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Effect of berberine on proliferation, biosynthesis of macromolecules, cell cycle and induction of intercalation with DNA, dsDNA damage and apoptosis in Ehrlich ascites carcinoma cells

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Abstract

Our primary aim was to study berberine, a potential anti-cancer drug, for its cytotoxic and antiproliferative activity in-vitro using Ehrlich ascites carcinoma (EAC) cells. Cytotoxicity was measured by the growth inhibition assay. We investigated the effect of berberine on the biosynthesis of macromolecules (DNA, RNA, proteins), cell cycle effects and induction of dsDNA damage and apoptosis in berberine-treated EAC cells. Our results showed that berberine acts cytotoxically on EAC cells. The cytotoxicity was directly concentration and time dependent. The highest cytotoxic concentrations (100 and 50 μg mL^{-1}) induced intercalation of berberine with DNA, formation of dsDNA breaks, inhibition of DNA synthesis and death of EAC cells. A concentration of 10 μg mL^{-1} induced clear apoptotic cell death, which was followed by inhibition of protein synthesis.

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Introduction

Berberine, an isoquinoline plant alkaloid widely used in traditional Chinese and Ayurvedic medicine, displays a varied pharmacological and biochemical activity. Berberine has demonstrated significant antimicrobial activity against a variety of organisms, including bacteria, fungi, viruses, chlamydia and protozoa (Amin et al 1969; Musumeci et al 2003). Extracts of berberine-containing plants have been used for many centuries in the treatment of diarrhoea, intestinal parasite infections and ocular trachoma infections (Ko et al 2000; Lee et al 2003). Several studies have indicated the usefulness of berberine in treating cardiovascular diseases, including hypertension, arrhythmias and heart failure (Huang et al 1989; Lau et al 2001). Berberine has also exhibited anti-inflammatory, antinociceptive and antipyretic effects (Küpeli et al 2002; Kuo et al 2004). It also has antibiotic (Ko et al 2000), antimotility (Lee et al 2003), antioxidant and antiradical (Račková et al 2004; Yokozawa et al 2004, 2005) and antimutagenic (Čerňáková et al 2002) activity. Berberine has been demonstrated to posses anti-cancer activity; significant cytotoxicity has been reported against some human cancer cell lines, murine leukaemia cells and L9 rat glioma cell line (Chen et al 1994; Kuo et al 1995; Yang et al 1996; Miura et al 1997; Iizuka et al 2000; Orfila et al 2000; Jantová et al 2003; Tang et al 2003; Letašiová et al 2004). It has also been shown that berberine exhibits the ability to induce apoptosis in HL-60 cells (Kuo et al 1995), Balb/c 3T3 cells (Yang et al 1996), murine thymocytes (Miura et al 1997), L1210 cells (Jantová et al 2003) and U937 cells (Letašiová et al 2004). By investigating the biochemical effects of berberine it has been found that berberine inhibits a number of enzymes (e.g. NADH oxidase, reverse transcriptase and diaminooxidase (Slaninová et al 2001), activator protein 1 and cyclooxygenase-2 (Fukuda et al 1999; Kuo et al 2004)); it interacts in-vitro with DNA, poly(A) fragments of mRNA and tRNA by the mechanism of intercalation (Slaninová et al 2001).

The Ehrlich ascites carcinoma cells (EAC), rapidly growing cancer cells, are widely used to investigate the antitumour properties of new agents. However, up to now, nothing is known about the action of berberine on EAC cells. Therefore, in this study, we examine the cytotoxic activity of berberine on EAC cells and its possible mechanism of action. We studied its antiproliferative effect in-vitro and in energy-requiring processes such as biosynthesis of

macromolecules. Furthermore, its effect on cell cycle, induction of damage in calf thymus DNA and DNA fragmentation were monitored.

Materials and Methods

Cell lines

EAC cells were maintained and propagated in ICR Swiss albino mice (Institute of Virology, Slovak Academy of Science, Bratislava, Slovakia), about 10 weeks old and 20–25 g body weight, as described previously (Miko et al 1991). EAC cells were transplanted at 7-day intervals by intraperitoneal injection of 0.2 mL of ascitic fluid collected under sterile conditions. The tumour cells were obtained from the peritoneal cavity and were packed by low-speed centrifugation (600 *g* for 10 min at 4°C). The cells were suspended in Krebs–Ringer phosphate buffer, pH 7.4, without calcium but with ascitic serum (2.5%, v/v) and glucose (final concentration, 3.0 mmol L^{-1}). The number of cells was adjusted to 5×10^6/mL of medium. L1210 cells grew in suspension and were subcultivated three times a week. For the study of the cell proliferation, EAC and L1210 cells were cultured in minimal Eagle medium (EAC) and RPMI 1640 (L1210) supplemented with 10% fetal bovine serum, penicillin G (100 μg mL^{-1}), streptomycin (100 μg mL^{-1}) and HEPES (10 mmol L^{-1}) (obtained from Biocom, Slovakia) at 37 °C in a humidified atmosphere containing 5% CO_2.

Berberine

Berberine (2,3-methylenedioxy-9,10-dimethoxyprotoberberine chloride, Figure 1), obtained from Sigma (Slovakia), was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO never exceeded 1% in either control or treated samples.

Cell growth inhibition assay

The starting inoculum of 3.76×10^5 EAC cells/mL in the exponential phase of growth was used. Five millilitres of the suspension were added to Petri dishes (diameter 60 mm). The EAC cells were exposed growing in suspension. Final concentrations of berberine added to the cells were 0.01, 0.1, 1, 10, 50 and 100 μg mL^{-1}. Control cells were treated with DMSO, the final concentration of which never exceeded 1%.

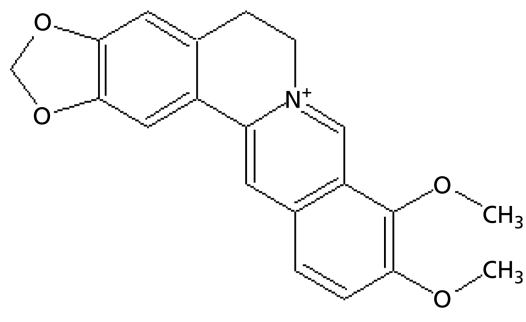

Figure 1 Chemical structure of berberine.

Cell growth and viability were assessed by direct counting of 0.4% trypan blue dye-excluding cells.

Relative inhibition of cell proliferation or degeneration of cell population was calculated by the formulae:

$$\% \text{ Inhibition} = [(K - E)/(K - K_0) \times 100] \quad (1)$$

$$\% \text{ Denegenaration} = 100 - [(E/K_0) \times 100] \quad (2)$$

where K_0 is the cell number at the time of berberine addition, K is the cell number after 12, 24, 36 or 48 h of cultivation with the solvent and E is the cell number after 12, 24, 36 or 48 h of cultivation with berberine.

Primary biochemical screening (cytotoxic assay)

Cells were incubated for 1 h in the presence of at least four selected concentrations of the substance, under defined conditions in-vitro, and the active synthesis of nucleic acids and proteins was followed. After 1 h of drug exposure, the test-tubes were transferred to an ice bath. [8-^{14}C]Adenine was added to the first series to a final concentration of 2.47 kBq/0.42 μg and L-[U-^{14}C] valine was added to the second series to a final concentration of 2.17 kBq/1.82 μg. Both series were again incubated for 1 h at 37°C. In control experiments only DMSO was used. The final concentration of DMSO was less than 1%, which does not affect the metabolic processes studied. Incorporation was terminated by adding 1 mL of 5% trichloroacetic acid (TCA) to each test-tube in an ice bath. The samples were filtered through synpor membrane filters, pore size 4 μm (Synthesia, Prague, Czech Republic), the precipitate washed with 10 mL of cold 2.5% TCA and 10 mL water and dried at 105°C. The radioactivity was measured by fluid scintillation computer (Rack Beta 1214/1219, Pharmacia, Finland) (Miko & Devinsky 1993). Precursors were obtained from the Institute for Research, Production and Use of Radioisotopes, Prague, Czech Republic.

Kinetics of biosynthesis of macromolecules

To define further the mechanism of action of berberine, the kinetics of DNA, RNA and protein synthesis were examined using isotope incorporation. The cells were incubated in a water bath at 37°C without shaking. At the indicated time intervals, samples of suspensions (1 mL) were analysed for radioactivity in acid-soluble material. Radioactivity was measured by fluid scintillation computer as in primary biochemical screening. In some cases, the nature of the labelled material was checked by alkaline-acids hydrolysis. In the case of adenine incorporation, 60.6% of the incorporated radioactivity corresponds to the RNA fraction and 39.4% corresponds to the DNA fraction. For thymidine, 90% of its incorporation was found in DNA. In uridine, 87.5% of the radioactivity was found in the RNA fraction (Miko & Devinsky 1993).

Detection of DNA damage by DNA-modified screen-printed electrode

A screen-printed electrode (SPE) based on a carbon paste with the surface modified by double-stranded (ds) calf thymus DNA (Merck, Germany) was prepared and applied to

study the effects of berberine in 1% DMSO solution on changes of integrity of surface attached to DNA. Interactions or intercalation of berberine with DNA were detected by differential pulse voltammetry (DPV) using $[Co(phen)_3]^{3+}$ as a DNA redox indicator (Labuda et al 2003). SPE assembly included a working carbon electrode, together with silver/silver chloride reference electrode and counter electrode. A working electrode was chemically modified by covering with 5 μL DNA stock solution (0.1 $\mu g\,mL^{-1}$) and leaving to dry overnight. After 2 min pretreatment in blank (pH buffer 7.0) solution before use, the final dsDNA layer fixed by adsorption was rather stable and could be exploited analytically with sufficient repeatability.

To detect the damage to DNA, the same DNA sensor was first incubated for 10, 60 and 120 min with berberine (concentration range 10–100 $\mu g\,mL^{-1}$) in 5 mmol L^{-1} phosphate buffer solution (PBS), pH 7.0, under stirring, and then rinsed with water. Then, the marker peak current (I) was obtained using DPV measurement/biosensor regeneration scheme and the normalized (relative) indicator signal I/I_o was calculated (I_o is the signal obtained before the incubation). As a positive control cisplatin (cisPt) (1.8 $\mu g\,mL^{-1}$) was used.

To detect intercalation of berberine, we used DPV of guanine moiety (Labuda et al 2000; de-los-Santos-Álvarez et al 2004). Before the first measurement, the DNA-modified electrode was immersed in the 5 mmol L^{-1} PBS, pH 7.0, for 5 min under stirring. Immediately, the anodic differential pulse voltammogram was recorded in blank PBS from 300 to 1100 mV using pulse amplitude 100 mV and scan rate 10 mV s^{-1}. The peak current was evaluated against a base-line.

Cell cycle analysis by flow cytometry

The control and berberine-treated EAC cells (0.5×10^6) at concentrations of 0.1, 1.0 and 10.0 $\mu g\,mL^{-1}$ were harvested, washed twice with PBS and exposed to 0.1% Triton X-100 in PBS supplemented with RNA-ase (50 $\mu g\,mL^{-1}$, Sigma) for 25 min at 37°C. Afterwards, DNA was stained by propidium iodide (50 $\mu g\,mL^{-1}$, Sigma) for 15 min at 4°C. Samples were analysed by a Coulter Epics XL (Beckman Coulter Company, FL, USA) flow cytometer with the use of System II software provided by the manufacturer. Excitation was elicited at 488 nm with the Argon laser.

Electrophoretic analysis of apoptosis

EAC cells (1×10^6) treated with 0.1, 1.0 and 10.0 $\mu g\,mL^{-1}$ of berberine were harvested, washed with 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 $\mu g\,mL^{-1}$) 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 $\mu g\,mL^{-1}$). Separated DNA fragments (DNA ladders) were visualized using a UV transilluminator (254 nm, Ultra-Lum Electronic UV Transilluminator, USA).

Statistical analysis

Results are shown as the arithmetic means ± s.d. (standard deviation) of the mean of three separate experiments (for each concentration of berberine five separate Petri dishes were used). Statistical analysis was performed with the Kruskal–Wallis one-way analysis of variance test for nonparametric measurements ($H>8.77$, $P<0.05$ was considered statistically significant). The effect of time and concentration on cell number and on counts min^{-1} was analysed by Friedman's nonparametric test ($P<0.002$ was considered statistically significant). Multiple-comparison procedure by the post-hoc test (Newman–Keuls test) was done on all measurements. The collected data were analysed using a statistical software package (Statgraphics Plus 5.0).

Results and Discussion

In our previous study, we have shown that berberine has antiproliferative and cytotoxic effects on the cancer cell lines murine melanoma B16, human cervix adenocarcinoma HeLa, murine leukaemia L1210 and human promonocytic U937 cells and the non-cancer cell line murine fibroblast NIH-3T3 (Jantová et al 2003; Letašiová et al 2004). For all cancer cell lines, the IC50 values (concentration causing 50% reduction of cell population) were 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 anti-cancer drug (Pisha et al 1995). Furthermore, berberine induced the cell cycle profile changes in the L1210 cells. No effect of berberine on the cell cycle of the U937, B16 and NIH-3T3 cells was detected. However, berberine induced apoptosis of in-suspension-growing U937 and L1210 cells. Cell lysis/necrosis of the berberine-treated adherent-growing B16 and NIH-3T3 cells was observed as a result of the integrity damage of cytoplasmic membrane.

In this study we tested the cytotoxic/antiproliferative activity of berberine on EAC cells in-vitro. Further, we investigated the effect of berberine on the synthesis of macromolecules (DNA, RNA, proteins), cell cycle effects and induction of apoptosis in berberine-treated EAC cells. The damage to calf thymus DNA was tested, too.

The proliferation of EAC cells during permanent exposure to berberine concentrations in the range 0.01–100 $\mu g\,mL^{-1}$ is shown in Figure 2. After 12 h of culturing, the three highest tested concentrations (100, 50 and 10 $\mu g\,mL^{-1}$) had an acute cytotoxic effect, manifested by a decrease in the number of viable EAC cells. After 24 h, part of the cell population proliferated, but after 36 and 48 h of culturing, viable EAC cell number decreased almost to zero. The antiproliferative effect of berberine at the next tested concentrations (1.0, 0.1 and 0.01 $\mu g\,mL^{-1}$) was directly proportional to the concentration and the time of exposure.

Table 1 shows the values of growth inhibitory concentrations (IC50, which inhibited the cell growth by 50%, and IC100, which inhibited the cell growth by 100%) of berberine for EAC cells. The IC50 values for berberine-treated EAC cells cultivated within 48 h were found to be below 1 $\mu g\,mL^{-1}$, which indicates that this compound can be classified as a potential anti-cancer drug, fulfilling the limit of IC50<4 $\mu g\,mL^{-1}$ submitted by the NCI (Pisha et al 1995).

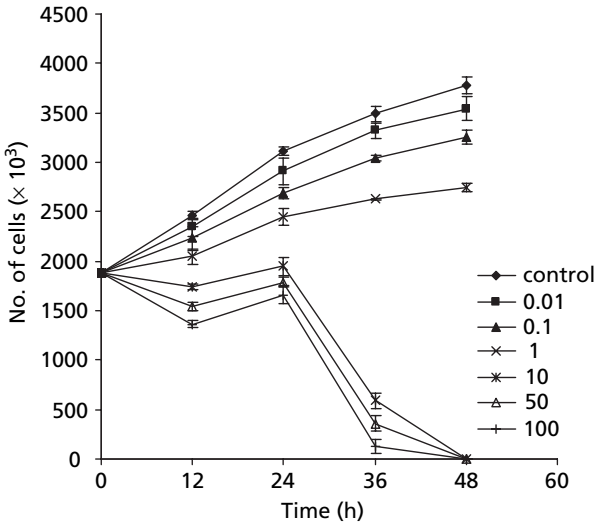

Figure 2 The proliferation of EAC cells during exposure for 48 h to berberine concentrations in the range 0.01–100 μg mL^{-1}. Each point represents the mean ± s.d. of three experiments.

Table 1 Inhibitory concentrations, IC50 and IC100 (μg mL^{-1}), of berberine that cause, respectively, 50 and 100% reduction of cell population for the EAC cells

Time (h)	IC50	IC100
12	0.358 ± 0.0201	10.0 ± 0.86
24	0.813 ± 0.0569	10.0 ± 0.79
36	0.870 ± 0.0466	5.0 ± 0.39
48	0.272 ± 0.0135	5.0 ± 0.31

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

Berberine, a protoberberine alkaloid, has been reported to possess significant cytotoxicity against HeLa cells (Orfila et al 2000; Slaninová et al 2001; Jantová et al 2003), L1210 cells (Jantová et al 2003), ovary carcinoma SVKO cells (Orfila et al 2000) and larynx carcinoma Hep-2 cells (Orfila et al 2000; Cordero et al 2004), giving LC50 values (concentration killing 50% of cells) for all cancer cell lines of 1.0–10 μg mL^{-1}. Berberine has also shown potent anti-tumour effects on colorectal cancer HT-29 cells, breast cancer MCF-7 cells and gastric cancer MKN-45 cells (Cordero et al 2004), with LC50 values < 0.05 mmol L^{-1} for all cancer cell lines, on six types of oesophageal cancer cells (YES-1 to YES-6) in a dose-dependent manner (Iizuka et al 2000), and on eight pancreatic cancer cell lines (Iizuka et al 2003).

The results from primary biochemical screening of the cytotoxic activity of berberine on EAC cells are summarized in Table 2. The inhibitory effect was characterized by IC50 value (molar concentration of compound required for 50% reduction of incorporation rate). The biosynthesis of proteins, indicated by the incorporation of [^{14}C]valine, was inhibited to a greater extent than the biosynthesis of nucleic acids, indicated by the incorporation of [^{14}C]adenine (Table 2).

It is convenient to use the IC50 adenine:IC50 valine ratio (R) as a suitable parameter to indicate the possible primary mode of action of the substance investigated. On the basis of the obtained IC50 values it was not possible to calculate R because the IC50 value for adenine was higher than the highest tested concentration.

The values from biochemical screening represent the first fundamental information about the cytotoxic activity of berberine. The data obtained in a relatively short time indicate whether the tested substance has cytotoxic activity at all, and perhaps also its possible mode of action (ratio). In a first approach to determine the mode of action of the cytotoxically active compound, the kinetics of DNA, RNA and protein synthesis inhibition were examined using isotope incorporation. Only when the time course is known is it possible to state at what time and concentration the inhibitory effect appears.

Figure 3 demonstrates the inhibitory effect of berberine upon the biosynthesis of macromolecules, indicated by incorporation of [^{14}C]adenine and [^{14}C]valine into TCA-insoluble material of EAC cells. The highest concentration (223.0 μg mL^{-1}) inhibited both incorporation of [^{14}C]adenine and [^{14}C]valine into appropriate macromolecules of EAC cells. It also reflects the decrease, in counts min^{-1}; in the case of adenine the counts min^{-1} decreases to 5787 in comparison with the control (9121). An even higher decrease is monitored in the case of [^{14}C]valine incorporation. While the control shows 3366 counts min^{-1}, the highest concentration (223.0 μg mL^{-1}) lowers this value to 610 counts min^{-1}. The concentration of 111.5 μg mL^{-1} and that of 55.75 μg mL^{-1} did not show marked difference in the case of [^{14}C]adenine incorporation, which is indicated by the counts min^{-1} values (7154 and 7642, respectively, versus 9121 for the control). [^{14}C]Valine incorporation presents a different case. The second highest concentration (111.5 μg mL^{-1}) lowered the counts min^{-1} from 3366 for the control to 970. Similarly, the third highest concentration (55.75 μg mL^{-1}) decreased the

Table 2 Primary biochemical screening of berberine

Mr (g mol^{-1})		Concentration of berberine (μg mL^{-1})					IC50	R
		0	27.875	55.75	111.5	223.0		
371.8	(a)	5163	4624 (11)	4485 (13)	3773 (27)	2655 (49)	>223.0	?
	(b)	4807	2907 (40)	1486 (69)	333 (93)	186 (96)	35.7	

The measure of the cytotoxic effect was the degree of inhibition of [^{14}C]adenine (a) and [^{14}C]valine (b) incorporation into the TCA-insoluble fraction of EAC cells after 2 h incubation in-vitro. The numbers represent counts min^{-1} with percentage of inhibition (in parentheses). Berberine was dissolved in DMSO shortly before experiments, R = IC50 adenine:IC50 valine.

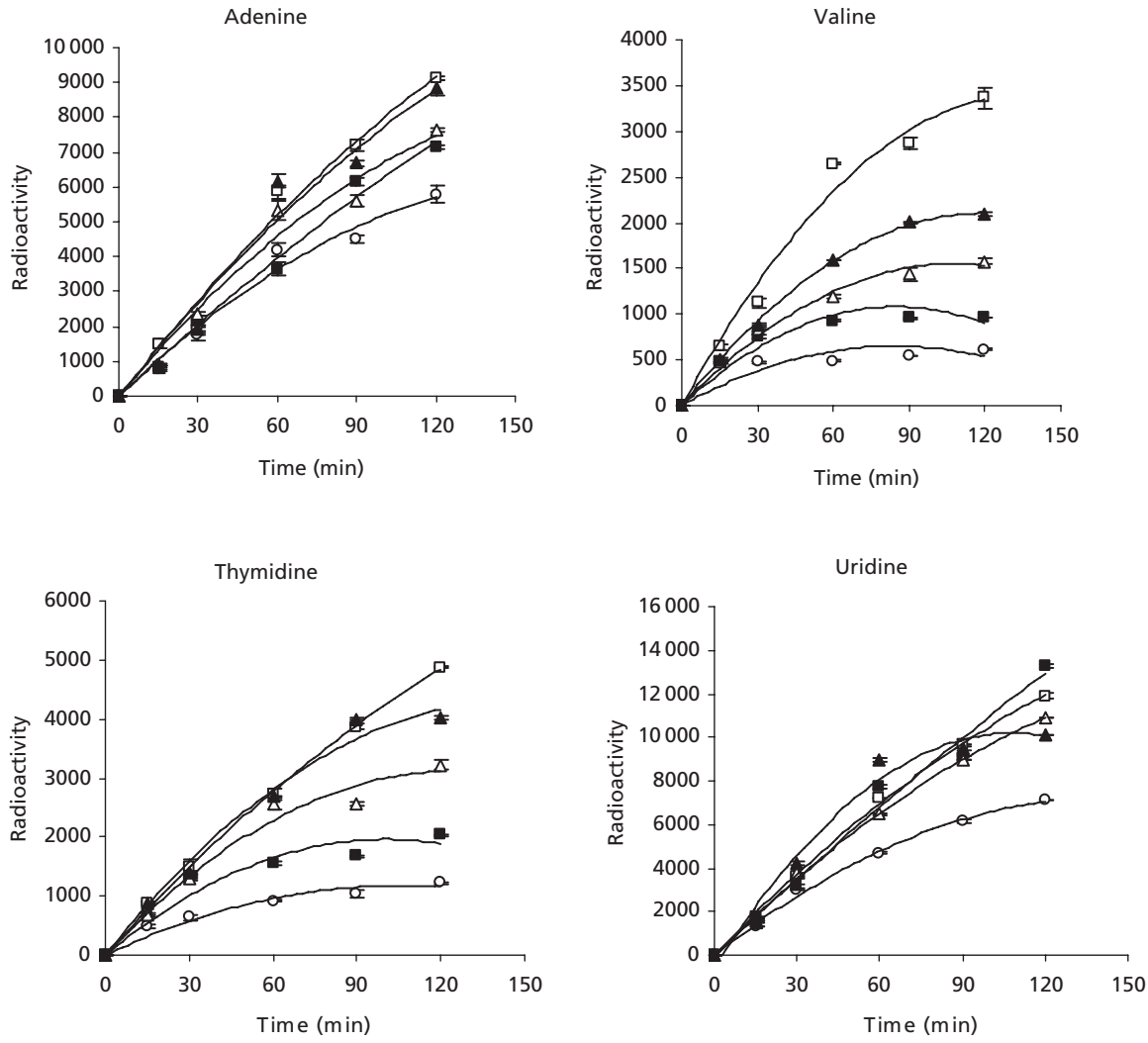

Figure 3 The effect of berberine on macromolecule synthesis of EAC cells. Incorporation of radioactive adenine (top left), valine (top right), thymidine (bottom left) and uridine (bottom right) into acid-insoluble fractions was determined by incubating cells with appropriate ^{14}C-labelled precursors. Radioactive precursors and berberine were added to the cells at the same time. The test-tubes were incubated at 37°C and 1-mL samples of suspension were analysed for radioactivity in acid-insoluble material. The results are expressed as counts $min^{-1}/5 \times 10^6$ cells. Concentrations: control (□); 223.0 (◇); 111.5 (■); 55.75 (△); 27.875 $\mu g\,mL^{-1}$ (▲).

counts min^{-1} by more than half (1581). The lowest concentration of berberine (27.875 $\mu g\,mL^{-1}$) in the case of [^{14}C]adenine showed only moderate inhibition, which reflects the counts min^{-1} (8825 versus 9121 for the control). However, in the case of [^{14}C]valine incorporation we monitored marked inhibition (37.9%), which was shown by the decrease in counts min^{-1} from 3366 for the control to 2091.

As [^{14}C] adenine is incorporated into both DNA and RNA, we determined which of these nucleic acids was more sensitive by the experiments presented in Figure 3. Berberine affected the incorporation of [^{14}C]thymidine (DNA synthesis), as well as the incorporation of [^{14}C]uridine (RNA synthesis), but the biosynthesis of DNA was inhibited more than the biosynthesis of RNA. The highest tested concentration of berberine in the case of [^{14}C]thymidine significantly reduced the counts min^{-1} from 4871 for the control to 1221 (75% inhibition) (Figure 3). In the case of [^{14}C]uridine the inhibition was also observed (11911 versus 7108 counts min^{-1}), which represents 40.3% inhibition. The second highest concentration (111.5 $\mu g\,mL^{-1}$) also markedly reduced the incorporation of [^{14}C]thymidine from 4871 to 2032 counts min^{-1} (58.3% inhibition). A different situation was found with incorporation of uridine, where moderate stimulation was observed, which was manifested by an increase in counts min^{-1} from 11911 to 13272. A berberine concentration of 55.75 $\mu g\,mL^{-1}$ in the case of thymidine reduced the counts min^{-1} from 4871 for the control to 3224 (33.8% inhibition). Moderate inhibition was observed in the case of incorporation of [^{14}C]uridine (11911 versus 10909 counts min^{-1}). After 90 min of incubation, inhibition of the incorporation of thymidine, as well as that of the incorporation of uridine, was observed at the lowest concentration of berberine (27.875 $\mu g\,mL^{-1}$).

Also, in this case, we monitored the higher inhibition of [^{14}C]thymidine incorporation (4020 versus 4871 counts min^{-1}, 17.4 % inhibition) than in the case of [^{14}C]uridine incorporation (10109 versus 11911 counts min^{-1}, 15.1% inhibition).

In both cases the inhibition was observed mainly after 90 min of berberine action. The significance of these results reposes in finding that berberine primarily affects the biosynthesis of DNA (incorporation of [^{14}C]thymidine) and that the inhibition of biosynthesis of proteins is the secondary consequence.

Every compound has a different mechanism of action. In general, compounds that primarily affect the production and utilization of energy, respectively, markedly inhibit the biosynthesis of all monitored precursors because it is an endergonic process (Miko et al 1979) (e.g. indoacetate).

Knowing that data from thymidine incorporation studies do not reflect absolute values of the extent of DNA synthesis, we favour the assumption that berberine interferes with DNA processes in EAC cells (Table 2, Figure 3).

The process of DNA synthesis is actually the culmination of many synthetic pathways. In the intact cell, interference with any of these pathways, as well as alterations and variation in the pool size of precursors, can alter the apparent rate of DNA synthesis and obscure specific drug effects. The rate of DNA synthesis is rapidly affected by a decreased level of any of the four deoxyribonucleotide triphosphates. Interference with the generation of high-energy phosphate bonds is one of the mechanisms available for the induction of nucleotide deficiency. A depletion of nucleotide pools can serve as an efficient tool to inhibit cellular growth and to induce cell death under some circumstances.

On the basis of our results (Figure 3), berberine could possibly be ranked into the group of the inhibitors of protein synthesis. Inhibitors of protein synthesis are known for their ability to induce apoptosis in cells, but the chronology of events therein and mechanism of apoptosis induction have not been delineated (Narayanan et al 2004). To such inhibitors can also belong actinomycin D, cyclohexamide, the plant toxin abrin (Narayanan et al 2004) and etoposide (Jeffrey et al 2002).

The inhibition of DNA biosynthesis (Figure 3) has probably been caused by interaction of berberine with DNA. Davidson et al (1977), Cushman et al (1979), Maiti & Chaudhuri (1981), Debnath et al (1989), Kumar et al (1992, 1993), Kuo et al (1995) and Schmeller et al (1997) reported that berberine binds to DNA by intercalation. However, the mode of binding of berberine to short oligonucleotide duplexes was re-examined by NMR and molecular modelling techniques and Mazzini et al (2003) concluded that the drug does not intercalate into DNA, but forms minor groove complexes. On the other hand, Kluza et al (2003) demonstrated that berberine and its structural analogue burasaine can intercalate into AT and GC sequences, without any marked selectivity. DNA-damaging agents, such as cisplatin, mytomycin-C and etoposide, can induce the inhibition of protein synthesis (Jeffrey et al 2002) and precede apoptosis (Tee & Proud 2000).

Therefore, in the next part of our study, we monitored the possible interaction of berberine with dsDNA on electrode surface. The DNA-based biosensor acts as an effective chemical toxicity sensor that can be simply used for the rapid detection of DNA damaging species and screening of DNA anti-cancer compounds. The possibility of investigating redox changes of DNA bases also is an advantage of the electrochemical DNA biosensor.

The DNA redox marker can be accumulated effectively from the $[Co(phen)_3]^{3+}$ solution within the DNA double helix on the electrode surface at a polarization of the modified electrode by a positive potential as well as an open circuit. Depending on the ionic strength of the medium, intercalation (predominantly at high ionic strength) and electrostatic forces (predominantly at low ionic strength) take part in binding of the marker particles. The interaction, as well as electrostatic binding, are equilibrium processes, which can be utilized for removal of $[Co(phen)_3]^{3+}$ from the DNA layer in solution without $[Co(phen)_3]^{3+}$ (Labuda et al 2000).

We have investigated the effects of berberine (10–100 μg mL^{-1}) on the surface attached to dsDNA. Values of the relative DPV signal of the DNA marker obtained after the biosensor incubation are summarized in Table 3. These data demonstrate practically no damage to DNA when the DNA/SPE sensor is treated by berberine for only 10 min (0.91 ± 0.02 (9%)) for the highest tested concentration (100 μg mL^{-1}), although some increase in the relative marker signal with decreasing concentration of berberine is observed. If, the DNA/SPE sensor is treated by berberine at a concentration of 100 μg mL^{-1} for 60 min, the data demonstrate damage to DNA (about 0.62 ± 0.02 (38%)). The trend in a signal is similar to that observed after the sensor treatment in berberine for 120 min. The damage to DNA was 0.39 ± 0.01 (61%) for 100 μg mL^{-1} of berberine. The reference I/I_0 value for 1.8 μg mL^{-1} cisplatin was 0.54 ± 0.04 for 10 min, 0.42 ± 0.03 for 60 min and 0.38 ± 0.03 for 120 min.

Damage to dsDNA has been proved also by a change in voltammetric signals of the DNA bases. The incubation of DNA/SPE in berberine solution for 10 min leads to significant decrease of the anodic peaks corresponding to the guanine moiety (about 700 mV) and adenine moiety (about 1100 mV). A new peak corresponding evidently to a modified nucleoside (the predominant production of 7-hydro-8-oxodeoxyguanosine has been reported (Jaiswal et al 1998; Oliveira-Brett et al

Table 3 Effect of berberine on dsDNA expressed by the relative DNA marker signal

Concentration of berberine (μg mL^{-1})	DNA marker signal (I/I_0) at DNA/SPE sensor		
	10 min incubation	60 min incubation	120 min incubation
1	1.01 ± 0.04	1.02 ± 0.04	1.04 ± 0.05
10	1.03 ± 0.03	0.98 ± 0.04	0.99 ± 0.04
50	1.01 ± 0.02	0.74 ± 0.02	0.59 ± 0.03
100	0.91 ± 0.02	0.62 ± 0.02	0.39 ± 0.01

Conditions: 10, 60 and 120 min incubation of the DNA/SPE sensor in berberine at 22°C, 5 mmol L^{-1} phosphate buffer solution, pH 7.0, under stirring. The reference I/I_0 value for 1.8 μg mL^{-1} cisplatin was 0.54 ± 0.04 for 10 min, 0.42 ± 0.03 for 60 min and 0.38 ± 0.03 for 120 min.

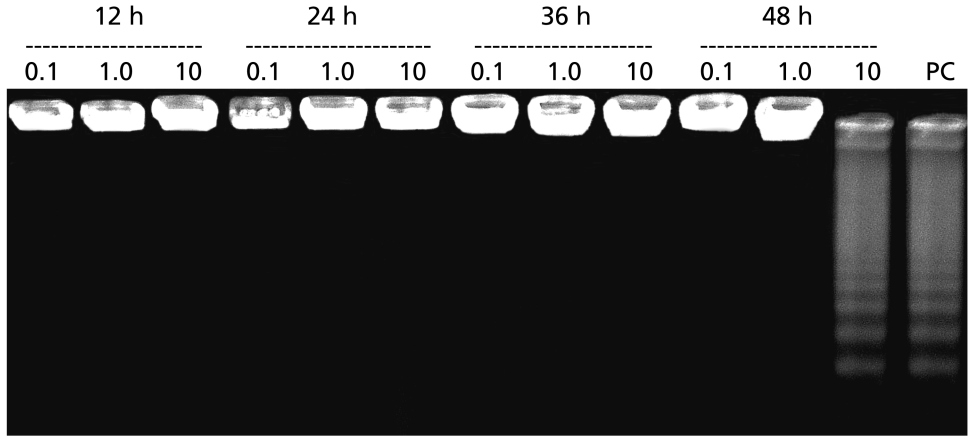

Figure 4 Agarose gel electrophoresis demonstrating apoptotic DNA fragmentation in EAC cells treated with 0.1, 1.0 and 10 μg mL^{-1} of berberine for 12, 24, 36 and 48 h. Concentration of berberine is expressed in μg mL^{-1}. PC, positive control (L1210 cells treated with 1.8 μg mL^{-1} cisplatin for 24 h).

2004)) appears at 400 mV with increasing concentration of berberine and time of incubation (60 and 120 min).

Oxidative DNA base damage by berberine (intercalation of berberine to DNA) in the presence of oxygen is indicated with the DNA-biosensor. The damage to DNA base (guanine) after 120 min was about 35%.

Our results obtained by DNA-modified screen-printed electrode confirmed that cytotoxic concentrations, 100 and 50 μg mL^{-1}, induce interaction of berberine with DNA by intercalation. Intercalation of berberine probably causes dsDNA breaks (Table 3) and then inhibition of DNA synthesis (Figure 3) and death of EAC cells (the decrease in viable cell number).

The effects of berberine at concentrations of 0.1, 1.0 and 10 μg mL^{-1} for 12, 24, 36 and 48 h on the cell cycle profile were evaluated by flow-cytometric analysis with propidium iodide staining (data not shown). Berberine induced an increase in the sub-Go fraction (A part), an indicator of apoptotic population, of the cells at concentration of 10 μg mL^{-1} for 48 h.

Agarose gel electrophoresis was used to demonstrate fragmentation of apoptotic DNA in EAC cells treated with 0.1, 1.0 and 10 μg mL^{-1} of berberine for 12, 24, 36 and 48 h. As indicated in Figure 4, apoptotic DNA fragmentation (DNA ladder) was observed in cells treated with 10 μg mL^{-1} of berberine for 48 h.

Induction of apoptosis by berberine was also monitored by other authors (Kuo et al 1995; Yang et al 1996; Miura et al 1997). We found the induction of apoptosis at short-term, as well as long-term, effects of a concentration of 100 μg mL^{-1} on murine leukaemia L1210 cells (Jantová et al 2003; Letašiová et al 2004).

Because of the significant in-vitro antiproliferative effects of berberine achieved, the preliminary investigations of anticancer activity in-vivo in ICR Swiss albino mice were done in the range of doses of 0.5–2 mg kg^{-1}. A definite reduction in mouse weight was observed at a dose of 2 mg kg^{-1}. It is known that the effect of the drug depends on the application dose, method of application and dose interval. Changing the method of application of berberine and its effect on anticancer activity will be the object of our next studies.

Conclusions

On the basis of the obtained results, it can be concluded that berberine acts cytotoxically on Ehrlich ascites carcinoma cells. The cytotoxicity was directly concentration and time dependent. The highest cytotoxic concentrations (100 and 50 μg mL^{-1}) induced intercalation of berberine with DNA, formation of dsDNA breaks, inhibition of DNA synthesis and death of EAC cells. A concentration of 10 μg mL^{-1} induced clear apoptotic cell death, which was followed by inhibition of protein synthesis.

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