

In Vitro* Cytotoxicity of Some Natural and Semi-Synthetic Isocoumarins from *Paepalanthus bromelioides

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Numerous natural compounds have a potential for therapeutic applications, but may have to be chemically modified to alter toxic side effects. We investigated structural parameters that could affect the cytotoxicity of isocoumarins similar to 9,10-dihydroxy-5,7-dimethoxy-1*H*-naphtho(2,3*c*)pyran-1-one (paepalantine 1). Paepalantine 1 has antimicrobial activity, as well as significant *in vitro* cytotoxic effects in the McCoy cell line. Two other natural and two semi-synthetic isocoumarins with similar structures obtained from the capitula of *Paepalanthus bromelioides* were tested on the same cell line by the neutral red assay. Substitution of the 9 and/or 10-OH group made these compounds less cytotoxic.

Introduction

Numerous compounds with biological activity have been investigated, however many of them are not suitable for therapeutic use due to their toxic, carcinogenic and mutagenic properties. Nowadays, it is possible to make modifications of active chemical structures, in order to synthesize compounds with improved therapeutic activity and reduced toxicity.

Isocoumarins are secondary metabolites derived from the acetate pathway, and are structurally related to the coumarins, but with an inverted lactone ring (Hill, 1986). They are produced by microorganisms, insects and some higher plants, and have a wide range of biological activities, including: antitumoral, antileuemic, antiviral and antimicrobial (Hill, 1986; Cañedo *et al.*, 1996; Whyte *et al.*, 1996). Although some isocoumarins have beneficial properties, they also have been reported to be toxic for eukaryotic cells. An example is ochratoxin A, a potent mycotoxin produced by *Aspergillus* and *Penicillium* species, which is hepatotoxic, nephrotoxic, teratogenic and carcinogenic in animals (Zepnik *et al.*, 2001).

Some isocoumarins have been isolated from species of the plant family *Eriocaulaceae*. This family is widespread in the region of the Serra do

Cipó, Minas Gerais state, Brazil. *Paepalanthus* is the largest genus of this family, with about 500 species, and more than 400 of these occur only in Brazil. The first isocoumarin isolated from *P. bromelioides*, 9,10-dihydroxy-5,7-dimethoxy-1*H*-naphtho(2,3*c*)pyran-1-one, named paepalantine 1, was found to have antimicrobial activity, as well as significant cytotoxicity in McCoy cell line and in Chinese hamster ovary cells (CHO₉) (Varanda *et al.*, 1997). Two other natural isocoumarins, paepalantine-9-O-β-D-glucopyranoside and paepalantine-9-O-β-D-allopyranosyl(1→6) glucopyranoside were isolated from an ethanolic extract of this plant species, and their structure was determined by spectrometric analysis (Vilegas *et al.*, 1998). The 9-OH-10-methylated and 9,10-acylated derivative were semi-synthesized through methylation with diazomethane and acetylation with acetic anhydride/pyridine, respectively (Vilegas *et al.*, 1990).

Actually, *in vitro* cytotoxicity assays with cultured cells are widely used to chemicals including cancer chemotherapeutics, pharmaceuticals, biomaterials, natural toxins, antimicrobial agents and industrial chemicals because they are rapid and economical (Barile, 1994; Ishiyama *et al.*, 1996). These cytotoxicity tests measure the concentration of the substance that damages components, structures or cellular biochemical pathways, and they

also allow direct extrapolation of quantitative data to similar *in vivo* situations (Freshney, 1994; Balls and Clothier, 1992; Bacon *et al.*, 1990).

The main objective of this investigation was to explore the structure-cytotoxicity relationships of some natural and semi-synthetic isocoumarins using the microculture neutral red assay.

Materials and Methods

Test compounds

The substances were obtained as described by Vilegas *et al.*, 1990 and Vilegas *et al.*, 1997 (Table I) and they were stored as stock solutions at 2.0 mg/ml in DMSO. Eagle medium with various concentrations of DMSO was used in the experimental design. The final concentration of DMSO (4%) used does not interfere cell viability. The purity of the compounds was proved by HPLC using column chromatography on C₁₈ reversed phase and ACN-H₂O (70:30 v/v) as the eluting solvent.

Reference substance

Cisplatin, a cytotoxic substance used in antineoplastic therapy, was evaluated in the same cellular system to obtain cytotoxicity parameters, allowing us to classify the isocoumarins according to their relative toxicity.

Experimental design

McCoy mouse fibroblast cell line (ATCC CRL-1696b) was maintained in Eagle medium with 7.5% fetal bovine serum. After trypsinization, 0.2 ml aliquots of medium containing approximately 10⁴ cells/ml were seeded into 96-well tissue-culture plates and incubated at 37 °C. After 24 hr, the Eagle medium was removed and the cells were placed into unmodified medium (control) or in

medium modified with various concentrations of test chemicals. After incubating for another 24 hr, the medium was removed and the plates were prepared for the neutral red (NR) assay. After brief agitation, the plates were transferred to a microplate reader (Spectra and Rainbow (Shell) Readers – Tecan, Austria) and the optical density of each well was measured using a 540 nm filter and a 620 nm reference wavelength (Borenfreund and Puerner, 1985). All experiments were performed at least four times, using three wells for each concentration of each chemical tested. The cytotoxicity data was standardized by determining absorbance and calculating the correspondent chemical concentrations. Linear regression analysis with 95% confidence limit was used to define dose-response curves and to compute the concentration of chemical agents needed to reduce absorbance of the NR by 50% (IC₅₀), the so called cytotoxic midpoint (Barile, 1994).

Results and Discussion

The isocoumarins are extremely variable in structure, due to the various types of substitutions in their basic structure, which can influence their biological activity (Hill, 1986). Varanda *et al.* (1997) and Tavares *et al.* (1999) suggest that alterations in the chemical structure of paepalantine 1 could change its cytotoxic properties. Relative cytotoxicity of isocoumarins for the McCoy cell line was: paepalantine 1 and 9,10-acylated derivative > paepalantine 9-0-β-D-glucopyranoside > paepalantine 9-0-β-D-allopyranosyl(1→6)glucopyranoside and its 9-OH-10-methylated derivative (Table II).

The paepalantines analyzed in this study have different chemical groups at position 9 and/or 10 of the aromatic ring. It is noteworthy that substitu-

Table I. Chemical structures of natural and semi-synthetic isocoumarins.

Isocoumarins	R ₁	R ₂
Paepalantine 1	H	H
Paepalantine 9-0-β-D-glucopyranoside	glc	H
Paepalantine 9-0-β-D-allopyranosyl(1→6)glucopyranoside	-glc ^α all	H
9,10-Acylated derivative	Ac	Ac
9-OH-10-Methylated derivative	H	Me

Table II. Cytotoxicity of isocoumarins against McCoy cells using neutral red assay.

Isocoumarins	IC ₅₀ * [µg/ml]
Paepalantine 1	37.8 ± 1.3
Paepalantine 9-0-β-D-glucopyranoside	50.2 ± 7.2
Paepalantine 9-0-β-D-allopyranosyl-(1→6)glucopyranoside	52.1 ± 7.5
9,10-Acylated derivative	43.3 ± 1.3
9-OH-10-Methylated derivative	62.7 ± 4.1

* Values are mean ± sd; *n* ≥ 3.

tion of either the 9-OH (R₁) or the 10-OH group (R₂), and both 9,10-OH groups (R₁, R₂) in paepalantine 1 reduced cytotoxicity. All compounds gave a significant cytotoxic index when compared to the IC₅₀ value of cisplatin (41.9 ± 5.2 µg/ml) used as the reference compound.

For coumarins, generally the *in vitro* structure-activity relationship studies have shown that cytotoxicity is found only with derivatives containing *ortho*-dihydroxy substituents (Kolodziej *et al.*, 1997). The higher cytotoxicity of paepalantine 1 compared with the other isocoumarins in this study seems to be related to the OH-9 and OH-10 groups, since they provide a catecholic-like system.

The chemical structure/biological activity study of the coumarins showed that the addition of a catecholic group to the basic structure induces increased cytotoxic activity in tumor cell lines (Kolodziej *et al.*, 1997). Paepalantine 1 has hydroxyls at positions 9 and 10 of the aromatic ring, making it similar to a catecholic system. Based on our results, the substitutions of the free hydroxyls at positions 9 and/or 10 reduced the cytotoxicity of the isocoumarins.

The different cytotoxic values found for the isocoumarins could be related to presence and the positions of the hydroxyls in their structures. The groups found at positions 1, 9 and 10 of paepalantine 1 permitted the formation of two intramolecu-

lar hydrogen bridges, making this molecule more lipophilic than the other isocoumarins in this study. The substitution of the free hydroxyl at position 9 of paepalantine 1 by other chemical groups resulted in the formation of only one hydrogen bridge between positions 1 and 10, reducing the hydrophobicity of the new substances (paepalantine-9-O-β-D-glucopyranoside and paepalantine-9-O-β-D-allopyranosyl (1→6) glucopyranoside). Catecholic systems should also be capable of forming intramolecular hydrogen bridges. The 9-OH-10 methylated derivative has a substitution at position 10, which impedes formation of the intramolecular hydrogen bridge, and therefore, theoretically, would be less hydrophobic, when compared to paepalantine 1.

Observations concerning the lipophilic nature of these compounds were confirmed by comparative thin layer chromatography. Another aspect was the simultaneous substitution of the OH groups at position 9 and 10 (9,10-acylated derivative), which impedes the formation of intramolecular links. Nevertheless, chromatography shows that this derivative is slightly less lipophilic than paepalantine 1. Paepalantine-9-O-β-D-glucopyranoside and paepalantine-9-O-β-D-allopyranosyl (1→6) glucopyranoside are less lipophilic than paepalantine 1, which could be due to their glycolized radicals.

The isocoumarins we studied have diverse biological properties and various effects on the different cellular systems. Other biological parameters should be evaluated to increase our understanding of mechanisms by which these isocoumarins act.

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