

ANTIPROLIFERATIVE ACTIVITY OF BERBERINE *IN VITRO* AND *IN VIVO*

Silvia Letašiová^a, Soňa Jantová^a, Marta Múčková^b, Marica Theiszová^a

^a Department of Biochemistry and Microbiology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, SK-81237 Bratislava, Slovak Republic

^b Drug Research Institute, Horná 36, SK-90001 Modra, Slovak Republic
e-mail: silvia.letasiova@stuba.sk

Received: June 10, 2005; Accepted: September 25, 2005

Key words: Berberine/B16 cells/C57BL/6 mice/Antiproliferative activity

Berberine, an isoquinoline plant alkaloid acted cytotoxically *in vitro* on tumour cell lines B16. Its anticancer activity *in vivo* was studied with the transplanted B16 line in the range of doses from 1 mg/kg to 10 mg/kg. The significant reduction of tumor volume was observed on day 16 at doses of 5 and 10 mg/kg. The dose of 1 mg/kg stimulated the tumor mass, but other tested concentration, 5 and 10 mg/kg, reduced the tumor weight.

INTRODUCTION

Berberine (2,3-methylenedioxy-9,10-dimethoxyprotoberberine chloride) is an isoquinoline alkaloid, which has a wide range of pharmacological and biochemical effects. It has demonstrated significant antimicrobial activity against a variety of organisms including bacteria, fungi, protozoans, viruses, chlamydia, and helminths. It can also be used as an antidiarrhea, antihypertension, antiarrhythmias and anti-inflammatory agent. Berberine is demonstrated to possess antitumor activity¹. The cell cycle analysis showed the accumulation of cells in the G₀/G₁ phase and relative decrease of the S phase. Chung et al.² reported that berberine induces inhibition of the N-acetyltransferase activity and 2-aminofluorene-DNA adduct formation in human leukemia cell in a dose-dependent manner. It has been shown that berberine induces apoptosis in the murine thymocytes and in the promyelocytic leukemic HL-60 cells. Dose-dependent effects of berberine on the cell cycle and apoptosis in the Balb/c 3T3 cells were also reported. The effect of berberine was demonstrated by flow cytometric analysis in the human hepatoma HepG2 cells. It has been shown that the S phase fraction is significantly reduced.

In the previous experiments the effect of berberine on the proliferation, cell cycle profile and apoptosis in the human tumor HeLa and murine leukemia L1210 cell lines was investigated. It has been shown that berberine induces apoptosis in L1210 cells³. In this study, the antiproliferative activity *in vitro* on murine melanoma B16 cells and *in vivo* on male C57BL/6 mice was studied.

MATERIAL AND METHODS

Murine melanoma cell line B16 (obtained from American Type Culture Collection, Rockville, MD, USA) grown in supplemented RPMI 1640 medium (Biocom

Bratislava) at 37 °C in a humidified atmosphere containing 5% CO₂ was used. Cell viability was assessed by 0.4% trypan blue staining. To study the antiproliferative activity *in vivo*, 5-6-week-old male C57BL/6 mice obtained from Velaz (Czech Republic) were used. Berberine obtained from Sigma Chemicals (St Louis, MD) was dissolved in dimethyl sulfoxide (DMSO). All other chemicals were obtained from Sigma Chemicals (St Louis, MD).

Antiproliferative activity *in vitro* was measured by the cell growth inhibition assay. The starting inoculum of 1.34×10^5 B16 cells/ml was used. After 24 h of incubation, berberine (concentrations of 0.001, 0.01, 0.1, 1, 5, 10, 25, 50, 75 and 100 µg/ml) was added to the cells. Control cells were treated with DMSO, its final concentration never exceeded 1%. After 24, 48 and 72 h of berberine treatment, B16 cells were harvested in triplicate, first washed with PBS, then harvested by 0.25% trypsin, and latter resuspended. Cell growth and viability were assessed by direct counting of 0.4% trypan blue dye-excluding cells.

When studying the antiproliferative activity *in vivo*, cell suspensions of B16 cells (5×10^4 /0.1 ml PBS / mouse) were injected into gastrocnemius muscle of mice. The day of cell implantation was designed as day 0. Tested compound, berberine, and vehicle, respectively were administered intraperitoneally every day within 15 days from the day of cancer cell injection. As a vehicle carboxymethylcellulose was used. The group treated with carboxymethylcellulose was designed as the control. Berberine was used at concentrations of 1,5 and 10 mg/kg. Doses were selected on the basis of the toxicity test. The weight of the animals was monitored twice per week. The physical activity of the mice was observed daily. Tumor growth was measured twice per week and tumor volume was estimated by the formula $\text{length} \times \text{width}^2/2$. At the end of the experiment, on day 16, the animals were sacrificed with cervical dislocation. The tumors were separated from the surrounding muscles and dermis, excised and weighed.

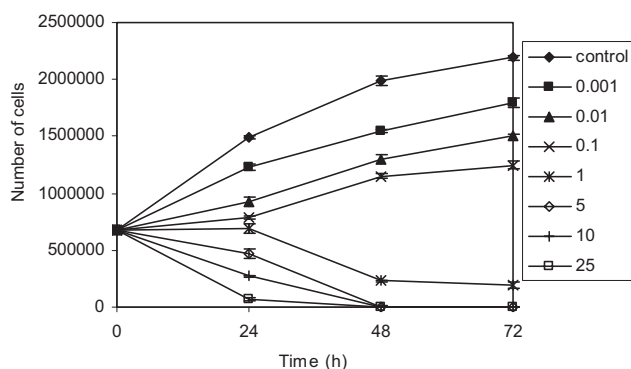


Fig. 1. Growth curve of the berberine treated B16 cells in the course of 72 h. Concentrations of berberine ($\mu\text{g/ml}$): control (◆), 0.001 (■), 0.01 (▲), 0.1 (×), 1 (⋈), 5 (◇), 10 (+), 25 (□). Each point represents the mean \pm SD of three experiments.

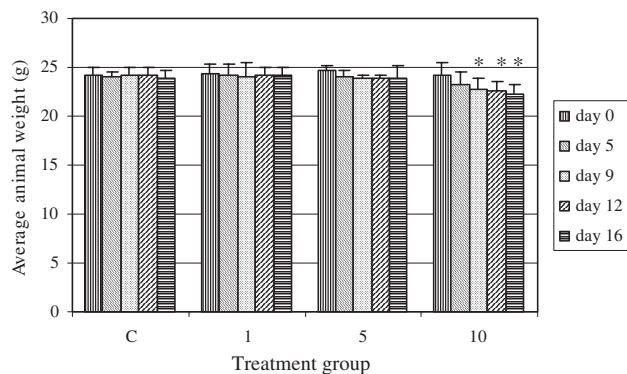


Fig. 2. Weight of animals after intramuscular implantation of B16 cells. Mice were intraperitoneally treated with carboxymethylcellulose as control (C) and tested compound, berberine, at concentrations of 1 (1), 5 (5) and 10 (10) mg/kg. The animals were weighed on day 0, 5, 9, 12 and 16. Data represent the arithmetic means \pm SE. Statistically significant differences with $P < 0.05$ are indicated with *.

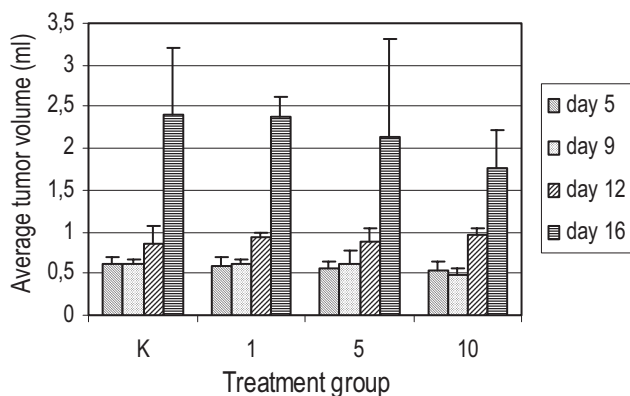


Fig. 3. Growth of tumors after intramuscular injection of B16 cells. Mice were intraperitoneally treated with carboxymethylcellulose as control (C) and berberine at concentrations of 1 (1), 5 (5) and 10 (10) mg/kg. The animals were measured on day 0, 5, 9, 12 and 16. Data represent the arithmetic means \pm SE. Statistically significant differences with $P < 0.05$ are indicated with *.

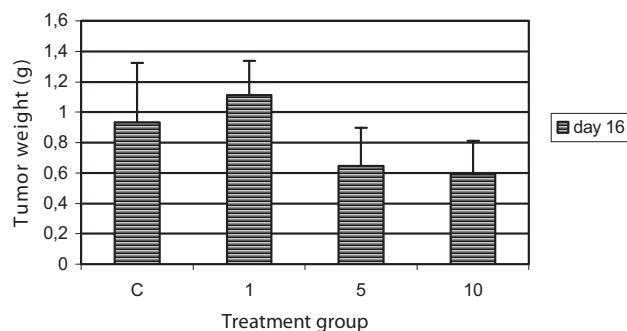


Fig. 4. Weight of tumors formed in mice 16 days after intramuscular injection of B16 cells. C57BL/6 mice were intraperitoneally treated with carboxymethylcellulose as control (C) and berberine at concentrations of 1, 5 and 10 mg/kg. Data represent the means \pm SE.

The data from *in vitro* and *in vivo* study were presented as means \pm s.e. (standard errors). The statistical significance of the results was evaluated by Student's t-test, with probability values of 0.05 being considered as significant.

RESULTS AND DISCUSSION

The growth of B16 cells exposed to berberine at concentrations ranging from 0.001 to 25 $\mu\text{g/ml}$ was monitored within 72 h of culturing (Fig. 1). As shown in Fig. 1, the addition of berberine to the medium reduced the B16 viable cell number. After 24 h, the highest tested concentrations (25, 10 and 5 $\mu\text{g/ml}$) had an acute cytotoxic effect

manifested by partial degeneration of the B16 cell populations. Berberine at concentrations of 1 $\mu\text{g/ml}$ for the B16 cells induced a delayed cytotoxic effect. After 24 h, part of the cell population proliferated, but after 48 h, 64.9 % of the cell degeneration was observed. The cytotoxic effect of berberine at the next tested concentrations (0.1, 0.01 and 0.001 $\mu\text{g/ml}$) was directly proportional to the concentration and the time of influence. The inhibition of cell proliferation was 32.7–86.1 %. These changes in viable cell number were also observed when aliquots of the cultures were examined by light microscopy.

The weight of the animals treated with 1, 5 and 10 mg/kg in comparison with control is shown in Fig. 2. The injected animals were weighed twice per week, on

day 0, 5, 9, 12 and 16. The weight of carboxymethylcellulose-injected animals did not change remarkably during the experiment. The weights of the animals treated with berberine at concentrations of 1 and 5 mg/kg did not differ from the weight of the control group. On the other hand, the weight of the animals treated with berberine at a concentration of 10 mg/kg was slightly decreased by 3.3 % on day 5 compared to the control, but in the next time interval, on day 9, the decrease was 5.8 % in comparison with the control on day 9. On day 12, the weight decrease between animals treated with 10 mg/kg and control was 6.6 %. In the next time interval, on day 16, the weight was inhibited by 6.7 %. The statistically significant differences were observed when comparing the highest tested concentration, 10 mg/kg, with the control on day 9, 12 and 16.

The injected animals were also examined twice per week for tumor growth. Five days following cell inoculation, the appearance of small solid tumors was observed. Concentrations 1, 5 and 10 mg/kg injection did not reduce tumor volume on day 5, 9 and 12 in comparison with the control (Fig. 3). On day 16, the dose of 1 mg/kg reduced tumor volume by 1.1 %, 5 mg/kg by 11.3 % and 10 mg/kg by 26.2 % compared to carboxymethylcellulose-injected animals. The statistically significant difference was observed when comparing the highest tested dose, 10 mg/kg, with the control on day 9 ($P = 0.046$).

By day 16, the group treated with berberine at a dose of 1 mg/kg grew slightly bigger tumors than carboxymethylcellulose-treated animals (Fig. 4). However, there was no statistically significant difference between the two groups ($P = 0.14$). Tumor mass in animals treated with 5 mg/kg was reduced by 30.8 % compared to the control group with statistically significant difference ($P = 0.014$). Injection of berberine at a concentration of 10 mg/kg resulted in similar tumor mass reduction as that induced by 5 mg/kg compared to the control group, reduction was 36.6 %. However, there was no statistically significant difference between the two groups ($P = 0.08$).

As it follows from the growth curves of the B16 cells (Fig. 1), berberine caused different cytotoxic effects depending on concentration and time of influence. The highest tested concentrations of berberine induced an acute cytotoxic effect which was manifested by the decrease of viable B16 cell number. Berberine at a concentration of 1 $\mu\text{g/ml}$ induced a delayed cytotoxic effect. While after the first 24 h of influence the increased cell number was monitored, in the next time interval the decrease of cell

number was found. The cytotoxic effect of berberine at the next tested concentrations (0.1, 0.01 and 0.001 $\mu\text{g/ml}$) was directly proportional to the concentration and the time of influence. Similar type of combined cytotoxic effect by monitoring the effects of berberine on proliferation of the HeLa and L1210 cells³ was observed. Because of the significant *in vitro* antiproliferative effects of berberine achieved, the antitumor activity *in vivo* was investigated. First of all the toxicity of the drug on C57BL/6 mice in the range of concentrations from 10 mg/kg to 50 mg/kg was studied and the LD_{50} , less than 50 mg/kg, (for intraperitoneal injection) value was determined. Then the cancerostatic activity *in vivo* was studied with the transplanted B16 line in the range of doses from 1 mg/kg to 10 mg/kg. Berberine administered intraperitoneally at doses of 1 and 5 mg/kg did not affect the weight of the animals. The dose of 10 mg/kg slightly reduced the weight of the animals, on day 16 the weight reduction was 6.7 % (Fig. 2). On the other hand, not even tumor volume was affected on day 5, 9 and 12 (Fig. 3). The significant reduction of tumor volume was first observed on day 16 at doses of 5 and 10 mg/kg. The tumor weight on day 16 is shown in Fig. 4. Concentration 1 mg/kg stimulated the tumor mass, but other tested concentrations, 5 and 10 mg/kg reduced the tumor weight. We can conclude that after long-term effect berberine has shown anticancer activity on C57BL/6 mice, while the tumor weight and tumor volume reduction are not at the expense of the animal weight.

It is known that the effect of the drug depends on the application dose, way of application and dose interval. The change of application way of berberine and its effect on anticancer activity will be the object of our next studies.

ACKNOWLEDGEMENT

This study was supported by the Slovak State Committee for Scientific Research VEGA, grant numbers 1/1173/04 and Science and Technology Assistance Agency under the contract No. APVT-20-007304 and APVT-51-019402.

REFERENCES

1. Shen YJ. Chinese Pharmacology. Shanghai Science and Technology Publishing Company, 1997. p. 50-2.
2. Iizuka N, Miyamoto K, Okita K, Tangoku A, Hayashi H, Yosino S, Abe T, Morioka 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.
3. Jantová S, Čipák L, Čerňáková M, Košťálová D. (2003) Effect of berberine on proliferation, cell cycle and apoptosis in HeLa and L1210 cells. *J Pharm Pharmacol* 55, 1143-9.