Identification of agents with antitumour activity can be facilitated by screening them against a large number of tumours of different histological types (Von Hoff *et al.*, 1985).

According to Thompson *et al.* (1993), phenolic compounds may be easily oxidized via an initial phenoxy radical to a more reactive quinone methide, which can subsequently alkylate cell proteins and/or DNA to induce cytotoxicity. In orsellinates, however, cytotoxicity may not be based on this mechanism, since the OH groups at C2 and C4 and the methyl group at C6 are located at a *meta* position. The structural difference between compounds **2**–**9** lies in their alkyl chains, which are responsible for differences in lipophilicity. The larger the value of log *P*, the greater is the interaction of phenol with the lipidic phase (cell membrane). As log *P* approaches infinity, drug interaction with membrane lipids increases to the point of preventing the drug from crossing the aqueous phase, remaining at the first lipophilic phase with which it comes into contact. As log *P* approaches zero, the drug becomes that water-soluble that it will not cross the lipidic phase, remaining in the aqueous phase (Silverman, 1992). A balance between lipophilicity and hydrophilicity is therefore crucial for drug activity at the cell membrane. The orsellinates **2**–**5** showed increasing activity against all cell lines tested, but the activity was decreased in *n*-pentyl orsellinate (**6**). These findings revealed that the cytotoxic activity of orsellinates against all the cells tested increases from methyl (**2**) to *n*-butyl (**5**), *i.e.*, it increases to the limit of linear-chain elongation. Chain branching can also interfere with receptor binding (Silverman, 1992). Having a log *P* value of 3.26 ± 0.33, *iso*-propyl orsellinate (**7**) was less active than *n*-propyl orsellinate (**4**), with a log *P* value of 3.44 ± 0.33, against HEp-2, MCF7, and 786-0 cells, but more active than *n*-propyl orsellinate against cells of the B16-F10 line. Similarly, *sec*-butyl orsellinate (**8**) (log *P* = 3.79 ± 0.33) and *tert*-butyl orsellinate (**9**) (log *P* = 3.31 ± 0.33) were less active than *n*-butyl orsellinate (**5**) against all cells tested.

Our results revealed that the orsellinate activity increases with chain elongation (from methyl to *n*-butyl), likely a consequence of an increase in lipophilicity, and also demonstrated that the structural modifications of lecanoric acid increases the cytotoxic activity of its derivatives.

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