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In vitro cytotoxicity of berberine against HeLa and L1210 cancer cell lines

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Received February 25, 2003, accepted September 5, 2003

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Pharmazie 59: 548–551 (2004)

Previous studies on anti-cancer activity of protoberberine alkaloids against a variety of cancer cell lines were extended to human uterus HeLa nad murine leukemia L1210 cell lines. Cytotoxicity was measured using *in vitro* techniques and cell morphology changes were examined by light microscopy in both cytostatic and cytocidal concentration ranges. The IC_{50} was found to be less than 4 μg/ml, a limit put forward by NCI for classification of the compound as a potential anti-cancer drug. The microscopy examination indicated that at cytocidal concentrations the HeLa and L120 cells died apoptotically. The comparative analysis revealed that berberine belongs to the camptothecin family of drugs characterized by the ability to induce DNA topoisomerase poisoning and hence apoptotic cell death. Although the cytotoxic potency of berberine was found to be several orders of magnitude lower compared to camptothecin, its significance may increase in future in view of the lack of unwanted side effects characteristic for camptothecin compounds currently in clinical use for treatment of cancer.

1. Introduction

Protoberberine alkaloids, such as berberine, constitute an important class of natural products, which have been used for many generations in Europe and Far Eastern countries as an antibiotic and to treat patients with gastrointestinal disorders. Berberine displays a wide range of biochemical and pharmacological actions and is relatively nontoxic to man; its antimicrobial activity has been demonstrated against many bacterial and fungal species (Amin et al. 1969; Okunade et al. 1994; Iwasa et al. 1997; Sarma et al. 1999). The drug was subsequently screened for anti-cancer activity following evidence of antineoplastic properties (Kuo et al. 1995; Anis et al. 1999; Anis et al. 2001). It has also been shown that berberine exhibits the ability to induce apoptosis in promyelocytic leukemia HL-60 and 3T3 fibroblast cells (Kuo et al. 1995; Yang et al. 1996). In addition, some protoberberines are highly effective as cytotoxic agents against several carcinoma such as HeLa, SVKO (Iwasa et al. 1997), Hep-2, primary culture from mouse embryos and human fibroblast cells (Orfila et al. 2000; Sanders et al. 1998); berberine showed consistently the highest cytotoxicity among the alkaloids tested. Very recently, the cytotoxicity of 24 protoberberines related to berberine was evaluated against a panel of 38 human cancer cell lines coupled with a drug sensitivity database (Iwasa et al. 2001). The results showed that the 50% inhibitory concentration of berberine varies within the range 1–10 μg/ml; the limit of 1 μg/ml can be overcome by introducing lipophilic substituents to the positions 9 and 13 of the isoquinoline nucleus, obviously due to improvement of the membrane permeability of the drug. It has also been reported that berberine possesses a dual topoisomerase I and II poisoning activity (Kobayashi et al. 1995; Li et al. 2000; Krishnan and Bastow 2000) and this was linked to its cytotoxic activity. Recently, we demonstrated that berberine elicited, at a very low dose (0.03 μg/ml), 90% suppresion of acridine orange-induced plastid mutagenicity in the *Euglena gracilis* test model (Čerňáková et al. 2002). In this study, two different techniques, viz. a (short-term) primary biochemical screening of Oyama and Eagle (1956) and a cell population counting assay, were employed to two distinct cancer cell lines to study the cytotoxic potential of berberine.

2. Investigations, results and discussion

First, a primary biochemical screening test (Oyama and Eagle 1956; Lowry et al 1951), based on inhibition of the total content of cell proteins, was applied to the two cancer cell lines. Following the 48 h treatment of either HeLa or L1210 cells with berberine, protein synthesis was inhibited in a dose-dependent manner (results not shown); computer-assisted analysis of the toxicity curve (a plot of percent inhibition of protein synthesis vs. drug concentration) showed that the data could best be fitted by a linear regression model, the corresponding inhibitory concentration, IC_{50} and IC_{100}, are shown in the Table.

Encouraged by this initial result, we examined the cytotoxicity of berberine more closely, namely in an *in vitro* assay, based on counting cell population of two different tumour cell lines treated at various times (24, 48 and 72 h) with berberine at concentrations ranging from 0.1 to 150 μg/ml. The resultant growth curves for the HeLa and L1210 cells are displayed in Figs. 1 and 2, respectively. A plot of percent growth inhibition vs. berberine concentra-

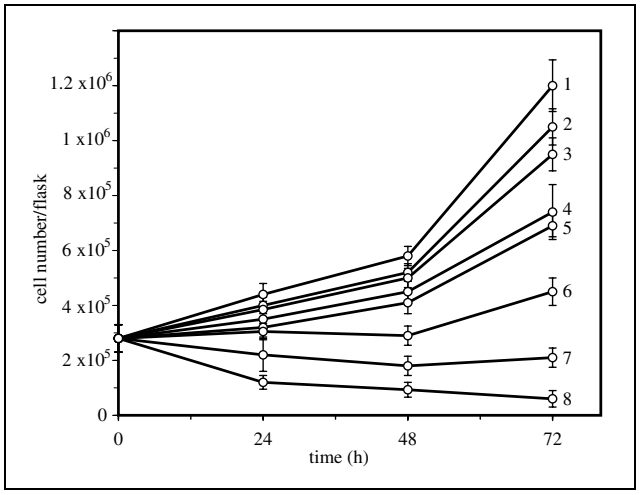

Fig. 1: Growth curve of the HeLa cell line, affected by berberine chloride in the course of 72 h. Concentration of berberine chloride (μg/ml): **1**−control, **2**−0.1, **3**−1, **4**−5, **5**−10, **6**−50, **7**−100, **8**−150

tion for both HeLa and L1210 cells showed an exponential relation (data not shown); the IC_{50} and IC_{100} values for the two cell lines are also given in the Table. As can be seen from the Table, the the IC_{50} and IC_{100} values determined by the two techniques for the 48 h exposure of the HeLa cells to berberine agree well to each other. Recently, the National Cancer Institute (NCI) (Pisha et al. 1995) has recommended that if the IC_{50} value is less than 4 μg/ml, then the compound can be considered as having a cytotoxic effect and hence can be classified as a potential anticancer-drug. As shown in the Table, the potency of berberine corresponds to the borderline of this criterion, with IC_{50} values being slightly higher or lower than the 4 μg/ml limit. Moreover, a comparison of the IC_{50} values (Table) for the two cell lines confirms the previous observation (Orfila et al. 2000; Sanders et al. 1998; Iwasa et al. 2001) that berberine can be regarded as a non-selective anti-cancer agent.

The microscopy examination of the berberine-treated HeLa and L1210 cells has shown that at cytostatic concentrations (below the IC_{100}) the cells are morphologically identical while in the cytocidal concentration range ($> IC_{100}$) the number of cells displaying morphological features typical for apoptosis (blebbing the plasma membrane and reduc-

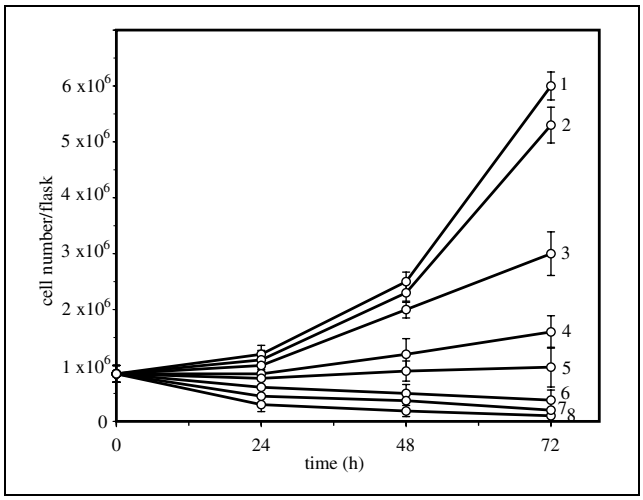

Fig. 2: Growth curve of the L1210 cell line, affected by berberine chloride in the course of 72 h. Concentration of berberine chloride (μg/ml): **1**−control, **2**−0.1, **3**−1, **4**−5, **5**−10, **6**−50, **7**−100, **8**−150

Table: Inhibitory concentrations, IC_{50} and IC_{100} (μg/ml) of berberine chloride which cause, respectively, 50 and 100% reduction of protein synthesis or cell population for the HeLa and L1210 cell lines

Time (h)	HeLa cells				L1210 cells			
	Protein inh. assay		Cell count assay		Protein inh. assay		Cell count assay	
	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}
24			6.1	68.2			2.7	16.5
48	4.8	58.5	7.2	62.5	2.8	32.2	3.5	26.8
72			4.8	74.6			1.0	27.2

The values represent means of 3 independent experiments

tion in cell size) proportionally increased with the time of exposure and the berberine concentration (data not shown). It has been reported (Yang et al. 1996) that treatment of Balb/c 3T3 cells with a low dose (∼100 μg/ml) of berberine causes arrest of the cell cycle in the G_2 phase while higher concentrations (ca. 200 μg/ml) lead to induction of apoptosis. Emerging evidence suggests that different cell types have varying apoptosis threshold; oncogenic transformation generally shifts the threshold to lower concentration levels which makes the tumour cells more sensitive to induction of apoptosis. As shown in the Table, our results provide further evidence of this trend; the threshold (IC_{100}) for berberine-induced apoptosis in the HeLa cell population is well below 100 μg/ml and approaches the 10 μg/ml limit for the leukemia L1210 cells.

Recently, we have initiated a comparative analysis of protoberberine alkaloids and other anticancer drugs so far reported in the literature in order to find a relationship between structure, molecular mechanisms of action (mode of binding to DNA) and cytotoxic activity. The analysis has revealed that protoberberines belong to the camptothecin family of compounds which exhibit antineoplastic acitivity against a broad spectrum of both ascites and solid tumours and some of them are currently in clinical use for the treatment of a variety of cancers (Pantazis et al. 1996); the cytotoxic activity of these drugs results from DNA-topoisomerase I (TOPO/I) poisoning, i.e. the ability to stabilize the ternary complex DNA-TOPO/I – drug in its cleavable state (Li et al. 2000; Pommier et al. 1995). It is well documented (Pommier et al. 1995) that camptothecin derivatives bind to the TOPO/I-DNA interface of the binary complex DNA-TOPO/I; accordingly, the structure of the TOPO/I inhibitors shares common features, viz. a planar (aromatic) polycyclic system able to intercalate between the base pairs of the DNA duplex and a less planar (or non-planar) portion localized in the minor groove and hence accessible to interactions with the enzyme. In the case of the berberine analogues it was found that the planar system of rings C and D is involved in the intercalative interaction with the DNA whereas rings A and B protrude out of the helix interior into the minor groove where they interact with TOPO/I (Li et al. 1995).

Among the camptothecin family of drugs a close correlation is discernible between the antineoplastic activity and the TOPO/I inhibitory potency (Keskin et al. 2000), especially the strength of the intercalative binding component which was shown to make a major contribution to the overall affinity (potency) of the drug (Chaires 1997). In general, tumour cells typically respond to the TOPO/I – induced DNA damage (fragmentation) by G_2 cell cycle arrest and finally by apoptosis, depending on the time of exposure and concentration of the drug.

The success of captothecin and its derivatives as anticancer drugs has spurred a search for additional agents acting by inhibition of TOPO/I; natural sources have also been extensively investigated in this respect. However, the cytotoxic potency of berberine analogues is 2–3 orders of magnitude lower relative to camptothecin, most likely due to weaker intercalative binding. Thus, to enhance the intercalative component of binding (e.g. by extension of the chromophore) would be desirable in order to increase the cytotoxic potency and hence the significance of the protoberberines as potential anti-cancer drugs.

In conclusion, from the results obtained in this study and previous work it can be concluded that although berberine, based on the NCI criteria, has sufficient cytotoxic potency to be classified as a potential anti-cancer drug, it does not offer any advantage over camptothecin and other TOPO/I poisons currently in clinical practise except if a markedly reduced cardiotoxicity was displayed following further biological investigations. The perspective is certainly not hopeless since the clinical use of camptothecin like anticancer drugs is seriously hampered by a number of problems, particularly a dose-related and irreversible cardiotoxicity, which is lacking for the berberine analogues even at high doses.

3. Experimental

3.1. Drug

Berberine (2,3-methylenedioxy-9,10-dimethoxyprotoberberine) chloride, obtained commercially from Merck, was pre-dissolved in DMSO and diluted with the medium so that its final concentration in the experiments did not exceed 2% (v/v). Cytotoxic activity was studied at seven concentrations, viz. 0.1, 1, 5, 10, 50, 100, and 150 μg/ml.

3.2. Cell lines

The human transformed cell line HeLa and murine leukemia cell line L1210 (both obtained from American Type Culture Collection, Rockville, MD, USA) were used in our experiments. The cells were grown in Eagle's medium (repeatedly sterilized by filtration through membrane filters MILIPOR) enriched with 6% fetal calf serum (both supplied by Institute of serum and inoculation materials, Prague, Czech Republic) and antibiotics at 37 °C in a humidified atmosphere containing 5% CO_2. After a uniform monolayer was formed, the cells were freed from the surface of the culture dish by a 0.25% trypsin solution. When a suitable cell concentration was reached, the suspension was used for experiments; the monitored cells were just in the exponential growth phase as described earlier (Stankovičová et al. 1995).

3.3. Biochemical screening according to Oyama and Eagle

A starting suspension of 3.5×10^4 cells/ml was prepared from 3-days-old HeLa cells after trypsination and dispersion in the culture medium; 2 ml of the suspension was inoculated into Leicht's flasks and after 24 h static cultivation at 37 °C in a 5% CO_2 humidified incubator, 20 μl of the drug solution at the above 7 concentrations was added to each culture flask. After 48 h incubation of the cells with berberine at 37 °C, the effect on HeLa cells morphology was first examined by light microscopy and then the cell growth evaluated by a colorimetric assay based on determination of the total content of cell proteins according to Lowry et al. (1951); the experiments were caried out in triplicate. Bovine serum albumin at concentrations 10–150 μg/ml was employed as standard and Folin reagent was used to colour the proteins so that the absorbance was recorded at 500 nm. The cytotoxic effect of the test compound was quantified by IC_{50} and IC_{100} defined as the concentrations of berberine which cause 50 and 100% reduction of the total amount of cell proteins, respectively. The IC_{50} and IC_{100} values were obtained from the curves of toxicity, i.e. plots of percentage of the total content of cell proteins (relative to the untreated cells) vs. concentration of berberine by simple regression (linear or exponential model) using Stat-Graphics version 5. For each concentration of the drug the percent inhibition was calculated according to the formula

$$\% \text{ of inhibition} = 100 \times [(C - T)/(C - T_0)] \quad (1)$$

where C is absorbance of the untreated (control) cells, T absorbance of the berberine-treated cells and T_0 absorbance at time 0 (addition of the drug). Similarly, the percent cell degradation was calculated by the fromula

$$\% \text{ degradation} = 100 - (T / T_0) \times 100 \quad (2)$$

3.4. Cultivation of cell population

Freshly trypsinized cell suspension 5 ml was seeded at proper density (5×10^4 for HeLa and 7×10^4 for L1210 cells) in Müller flasks (HeLa) or plastic Petri dishes (L1210 cells). After 24 h cultivation in a 5% CO_2 humidified incubator at 37 °C, 50 μl of new media (control) or berberine solution at seven concentrations was added to each cultivation flask. After further 24, 48, and 72 h cultivation, the cells were first examined under light microscope and, at the same time, the number of viable cells (dye exclusion) were counted after mixing with 0.4% trypan blue in a Bürker chamber. The IC_{50} and IC_{100} values were determined graphically from the toxicity curves using the same procedure as described above for the protein inhibition assay. The cell growth inhibition data (required to construct the toxicity curves) were calculated by the formula:

$$\% \text{ inhibition} = 100 - [(T - T_0)/(C - T_0)] \times 100 \quad (3)$$

$$\% \text{ degradation} = 100 - (t/T_0) \times 100 \quad (4)$$

where T is the number of berberine-treated cells, C number of control cells and T_0 number of control cells at the time of the addition of the drug.

Acknowledgements: This research was supported by the Grant Agency of the Slovak Republic, Project Nos. 1/8216/01, 1/8215/01 and 2/2055/22.

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