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Structural modification of berberine alkaloids in relation to cytotoxic activity in vitro

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Abstract

The cytotoxicity of two protoberberine alkaloids: berberine and lincangenine, their 8-hydroxy-7,8-dihydro-derivatives and tetrahydroprotoberberine:thaicanine, was evaluated. The cellular responses through the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT) method were measured in Hela (uterus carcinoma), $SVKO_3$ (ovary carcinoma), Hep-2 (larynx carcinoma), primary culture from mouse embryon, and human fibroblast cells at the concentration: 10–1000 ppm (μg/ml) for 24 h. Berberine showed the highest cytotoxicity among the compounds tested, giving LC_{50} values for all cell lines at the concentration of 10 ppm. The results indicated that the cytotoxicity was notably decreased by structural changes, i.e. by modulation of the planarity caused by the introduction of hydroxyl group at C-8 and concomitant saturation of double bond between N-C8 in protoberberine molecules. In the case of berberine, the cytotoxic effect changed from 98.8 (berberine) to 39% for 8-hydroxydihydroberberine at the concentration of 100 ppm in Hela cells line. The same effect was observed with lincangenine and 8-OH-lincangenine (cytotoxicities 70 and 25%, respectively, at 1000 ppm in $SVKO_3$ cells). On the other hand, these compounds showed a low selectivity for the different human cancer cell lines tested.

Keywords: Cytotoxicity; Cell growth; MTT; Human tumor cells; Berberine alkaloids

1. Introduction

In vitro model systems with human cells are increasingly used to investigate mechanisms of chemical-induced toxicity and for toxicity screening of new drug families (Carmichael et al., 1987; Bata et al., 1995; Dolara et al., 1995).

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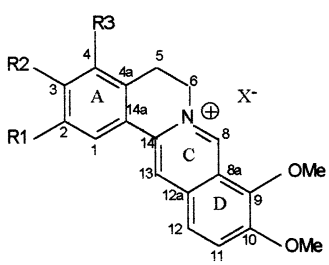
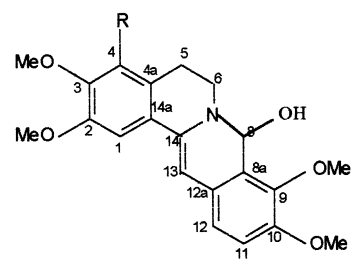
Cell culture systems are generally devoid of many of the variables inherent in whole animal studies, such as: blood flow, hormonal factors, and nervous system controls. The use of human and rodent culture cells (Bata et al., 1995), allows the maintenance of a uniform chemical and physical environment. Cells in culture, have a high creased accessibility to chemicals, placed in the culture medium, and are useful for studies with more accurate xenobiotic dosing concentrations, and durations of exposure (Acosta et al., 1985; Melzig and Dienwiebel, 1990; Koulman et al., 1996).

As a part of bioactivity-directed phytochemical studies on annonaceous plants from Amazonian regions of Venezuela, some protoberberine alkaloids were isolated from *Guatteria schomburgkiana* Mart. (*G. sessilis* R.E. Fries).). Two alkaloids lincangenine and thaicanine have been isolated from other plant sources (Ruangrungsi et al., 1986; Yan et al., 1991).

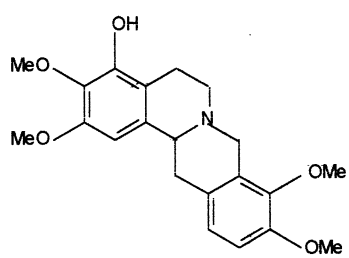
Regarding the biological activities, the protoberberine coralyne and its derivatives have been investigated as antitumor agents (Zee-Chang et al., 1974; Zee-Cheng and Cheng, 1976), and DNA topoisomerase I was identified as the cytotoxic target of these alkaloids (Gatto et al., 1996; Pilch et al., 1997). Also, antileukemic activity of berberine was discussed on the basis of its DNA-binding action mechanism (Cushman and Dekow, 1979; Kuo et al., 1995). Besides the works above mentioned, there are relatively few investigations reported about the antitumor activity of protoberberine alkaloids. In this paper, the cytotoxic activity of lincangenine, and berberine analogue, with commercial berberine sulfate, and their 8-hydroxydihydro derivatives and thaicanine (tetrahydrolincangenine) were evaluated, using human cancer cell lines.



1 R1=R2= OMe, R3 = OH, X= Cl -, lincangenine
3 R1=R2= OCH_2O, R3=H X= SO_4-, berberine



2 R1=R2= OMe, R3 = OH, X= Cl -, 8-hydroxilincangenine
4 R1=R2= OCH_2O, R3=H X= SO_4-, 8-hydroxiberberine



5 Thaicanine

Fig. 1. Berberine alkaloids.

2. Materials and methods

2.1. Drugs (Fig. 1)

2.1.1. Lincangenine **1**, (MW 368g)

(4-hydroxy-2,3,9,10-tetramethoxyprotoberberine): proto berberine alkaloid isolated from the leaves of *G. schomburgkiana* Mart. (*G. sessilis* R.E. Fries) of the family Annonaceae, identified by comparison of their spectral data with those reported in the literature (Yan et al., 1991).

2.1.2. 8-Hydroxydihydrolincangenine **2**, (MW 385g)

(4-hydroxy-2,3,9,10-tetra methoxy dihydro proto berberine): was obtained by treatment with 2 mg of lincangenine with NaOH, and then identified by spectroscopic data.

2.1.3. Berberine **3**, (MW 336g)

(2,3-methylenedioxy-9,10-dimethoxyprotoberberine) sulfate: commercially obtained from Merck.

2.1.4. 8-hydroxydihydroberberine **4**, (MW 353g)

derived from berberine sulfate: was obtained by treatment with 2 mg of berberine with NaOH, and then identified by spectroscopic data.

2.1.5. Thaicanine **5**, (MW 371g)

(tetrahydrolincangenine) (4-hidroxy-2,3,9,10-tetrametoxy-tetrahydroprotoberberine): isolated from the leaves of *G. schomburgkiana* Mart. identified by comparison of their spectral data with those reported in the literature (Ruangrungsi et al., 1986).

The purity of the isolated alkaloids and related compounds was confirmed by their spectroscopic data.

The plant was collected and identified by Dr Anibal Castillo, botanist of the Instituto de Biologia Experimental, Facultad de Ciencias, Universidad Central de Venezuela. A voucher specimen *G. schomburgkiana* Mart. (*G. sessilis* R.E. Fries) has been deposited in Herbario Nacional de Venezuela (VEN), Caracas Botanical Garden (No. 293.530).

2.1.6. Extraction and isolation of lincangenine and thaicanine

The dried and pulverized leaves (2.4 kg) were percolated with 5% HCl at room temperature. The acidic aqueous solution was basified with 10% NH_4OH and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with water, dried with anhyd. $MgSO_4$, and evaporated to dryness to yield 5.93 g of crude alkaloidal fraction, representing 0.25% of dry plant material. This alkaloidal fraction was chromatographed on a silica gel column using $CHCl_3$ and $CHCl_3$–MeOH mixtures with increasing polarity to yield 37 fractions. The intermediate fractions (6–13, 4.13 g), positive to Dragendorff's reagent, were combined and passed through a Sephadex LH-20 column using MeOH as eluent to give six collective fractions A–F. Three alkaloid-positive fractions D, E, and F were combined and subjected to silica gel column chromatography initiating elution with $CHCl_3$ and increasing polarity by addition of MeOH and NH_4OH. Final separations of the less complicated fractions by repeated preparative layer chromatography on silica gel, developing with the mixture of $CHCl_3$–MeOH–NH_4OH (50:10:1), enabled one to isolate two following alkaloids: lincangenine (**1**, 500 mg), and thaicanine (**3**, 250 mg).

2.2. Cell culture

Different human cancer cell lines were used: Hela (human uterus carcinoma), $SVKO_3$ (human ovary carcinoma), Hep-2 (human larynx carcinoma), Fadu (human pharynx carcinoma) and primary culture from mouse embryon and human fibroblast. The cell line used in this study was kindly provided by Dr M.F. Poupon (Institut Curie, Paris-France). Growth medium was Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin and 50 μg/ml of gentamycine in an atmosphere of 5% CO_2.

Table 1
Cytotoxicity evaluation (LC_{50}) of isoquinoline alkaloids in normal and tumor cells (ppm)[a]

Cells type	Lincangenine		8-Hydroxy-dihydro-lincangenine		Berberine		8-Hydroxy-dihydro-berberine		Thaicanine	
	ppm	μM	ppm	μM	ppm	μM	ppm	μM	ppm	μM
SVKO3	712	1.93	1912	4.97	<10	<0.03	641	1.82	<100	<0.27
Fadu	834	2.27	931	2.42	<10	<0.03	<10	<0.03	500	1.35
Hep2	924	2.51	2003	5.20	10	0.03	650	1.84	<100	<1.35
Human fibroblast	<10	<0.03	<10	<0.03	<10	<0.03	<10	<0.03	<10	<0.03
Mouse fibroblast	483	1.31	NE	NE	<10	<0.03	NE	NE	<100	<0.27
Hela	1418	3.85	2519	6.54	10	0.03	340	0.96	<100	<0.27

[a] NE = not evaluated; ppm = μg/ml.

2.3. Cytotoxicity evaluation

In order to evaluate the in vitro cytotoxic activity, lincangenine, 8-hydroxidihydrolincangenine, berberine, 8-hydroxidihydroberberine and thaicanine, were assayed in six human cancer cells lines. Individual wells of a tissue culture 96-well microtiter plate were inoculated with 0.2 ml of medium (DMEM + 10% FBS) containing sufficient cells to provide approximately 70% confluence after 24–48h of incubation. After 24 h of exposure to the drug at different concentrations (10–100–1000 ppm), these cells were used for the cytotoxicity evaluation.

The cytotoxicity assays were performed with the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT) method, using tetrazolium salt (Mosman, 1983; Denizot and Lang, 1986; Rahn et al., 1991; Vistica et al., 1991) This is a colorimetric assay, based on the ability of live but not dead tumor cells, to reduce a tetrazolium based compound (MTT) to a bleu formazan product (Carmichael et al., 1987). Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzyme. Cells in microtiter plate were incubated (37°C) with the MTT dye for 3 h, washed with phosphate buffered saline (PBS), and then, the formazan was dissolved in DMSO. The absorbance was recorded in a microplate reader (Cambridge Technology) at the wavelength of 570 nm. The LC_{50} value was defined as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells in the MTT assay.

2.4. Statistic

Results are expressed as mean ± S.D. Student's *t*-test was used to compare means from the different treatment and the control and analysis of variance (ANOVA) were followed by the Newman–Keuls test. Differences at the 5% level or less were considered as statistically significant.

3. Results and discussion

The cytotoxicity of two series of protoberberine alkaloids: (1) lincangenine, 8-hydroxydihydrolincangenine, and thaicanine (tetrahydrolincangenine); and (2) berberine and 8-hydroxydihydroberberine, was evaluated in six cell lines: Hela (human uterus carcinoma), $SVKO_3$ (human ovary carcinoma), Hep-2 (human larynx carcinoma), Fadu (human pharynx carcinoma) and primary culture from mouse embryon and human fibroblast.

The cytotoxic activity is expressed in terms of relative absorbance of drug-treated cells, in comparison to control cells. It was necessary to use high concentrations as up to the limit of solubility of the compounds, to obtain significant cytotoxic values. The duration of the treatment was 24 h.

The results are presented in Table 1 (LC_{50} at 24 h) and Figs. 2–6. The results show a dose-dependent cytotoxic effect relationship for all the alkaloids and all the cell lines evaluated.

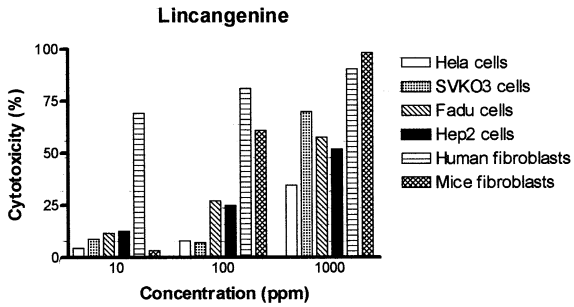


Fig. 2. Cytotoxic effect (%) of lincangenine in tumoral and normal cells. The cytotoxic levels varied with the cells type. Lincangenine as much as important cytotoxicity effect in human fibroblast, but is less important in mouse fibroblast (10 ppm) and Hela cells (all concentrations).

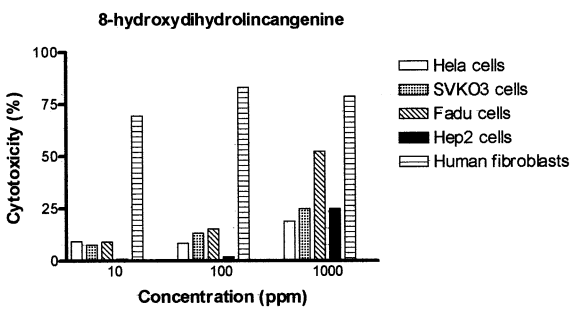


Fig. 3. Cytotoxic effect (%) after introduction of hydroxyl group in the lincangenine (8-hydroxydihydrolincangenine) molecule in tumoral and normal human cells.

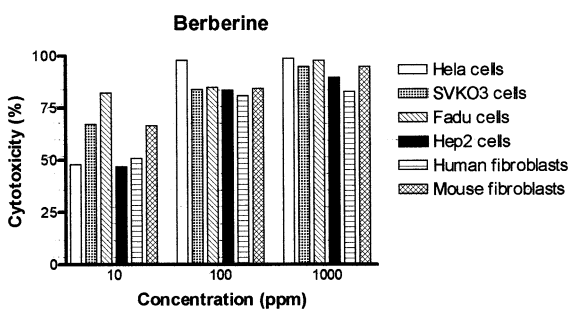


Fig. 4. Cytotoxic effect (%) of berberine in tumoral and normal human cells. The cytotoxic level is very important with all cells types.

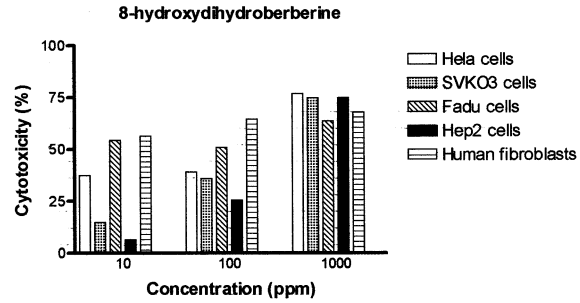


Fig. 5. Cytotoxic effect (%) after introduction of hydroxyl group in the berberine (8-hydroxydihydroberberine) molecule in tumoral and normal human cells.

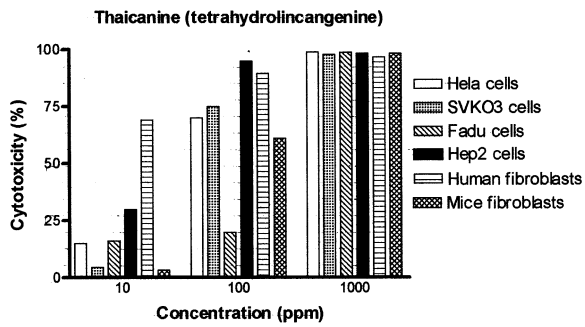


Fig. 6. Cytotoxic effect (%) of thaicanine in tumoral and normal human cells. The cytotoxic levels are very important after 100 ppm with exception of Fadu cells.

Fig. 2 shows the % cytotoxicity results obtained from the three concentrations of lincangenine with respect to the control. These results indicate that the cytotoxicity is roughly proportional to the dose, for all types of tumoral cells, and mouse normal fibroblast, neverthless lincangenine is very toxic for the human normal cells.

Fig. 3 shows the results obtained from 8-hydroxydihydrolincangenine. The cytotoxic effect is less for all cell types in comparison with lincangenine, except for human fibroblast cells. The cytotoxic effect of berberine is shown in Fig. 4. At any concentration in the study berberine is very toxic for all cell types. As shown in Fig. 5, 8-hydroxydihydroberberine is less toxic than berberine for almost all cell types studied, but it is more toxic for normal human fibroblast at 10 ppm. Fig. 6 shows the results obtained from thaicanine (tetrahydrolincangenine). The cytotoxic effect had

high values at all concentrations and against all cell types, except for Fadu cells at the concentration of 100 ppm.

The cytotoxicity of the two protoberberine alkaloids (lincangenine and berberine) have been compared with those of their 8-OH-dihydro derivatives, and tetrahydroprotoberberine (thaicanine) in vitro, to establish a relationship between the chemical structure changes of protoberberines to 8-OH-dihydro derivatives and their cytotoxic effects on tumor and normal cells. Under the experimental conditions, the different compounds evaluated showed a selective cytotoxicity against several human tumor cells. The lowest concentration (10 ppm) of lincangenine and 8-OH-lincangenine showed low toxicity compared with the control. However, berberine was highly toxic even at the concentration of 10 ppm. The LC_{50} at 24 h is shown in Table 1. The order of cytotoxic effects of the evaluated compounds is: berberine > 8-OH-dihydroberberine > thaicanine > lincangenine > 8-OH-dihydrolincangenine.

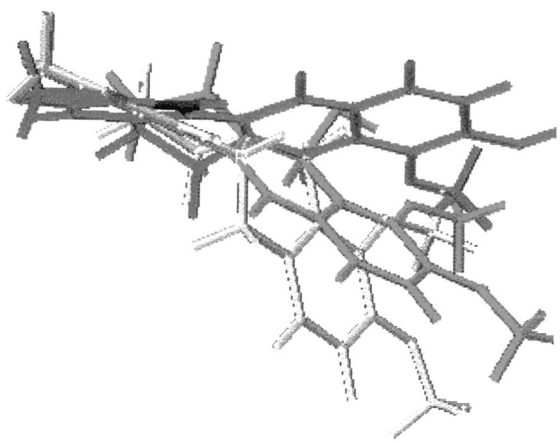


Fig. 7. Berberine alkaloids. Green, protoberberine alkaloids; purpure, 8-hydroxiderivates; and yellow, tetraprotoberberine alkaloids.

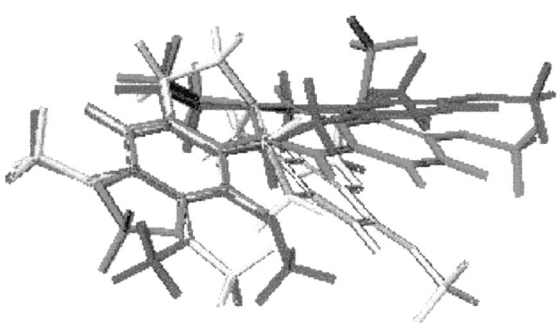


Fig. 8. Berberine alkaloids. Molecular shape and perpendicularity for protoberberine (red), 8-hydroxiderivates (green) and tetrahydroprotoberberine alkaloids (yellow).

All compounds demonstrated significant cytotoxicity in normal human cells. Comparing berberine and lincangenine, berberine is much more toxic than lincangenine against all the cell lines, except human fibroblast (Figs. 2 and 4). Structural difference of these compounds resides in ring A, instead of one methylenedioxy group at C-2 and -3 in berberine, lincangenine has two methoxy groups at these positions and one hydroxy group at C-4 (Fig. 1). Molecular modeling on protoberberine alkaloids, shows that lone pair electron orbitals of oxygen atoms of methylenedioxy in berberine, are perpendicular to this aromatic ring A (Figs. 1, 7 and 8), while vicinal methoxy groups at the same positions are some out of the plane of ring A to avoid steric compression. This molecular shape difference and perpendicularity of oxygen atom orbitals around the ring A, may be in relation to intercalative ability of these alkaloids to bind to duplex DNA in preference of berberine to lincangenine. The introduction of the hydroxyl group in the ring C of lincangenine and berberine molecules, resulted in decreasing of cytotoxic effect in human tumor cells, but not in normal human cells. The introduction of the hydroxy group at C-8 in aromatic C-ring shows a modulating effect, because the LC_{50} has remarkably decreased more than 50%. In the case of berberine, the cytotoxic effect changes from 98.8 to 39% at 100 ppm against Hela cell lines. Similarly, the cytotoxic effect of lincangenine changes from 70 to 25% in 8-OH-dihydrolincangenine at 1000 ppm against $SVKO_3$ cells. The difference of cytotoxicity could be analyzed from the following chemical structure changes: (1) positively charged quaternary nitrogen atom (in berberine and lincangenine) to unshared electron-bearing tertiary one; (2) saturation of double bond between N-7 and C-8 and consequent change into molecular planarity; (3) introduction of hydroxy group on C-8. Rela-

tively high toxicity of thaicanine could be explained by its *trans*-quinolizidine planar conformation (Cushman and Dekow, 1979), this is evidenced by infrared and proton magnetic resonance spectroscopic data of thaicanine. On the basis of these results, the semiplanar structure of 8-OH-dihydroprotoberberines could be responsible for the modulation of the cytotoxic activity of these compounds. Lincangenine, 8-OH-dihydrolincangenine, berberine, 8-OH-dihydroberberine and thaicanine were inhibitors in growing normal and tumoral cells at concentrations below LC_{50}; the same as the cytotoxic effect, the growth inhibition against tumoral cells, was more remarkable for protoberberines in comparison with their 8-OH-dihydro derivatives (Unpublished results). These conclusions are in accordance with Zee-Chang et al. (1974). These authors argued that antileukemic activity against leukemia L 1210 and P 388 in mice showed changes in the structure of coralyne and related compounds. The structural requirements concerning antitumoral activity are in connection with the planarity and rigidity of molecules, alkyl substituting on C-8, as well as the presence of methoxy or methylenedioxy groups on A and D rings. Cushman and Dekow (1979) pointed out the importance of *trans*-quinolizidine conformation of tetrahydroprotoberberine over *cis*-conformation from the point of view of DNA-binding properties of the compounds.

In conclusion, the compounds tested showed significant cytotoxic activity against different human tumor cell lines, but were also very toxic for normal human cells. Similar results were observed about cell growth inhibition for both normal and tumoral cells.

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