JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 1143–1149
© 2003 The Authors
Received April 17, 2003
Accepted April 17, 2003
DOI 10.1211/0022357021422
ISSN 0022-3573

Effect of berberine on proliferation, cell cycle and apoptosis in HeLa and L1210 cells

Soňa Jantová, L'uboš Čipák, Marta Čerňáková and Daniela Košt'álová

Abstract

Previous studies on the anticancer activity of protoberberine alkaloids against a variety of cancer cell lines were extended to human tumour HeLa and murine leukemia L1210 cell lines. An attempt was also made to investigate the relationship between the cytotoxic activity of berberine and its molecular mechanism of action. Cytotoxicity was measured in-vitro using a primary biochemical screening according to Oyama and Eagle, and the growth inhibition assay. The in-vitro cytotoxic techniques were complemented by cell cycle analysis and determination of apoptotic DNA fragmentation in L1210 cells. Berberine acted cytotoxically on both tumour cell lines. The sensitivity of leukemia L1210 cells to the berberine was higher than that of HeLa cells. The IC_{100} was below 100 $\mu g\,mL^{-1}$ for HeLa cells and approached a 10 $\mu g\,mL^{-1}$ limit for the leukemia L1210 cells. For both cell lines the IC_{50} was found to be less than 4 $\mu g\,mL^{-1}$, a limit put forward by the National Cancer Institute (NCI) for classification of the compound as a potential anticancer drug. In L1210 cells treated with 10–50 $\mu g\,mL^{-1}$ berberine, G_0/G_1 cell cycle arrest was observed. Futhermore, a concentration-dependent decrease of cells in S phase and increase in G_2/M phase was detected. In addition, apoptosis detected as sub-G_0 cell population in cell cycle measurement was proved in 25–100 $\mu g\,mL^{-1}$ berberine-treated cells by monitoring the apoptotic DNA fragmentation (DNA ladder) using agarose gel electrophoresis.

Department of Biochemistry and Microbiology, Faculty of Chemical and Food Technology, Slovak University of Technology, SK-81237 Bratislava, Slovak Republic

Soňa Jantová

Cancer Research Institute, Slovak Academy of Sciences, Vlárska 7, SK-83391 Bratislava, Slovak Republic

L'uboš Čipák

Department of Enviromental Sciences, Faculty of Chemical and Food Technology, Slovak University of Technology, SK-81237 Bratislava, Slovak Republic

Marta Čerňáková

Department of Pharmacognosy and Botany, Faculty of Pharmacy, Comenius University, SK-83232 Bratislava, Slovak Republic

Daniela Košt'álová

Correspondence: S. Jantová, Department of Biochemistry and Microbiology, Faculty of Chemical and Food Technology, Slovak University of Technology, SK-81237 Bratislava, Slovak Republic. E-mail: jantova@chtf.stuba.sk

Funding: This study was supported by the Grant Agency for Science Research of the Ministry of Education of the Slovak Republic , VEGA numbers 2/2094/22.

Introduction

Berberine, an isoquinoline plant alkaloid, belongs to the structural class of protoberberines. Protoberberine alkaloids constitute an important class of natural products, which have been used for many generations in Europe and the Far East 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 humans; its antimicrobial activity has been demonstrated against a variety of organisms, including bacteria, viruses, fungi, protozoans and chlamydia (Amin et al 1969; Okunade et al 1994; Iwasa et al 1997; Sarma et al 1999; Jeon et al 2002). The drug was subsequently screened for anticancer activity (Kuo et al 1995; Anis & Kuttan 1999; Anis et al 2001; Tang et al 2002). It has also been shown that berberine effectively inhibits cyclo-oxygenase-2 transcriptional activity in human colon cancer cells (Fakuda et al 1999) and has the ability to induce apoptosis in promyelocytic leukemia HL-60 and 3T3 fibroblast cells (Kuo et al 1995; Yang et al 1996). Berberine has been identified as a potential inhibitor for caspase 3, a member of the cysteine protease family, which is well known as a major apoptosis effector (Kim et al 2002). In addition, some protoberberines are highly effective as cytotoxic agents against several carcinomas, such as HeLa, SVKO (Iwasa et al 1997), Hep-2, primary culture from mouse embryos and human fibroblats cells (Sanders et al 1998; Orfila et al 2000); 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 at al 2001). The results showed that the 50% inhibitory concentration of berberine varies within the range 1–10 $\mu g\,mL^{-1}$; the limit of 1 $\mu g\,mL^{-1}$ can be overcome by introducing lipophilic substituents to positions 9 and 13 of the isoquinoline nucleus, obviously because of improvement in the membrane permeability of the drug. Sanders et al (1998) reported

that some protoberberines, unlike many clinically used anticancer drugs tested, exhibited selective cytotoxicity against certain solid tumour-derived cells, including SF-268 glioblastoma cells, as compared with leukemia cells. 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 & Bastow 2000; Mazzini et al 2003) and this was linked to its cytotoxic activity. Chang et al (1990) reported that berberine down-regulated *K-Ras2* gene expression associated with morphologic differentiation in human embryonal carcinoma cells. Berberine was also found to be a DNA triplex binder (Ren & Chaires 1999) and to enhance the cytotoxicity of clinically used antitumour agents, such as nucleotides (Lee et al 1995) and nitrosoureas (Zhang et al 1990) in-vitro. It has also been reported that berberine up-regulated the multidrug-resistant transporter (pgp-170) expression in two oral (KB, OC2), two gastric (SC-M1, NUGC-3) and two colon (COLO 205, CT 26) cancer cell lines (Lin et al 1999a, 1999b). Recently, we demonstrated that berberine elicited, at a very low dose (0.03 $\mu g\,mL^{-1}$), 90% suppression of acridine orange-induced plastid mutagenicity in a *Euglena gracilis* test model (Čerňáková et al 2002).

In this paper, two different techniques, viz the (short-term) primary biochemical screening of Oyama and Eagle (1956) and the growth inhibitory assay, were employed for two distinct cancer cell lines to study the cytotoxic potential of berberine. Furthermore, cell cycle effects and apoptosis in L1210 cells induced by berberine were monitored by flow cytometry and agarose gel electrophoresis (internucleosomal DNA fragmentation) respectively.

Materials and Methods

Cell lines

The human tumour cell line HeLa and murine leukemia cell line L1210 (both obtained from American Type Culture Collection, Rockville, MD, USA) were used. These cells were grown in minimal Eagle (HeLa) or RPMI medium (L1210) in 5% CO_2 at 37 °C. The medium was enriched with penicillin and streptomycin (100 mg L^{-1}), 10% (HeLa) inactived bovine serum or 10% (L1210) fetal bovine serum (from Biocom, Slovakia). Before a uniform monolayer of HeLa cells was formed, cells were freed from the surface of the culture dish by a 0.25% solution of trypsin and were subcultivated two to three times a week. The L1210 cells grew in suspension and were subcultivated three times a week. The suspension of these cells was then blown and the total number of cells was stated as their viability. Cell viability was determined by 0.4% trypan blue staining.

Berberine

Berberine (2,3-methylenedioxy-9,10-dimethoxyprotoberberine chloride, Figure 1), obtained commercially from Merck, was dissolved in dimethyl sulfoxide (DMSO); its final concentration never exceeded 1% (v/v) in either control or treated samples. Cytotoxic activity was evaluated at seven concentrations in the range 0.1–150 $\mu g\,mL^{-1}$. The cell cycle effect was measured at concentrations ranging from 10 to 50 $\mu g\,mL^{-1}$. The induction of apoptosis was studied at concentrations of 25–100 $\mu g\,mL^{-1}$.

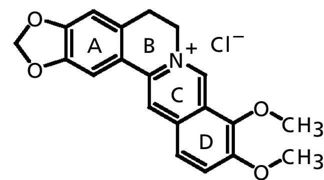

Figure 1 Chemical structure of berberine chloride.

Primary biochemical screening

Human tumour cells HeLa were seeded at a density of 7×10^4 cells per Leighton flask 24 h before treatment. After 24 h of culturing at 37 °C in a 5% CO_2 humidified incubator, 20 μL of one of the solutions in the range 0.1–150 $\mu g\,mL^{-1}$ was added to the cells. After 48 h of incubation of the cells treated with berberine at 37 °C, the intensity of cell growth was assessed using the Lowry method (Lowry et al 1951) for the determination of total cell protein content with bovine serum albumin as the standard. The cytotoxic activity of the berberine was determined from the inhibitory concentrations IC_{50} and IC_{100} (i.e. concentrations of berberine which, in comparison to the control, inhibited the contents of total cell proteins by 50% or 100%, respectively). The IC_{50} and IC_{100} values were obtained from the toxicity curves, i.e. plots of the percentage of the total content of cell proteins (relative to the untreated cells) vs the concentration of berberine, by simple regression using Stat-Graphics version 5. For each concentration of the drug the percentage inhibition was calculated according to the formula:

$$\%\ \text{inhibition} = 100 - [(E_B - E_0/E_K - E_0) \times 100]$$

where E_K is absorbance of the untreated (control) cells, E_B is the absorbance of the berberine-treated cells and E_0 is the absorbance at time 0 (addition of the drug). Similarly, the percentage cell degeneration was calculated by the formula:

$$\%\ \text{degeneration} = (E_B/E_0) \times 100$$

The growth inhibition assay

A starting inoculum of 3.5×10^4 HeLa cells mL^{-1} and 8×10^4 L1210 cells mL^{-1} in an exponential phase of growth was used. Five millilitres of the suspension were added to glass Petri dishes (diameter 60 mm). After 24 h of incubation in a 5% CO_2 humidified incubator at 37 °C, 50 μL of berberine at concentrations of 150, 100, 50, 10, 5, 1 and 0.1 $\mu g\,mL^{-1}$ were added to the cells. Control cells were treated with the same amount of DMSO. The treated

and control HeLa cells were then washed once with phosphate-buffered saline (PBS) to remove fragments and dead cells. The dishes were harvested in triplicate with 0.25% trypsin, washed once with PBS and resuspended in the latter. The cells were recognized by their ability to exclude trypan blue stain (0.4%). Unstained cells were counted in a counting chamber. L1210 cells were exposed growing in suspension. The number of cells per culture dish was counted in a Bürker chamber and viable treated and control cells were determined by 0.4% trypan blue staining.

Cytotoxic effects were evaluated in terms of inhibition of cell proliferation. Relative inhibition (degeneration) was calculated using the formulae:

$$\% \text{ inhibition} = 100 - [(K - E/K - K_o) \times 100]$$

$$\% \text{ degeneration} = (E/K_o) \times 100$$

where K_o is the cell count at the time of the addition of the berberine, K is the cell count after 24, 48 and 72 h of cultivation with the solvent and E is the cell count after 24, 48 and 72 h of cultivation with the berberine.

Cell cycle measurements

Cells (0.5×10^6) treated with berberine (10–50 μg mL^{-1}) for 24 and 48 h were harvested, washed twice in PBS and exposed to 0.1% Triton X-100 in PBS supplemented with RNA-ase (50 μg mL^{-1}) for 25 min at 37 °C. Afterwards, DNA was stained by propidium iodide (50 μg mL^{-1}) for 15 min at 4 °C. Samples were analysed by FACStar (A Beckman Coulter Company, Miami, Florida, USA) with the use of EXPO^{TM}32 MultiCOMP software provided by the manufacturer. A minimum of 10 000 cells per sample was analysed at a flow rate of 200 cells s^{-1}.

Electrophoretic determination of apoptosis

The untreated L1210 cells (control) and the cells (1×10^6) treated with 25–100 μg mL^{-1} berberine for 24 and 48 h were harvested, washed in PBS and then lysed in 100 μL of solution (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100) supplemented with proteinase K (1 mg mL^{-1}). Samples were then incubated at 37 °C for 1 h and heated at 70 °C for 10 min. Following lysis, RNA-ase (200 μg mL^{-1}) was added and repeated incubation at 37 °C for 1 h followed. The samples were subjected to electrophoresis at 40 V for 3 h in 2% (w/v) agarose gels complemented with ethidium bromide (1 μg mL^{-1}). Separated DNA fragments were visualized using a UV transilluminator (254 nm, Ultra-Lum Electronic UV Transilluminator, USA).

Quantification of DNA fragmentation

The extent of DNA fragmentation of the cellular DNA of treated cells was determined by the method of Rauko et al (Rauko et al 2001). Equal amounts of DNA sample (from 1×10^6 cells) were electrophoresed and visualized as described above. Photographs of gels were made using a digital Olympus C-W95 camera. Determination of a relative DNA intensity at the area of DNA ladders (width of area > 200 to < 1200 bp) was performed using the software UTH-SCSA Image Tool for Windows (version 1.28). The values of DNA ladder intensities are presented as multiples of the DNA ladder intensity of non-treated cells.

Statistics

The growth curves in Figure 2 represent the results obtained from three separate experiments (for each concentration of berberine five separate Petri dishes were used). The individual data points are presented as the arithmetic means ± s.d. (standard deviation). The statistical significance of the results was evaluated by Student's t-test, with probability values of 0.05 being considered significant.

Results and Discussion

As noted above, two different assays were used here to demonstrate the cytotoxic activity of berberine chloride. First, a primary biochemical screening test (Lowry et al 1951; Oyama & Eagle 1956) based on inhibition of the

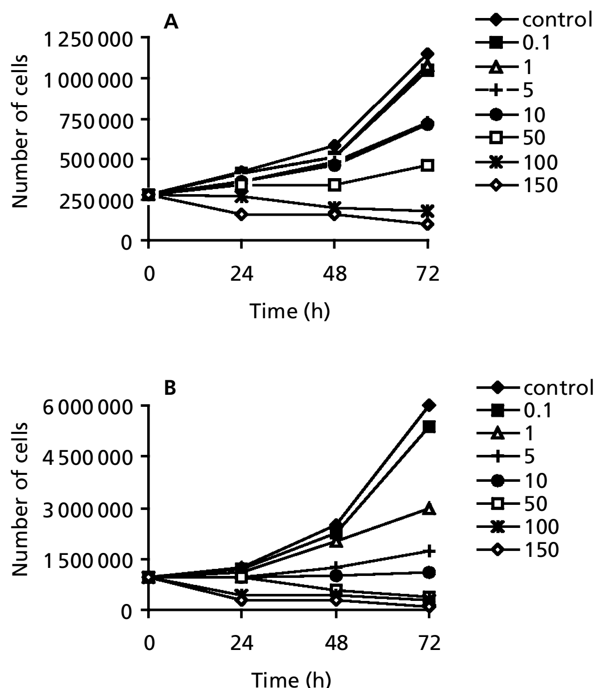

Figure 2 Growth curve of the (A) HeLa and (B) L1210 cells treated by berberine chloride in the course of 72 h. Concentration of berberine chloride (μg mL^{-1}): control (◆), 0.1 (■), 1 (Δ), 5 (+), 10 (●), 50 (□), 100 (×), 150 (◇). Each point represents the mean ± s.d. of three experiments. s.d. is < 10%.

Table 1 Inhibitory concentrations, IC_{50} and IC_{100} ($\mu g\,L^{-1}$) of berberine chloride which cause, respectively, 50 and 100% reduction of protein synthesis (a) or cell population (b) for the HeLa and L1210 cell lines.

Time (h)	HeLa cells Protein inhibition assay (a)		HeLa cells Growth inhibition assay (b)		L1210 cells Growth inhibition assay (b)	
	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}
24			6.1 ± 0.5	68.2 ± 5.1	2.7 ± 0.1	16.5 ± 1.1
48	4.8 ± 0.3	58.2 ± 4.3	7.2 ± 0.3	62.5 ± 4.8	3.5 ± 0.2	26.8 ± 2.0
72			4.8 ± 0.2	74.6 ± 5.1	1.0 ± 0.05	27.2 ± 2.1

The values represent means ± s.d. of three independent experiments.

total content of cell proteins was applied to the human tumour cell line HeLa. Table 1 shows the values of inhibitory concentrations IC_{50} and IC_{100} of the berberine.

Encouraged by this initial result, we examined the cytotoxicity of berberine more closely using an in-vitro growth inhibition assay based on counting the cell population of two different model tumour cell lines treated at various times (24, 48 and 72 h) with berberine at concentrations ranging from 0.1 to 150 $\mu g\,mL^{-1}$. The resultant growth curves for the HeLa and L1210 cells are displayed in Figure 2. After 24 h, the two highest concentrations tested (50 and 100 $\mu g\,mL^{-1}$) had an acute cytotoxic effect manifested by degeneration of certain parts of the HeLa and L1210 cell populations. In the next time intervals the degeneration of the cell population increased. Although the HeLa cells treated by a berberine concentration of 50 $\mu g\,mL^{-1}$ did not proliferate during the first 48 h interval, in the next 24 h, 21% of the cell population proliferated. This concentration, however, induced the total inhibition of L1210 cell proliferation after 24 h of exposure. In the other two time intervals, the degeneration of part of the cell population occurred. The other three concentrations (10, 5, 1 $\mu g\,mL^{-1}$) of berberine induced a toxicity that was indirectly time dependent. The HeLa and

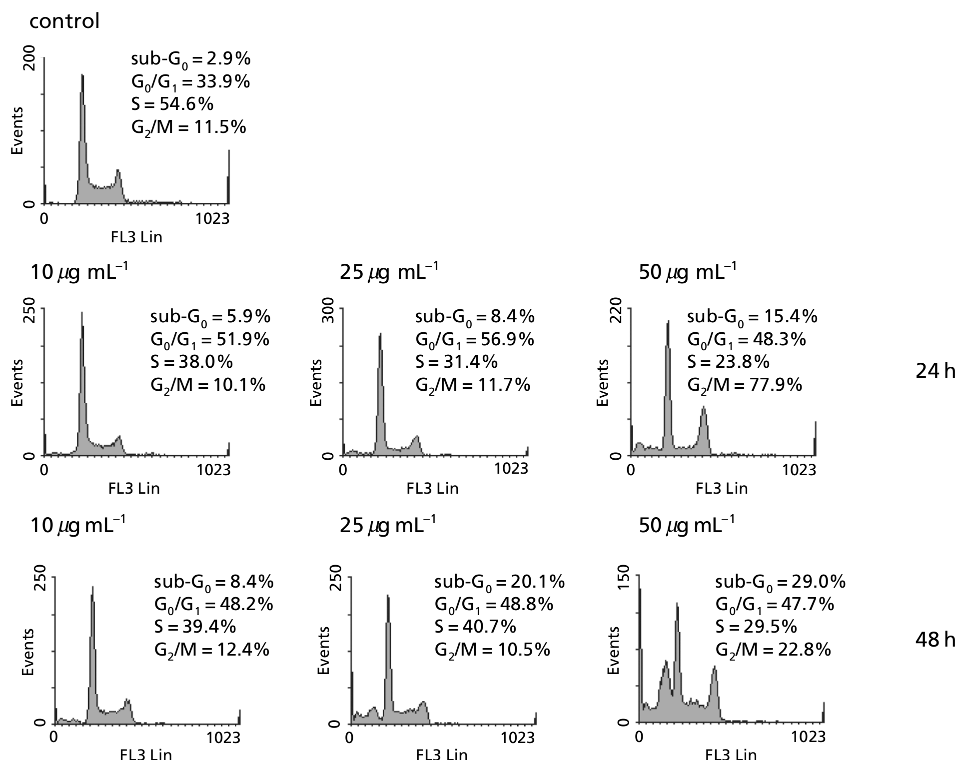

Figure 3 Cell cycle analysis of berberine-treated L1210 cells. Cells were exposed to 10–50 $\mu g\,mL^{-1}$ berberine for 24 and 48 h. Cell cycle profile was evaluated by propidium iodide staining using flow cytometry as described in Materials and Methods. This figure is representative of three separate experiments. FL3 Lin, DNA content; Events, cell number.

L1210 cells treated with the lowest concentration ($0.1 \mu g\,mL^{-1}$) grew comparably to the control cells.

The cytotoxicity of berberine determined by the MTT method on HeLa cells has been evaluated by Orfila et al (2000). Berberine showed high cytotoxicity, giving a LC_{50} value at a concentration of $10 \mu g\,mL^{-1}$. A berberine concentration of $100 \mu g\,mL^{-1}$ induced a 98.8% cytotoxic effect. Using human $HepG_2$ hepatoma cells, Chi et al (1994) found that continuously exposed cells (to concentrations in the range 1–50 μM) resulted in growth inhibition in a dose-dependent manner. These results correlate with our results. Berberine was reported to possess significant cytotoxicity against some human cancer cell lines, P388 murine leukaemia cells (Dai et al 1993), and 9L rat glioma cell line (Chen et al 1994). Recently, Iizuka et al (2000) have reported that extract of Rhizoma Coptidis and berberine significantly inhibited the proliferation of six oesophageal cancer cell lines in-vitro in a concentration-dependent manner.

Table 1b shows the values of growth inhibitory concentrations IC_{50} and IC_{100} of berberine. The values were obtained from the growth curve of HeLa and L1210 cells treated for 72 h with berberine. The IC_{100} values show that the sensitivity of the L1210 cells was increased in comparison to the HeLa cells (the values were 2.3–4.1 times lower). The higher sensitivity of L1210 cells in comparison to the HeLa cells was found when we evaluated the biological activity of 9-bromo-5-morpholino-tetrazolo[1,5-c] quinazoline (Jantová et al 2003). Recently, the National Cancer Institute (NCI) (Pisha et al 1995) have recommended that if the value is less than $4 \mu g\,mL^{-1}$, the compound can be considered as having a cytotoxic effect and hence can be classified as a potential anticancer drug. As shown in Table 1, the potency of berberine corresponds to the borderline of this criterion, with the IC_{50} values being slightly higher and lower, respectively, with respect to the $4 \mu g\,L^{-1}$ limit. Moreover, a comparison of the IC_{50} values (Table 1) for the two cell lines confirms the previous observation (Sanders et al 1998; Orfila et al 2000; Iwasa et al 2001) that berberine can be regarded as a non-selective anticancer agent.

To study the effects of berberine on cell cycle progression we monitored the effects of $10–50 \mu g\,mL^{-1}$ berberine in L1210 cells for 24 and 48 h. As indicated in Figure 3, berberine increased the percentage of cells in the G_0/G_1 phase (47–57%) in comparison with the control (34% of cell population in G_0/G_1 phase). The ability of berberine to induce G_0/G_1 block was not time and concentration dependent. In contrast to the G_0/G_1 effect of berberine, its effects on the S and G_2/M phases were time and concentration dependent, i.e. berberine decreased the S phase and increased the G_2/M phase of treated cells (see Figure 3). Additionally, cell cycle analysis showed that berberine induced a time- and concentration-dependent increase of the sub-G_0 cell fraction (an indicator of apoptotic population). Thus we monitored apoptotic DNA fragmentation by agarose gel electrophoresis in cells treated with $25–100 \mu g\,mL^{-1}$ of berberine. DNA fragmentation was first detected in cells treated with $50 \mu g\,mL^{-1}$ berberine for 24 h, and in cells treated with $25 \mu g\,mL^{-1}$ berberine for 48 h. Berberine induced a concentration- and time-dependent increase of apoptotic DNA fragmentation (Figure 4A). Values of relative intensity of DNA ladders (Figure 4B) correlate with the observed sub-G_0 cell population determined in cell cycle measurements (see Figure 3).

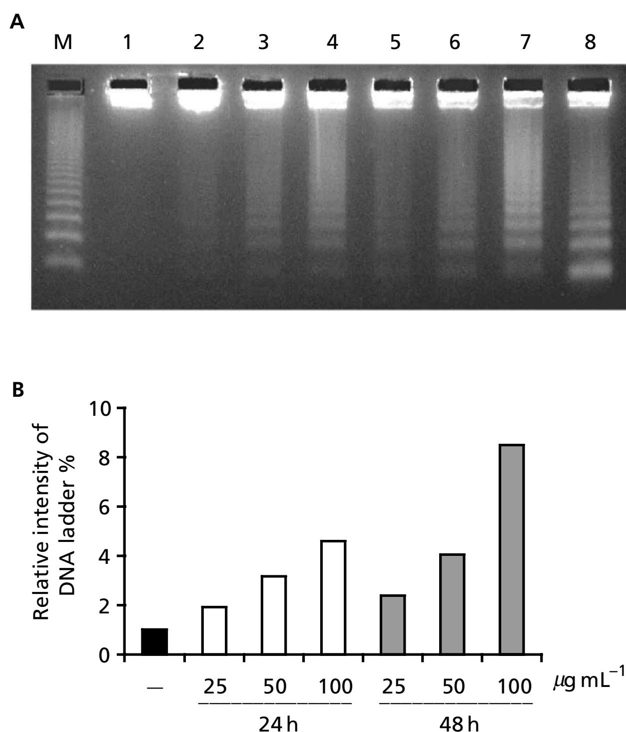

Figure 4 (A) Detection of apoptotic DNA fragmentation in treated L1210 cells by agarose gel electrophoresis. M, size marker DNA (super ladder-Mid2, 200 bp); lane 1, negative control (non-treated cells); lanes 2, 3 and 4, cells treated with 25.0, 50.0 and $100.0 \mu g\,mL^{-1}$ berberine for 24 h; lanes 5, 6 and 7, cells treated with 25.0, 50.0 and $100.0 \mu g\,mL^{-1}$ berberine for 48 h; lane 8, positive control for apoptosis (cells treated with 6 μM cisplatin for 24 h). (B) Densitometric evaluation of DNA ladder content obtained after treating L1210 cells with berberine (25, 50, $100 \mu g\,mL^{-1}$). Intensity of DNA ladder was quantified as described in Materials and Methods. The values of DNA intensities are presented as the multiples of the DNA ladder intensity of non-treated cells.

The flow cytometry study of the effect of berberine on human hepatoma $HepG_2$ cells has been reported by Chi et al (1994). Berberine treatment caused a significant reduction of the S phase fraction of $HepG_2$ cells and an arrest of gastric cancer cells in the G_2/M phase. Oesophageal cancer cells treated with the extract of Rhizoma Coptidis showed an accumulation in the G_0/G_1 phase and a relative decrease in the S phase (Iizuka et al 2000). Concentration-dependent effects of berberine were also reported on cell cycle and apoptosis in Balb/c 3T3 cells (Yang et al 1996). Furthermore, berberine and some related protoberberine alkaloids induced apoptosis in murine thymocytes (Miura et al 1997) and in promyelocytic leukaemia HL-60 cells (Kuo et al 1995).

Our results, which are similar to those of Kuo et al (1995), suggest two pathways for inducing apoptosis, one in which there is a rapid, presumably more direct, effect

on cell growth and death, and a later indirect pathway which may be associated with the transition through the S and G_2/M phases of the cell cycle. Whether or not the pathways for berberine-induced apoptosis are associated with modulation of the coupled cell growth/cell death control needs further investigation. However, Kuo et al (1995) have reported that some important cellular processes other than the intracellular DNA-interacting action of berberine may be involved in the berberine-induced apoptosis in HL-60 cells.

Conclusions

The results described in this study demonstrate that berberine acts cytotoxically on tumour HeLa and L1210 cell lines. Flow cytometric analysis shows G_0/G_1 cell cycle arrest and a concentration- and time-dependent decrease in the S phase and increase in the G_2/M phase in L1210 cells. Apoptosis detected as sub-G_0 cell population in cell cycle measurement correlated in berberine-treated cells with apoptotic DNA fragmentation (DNA ladder) monitored by agarose gel electrophoresis. In conclusion, from the results obtained, berberine, based on the NCI criteria, has sufficient cytotoxic potency to be classified as a potential anticancer drug. However, it does not offer any advantage over camptothecin and other topoisomerase I poisons currently in clinical practice except if a markedly reduced cardiotoxicity is displayed following further biological investigations.

References

Amin, A. H., Subbaiah, V., Abbasi, K. M. (1969) Berberine sulphate: antimicrobial activity, bioassay, and mode of action. *Can. J. Microbiol.* **15**: 1067–1076

Anis, K. V., Kuttan, R. (1999) Role of berberine as an adjuvant response modifier during tumour therapy in mice. *Pharm. Pharmacol. Commun.* **5**: 697–700

Anis, K. V., Rajeshkumar, N. V. (2001) Inhibition of chemical carcinogenesis by berberine in rats and mice. *J. Pharm. Pharmacol.* **53**: 763–768

Čerňáková, M., Košťálová, D., Kettmann, V., Plodová, M., Tóth, J., Dřímal, J. (2002) Potential antimutagenic activity of berberine, a constituent of *Mahonia aquifolium*. *BMC Complement. Altern. Med.* **2**: 1–6

Chang, K. S., Gao, C., Wang, L. C. (1990) Berberine-induced morphologic differentiation and down-regulation of c-Ki-ras2 protooncogene expression in human teratocarcinoma cells. *Cancer Lett.* **55**: 103–108

Chen, K. T., Hao, D. M., Liu, Z. X., Chen, Y. C., You, Z. S. (1994) Effect of berberine alone or in combination with argon ion laser treatment on the 9L rat glioma cell line. *Chin. Med. J. (Engl)* **107**: 808–812

Chi, C. W., Chang, Y. F., Chao, T. W., Chiang, S. H., P'eng, F. K., Lui, W. Y., Liu, T. Y. (1994) Flowcytometric analysis of the effect of berberine on the expression of glucocorticoid receptors in human hepatoma HepG2 cells. *Life Sci.* **54**: 2099–2107

Dai, J. R., Chai, H., Pezzuto, J. M., Konghorn, A. D., Tsauri, S., Padmawinata, K. (1993) Studies on Indonesian plants. V. Cytotoxic constituents of the roots of the Indonesian medicinal plant *Fibraurea chloroleuca*. *Phytotherapy Res.* **7**: 290–294

Fakuda, K., Hibiya, Y., Mutoh, M., Koshiji, M., Akao, S., Fujiwara, H. (1999) Inhibition by berberine of cyclooxygenase-2 transcriptional activity in human colon cancer cells. *J. Ethnopharmacol.* **2**: 227–233

Iizuka, N., Miyamoto, K., Okita, K., Tangoku, A., Hayashi, H., Yosino, S., Abe, T., Marioka, T., Hazama, S., Oka, M. (2000) Inhibitory effect of Coptidis Rhizoma and berberine on the proliferation of human esophageal cancer cell lines. *Cancer Lett.* **148**: 19–25

Iwasa, K., Kamigauchi, M., Sugiura, M., Nanba, N. (1997) Antimicrobial activity of some 13-alkylsubstituted protoberberinum salts. *Planta Med.* **63**: 196–198

Iwasa, K., Moriyasu, M., Yamori, T., Turuo, T., Lee, D. U., Wiegrebe, W. (2001) *In vitro* cytotoxicity of the protoberberine-type alkaloids. *J. Nat. Prod.* **64**: 896–898

Jantová, S., Čipák, L., Slameňová, D., Horváth, V., Rauko, P. (2003) Induction of cytotoxicity and ssDNA breaks by 9-bromo-5-morpholino-tetrazolo[1,5-c]quinazoline in tumor cells cultured in vitro. *Toxicol. in Vitro.* In press.

Jeon, Y.W., Jung, J. W., Kang, M., Chung, I. K., Lee, W. (2002) NMR studies on antitumor drug candidates, berberine and berberrubine. *Bull. Korean. Chem. Soc.* **3**: 391–394

Kim, E. S., Yoo, S., Yi, K. Y., Lee, S., Noh, J., Jung, Y. S., Kim, E., Jeong, N. (2002) Design, syntheses and biological evaluations of nonpeptidic caspase 3 inhibitors. *Bull. Korean. Chem. Soc.* **7**: 1003–1010

Kobayashi, Y., Yamashita, Y., Fujii, N., Takaboshi, K., Kawakami, T., Kawamura, M., Mizukami, T., Nakano, H. (1995) Inhibitors of DNA topoisomerase I and II from Coptis rhizomes. *Planta Med.* **61**: 414–418

Krishnan, P., Bastow, K. F. (2000) The 9-position in berberine analogs is an important determinant of DNA topisomerase II inhibition. *Anti-cancer Drug Design* **15**: 255–264

Kuo, C. L., Chou, C. C., Yung, B. Y. M. (1995) Berberine complexes with DNA in the berberine-induced apoptosis in human leukemic HL-60 cells. *Cancer Lett.* **93**: 193–200

Lee, S. J., Kim, J. B., Lee, S. W., Kim, J. H. (1995) Enhanced cytotoxicity of berberine and some anticancer nucleotides against tumor cell-lines. *Arch. Pharm. Res.* **18**: 138–139

Li, T. K., Bathory, E., LaVoie, E. J., Srinivasa, A. R., Olson, W. K., Sauers, R. R., Liu, L. F., Pilch, D. S. (2000) Human topoisomerase I poisoning by protoberberines: potential roles for both drug–DNA and drug–enzyme interactions. *Biochemistry* **39**: 7107–7116

Lin, H. L., Liu, T. Y., Wu, C. V., Chi, C. W. (1999a) Berberine modulates expression of mdr1 gene product and the responses of digestive tract cancer cells to Paclitaxel. *Br. J. Cancer* **81**: 416–422

Lin, H. L., Liu, T. Y., Lui, W. Y., Chi, C. W. (1999b) Up-regulation of multidrug resistance transporter expression by berberine in human and murine hepatoma cells. *Cancer* **85**: 1937–1942

Lowry, O. H., Roesbrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 256–262

Mazzini, S., Bellucci, M. C., Mondelli, R. (2003) Mode of binding of the cytotoxic alkaloid berberine with the double helix oligonucleotide d(AAGAATTCTT)(2). *Bioorg. Med. Chem.* **4**: 505–514

Miura, N., Yamamoto, M., Ueki, T., Kitani, T., Fukuda, K., Komatsu, Y. (1997) Inhibition of thymocyte apoptosis by berberine. *Biochem. Pharmacol.* **53**: 1315–1322

Okunade, A. L., Hufford, C. D., Richardson, M. D., Petterson, J. R., Clark, A. M. (1994) Antimicrobial properties of alkaloids from Xanthoriza simplicissima. *J. Pharm. Sci.* **83**: 404–406

Orfila, L., Rodriguez, M., Colman, T., Hasegawa, M., Merentes, E., Arvelo, F. (2000) Structural modification of berberine in relation to cytotoxic activity *in vitro*. *J. Ethnopharmacol*. **71**: 449–456

Oyama, V. I., Eagle, H. (1956) Measurement of cell growth in tissue with a phenol reagent (Folin–Ciocalteau). *Proc. Soc. Exp. Biol. Med*. **91**: 305–307

Pisha, E., Chai, H., Lee, I. S., Chagwedera, T. E., Farnsworth, N.R., Cordell, G. A., Beecher, C. W., Fong, H. H., Kinghorn, A. D., Brown, D. M. et al (1995) Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nature Med*. **1**: 1046–1051

Rauko, P., Novotny, L., Dovinova, I., Hunakova, L., Szekeres, T., Jayaram, H. N. (2001) Antitumor activity of benzamide riboside and its combination with cisplatin and staurosporine. *Eur. J. Pharm. Sci*. **12**: 387–394

Ren, J., Chaires, J. B. (1999) Sequence and structural selectivity of nucleic acid binding ligands. *Biochemistry* **38**: 16067–16075

Sanders, M. M., Liu, A. A., Li, T. K., Wu, H. Y., Desai, S. D., Mao, Y., Rubin, E. H., LaVoie, E. J., Makhey, D., Liu, L. F. (1998) Selective cytotoxicity of topoisomerase-directed protoberberines against glioblastoma cells. *Biochem. Pharmacol*. **56**: 1157–1166

Sarma, B. K., Pandey, V. B., Mishra, G. D., Singh, U. P. (1999) Antifungal activity of berberine iodide, a constituent of *Fumaria indica*. *Folia Microbiol*. **44**: 164–166

Tang, W., Hemm, I., Bertram, B. (2002) Recent development of antitumor agents from Chinese herbal medicine; Part I. Low molecular compounds. *Planta Med*. **69**: 97–108

Yang, I. W., Chou, C. C., Yung, B. Y. M. (1996) Dose dependent effects of berberine on cell cycle pause and apoptosis in Balb/c 3T3 cells. *Naunyn–Schmiedebergs Arch. Pharmacol*. **354**: 102–108

Zhang, R. X., Dougherty, D. V., Rosenblum, M. L. (1990) Laboratory studies of berberine used alone and in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea to treat malignant brain tumors. *Chin. Med. J. (Engl)* **103**: 658–665