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Characterization of Berberine on Human Cancer Cells in Culture

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Abstract: Berberine originates from a Chinese herbal medicine and possesses a wide variety of anti-cancer activities. In this study, the killing effect of berberine on nasopharyngeal carcinoma cells (NPC/HK1) was investigated. The trypan blue exclusion assay was used to assess the cytotoxic effect of berberine in this cell line. Berberine, at 5-200 μ M, induced cell death in a dose-dependent manner. Treatment of cells with 200 μ M berberine for 5 h yielded a lethal dose of 50% (LD₅₀). The Comet Assay was employed to evaluate the extent of DNA

damage and repair after berberine treatment (0 -100 μ M). DNA damage was evident within 30 min and was more pronounced after 1.5 h. The damaged cells were not able to be repaired, as indicated by the increase in tail DNA content. However, the repair of H₂O₂ mediated DNA damage on this cell line occurred within 1.5 h, indicating that DNA repair inhibition may have contributed to the high efficacy of cell killing by berberine.

Key Words: Cytotoxicity, DNA damage, repair, cellular localization, berberine

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Introduction

Traditional Chinese drugs have been used in China for many years. However, they have not been merged into the mainstream of Western medicine, even in the developed cities in Southeast Asia. This is because there is not sufficient scientific evidence of the therapeutic effects. Recently, these therapeutic agents have been studied systematically and are now being tried clinically in several Western research centers (1-3).

Nasopharyngeal carcinoma (NPC) encompasses any squamous cell carcinoma arising in the nasopharynx, a tubular space situated at the base of the skull. This cancer is relatively rare in Caucasian populations, but is prevalent among the Southern Chinese, especially in Guangdong and Guangxi Provinces (4-6). The primary form of treatment of NPC is radiotherapy. The efficacy of this treatment depends on the radiosensitivity of the tumor and the ability to deliver an adequate radiation dosage to all affected areas, while sparing normal tissues. Although radiotherapy can eradicate the majority of the primary tumor, recurrent or residue disease occurs in 13-31% of patients. The combination of chemotherapy with secondary dose radiotherapy was given for relapse

patients. Nevertheless, all cytotoxic agents create adverse reactions, including bone marrow suppression, cardiotoxicity and renal damage, which justify more effective therapeutic regimes (7-10).

Berberine is an alkaloid isolated from the root of *Coptis chinensis*, a Chinese herbal plant (11). Numerous pharmacological activities have been reported, e.g. anti-bacterial activity (12,13), including antimalarials (14), and antileishmanial (15). The anti-tumor properties of berberine are now becoming recognized by researchers and clinical oncologists. The effects of berberine on human malignant brain tumor, esophageal cancer, and human leukemic and human colon cancer cell lines were tested; a significant killing effect was achieved (16-19). In this study, we investigated the cytotoxicity and the DNA damage effect on NPC/HK1 cells after berberine treatment and evaluated the possibility of DNA repair in this cell line. To further understand the biological action of berberine, we also examined the relationship between drug uptake and cellular localization. Finally, the molecular mechanisms of berberine on NPC/HK1 cell death were explored.

Materials and Methods

Chemicals

Berberine chloride, DAPI (4,6-diamidine-2,phenylindole dihydrochloride) stain, D-mannitol, and catalase were purchased from Sigma, St. Louis, MO, USA. The stock solution of berberine was prepared by dissolving the berberine powder in phosphate-buffered saline (PBS). A further dilution with PBS was made of this solution as required just before testing. Trolox was purchased from Aldrich, Dorset, UK.

Cell line and culture conditions

The NPC/HK1 cell line used in this study is a well-differentiated squamous cell carcinoma originally derived from a Chinese male patient (20). The cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotic PSN (50 IU/mL penicillin, 50 µg/mL streptomycin and 100 µg/mL neomycin)(Gibco) at 37 °C in a humidified 5% CO₂ incubator.

Cell viability determination

The cell viability was evaluated by a trypan blue exclusion assay. The NPC/HK1 cells were trypsinized with 0.25% trypsin. The cells was washed with PBS and then resuspended in PBS. Ten microliters of cells were transferred to microcentrifuge tubes with 1 ml of different concentrations of berberine (0-200 µM) for various treatment times (ranging from 1 to 5 h) at 37 °C. After incubation, the treated and untreated cells were washed with PBS and mixed with equal volumes of 0.2% of trypan blue solution. The cells were examined in a counting chamber with a light microscope. Viable and non-viable cells were recorded. Each assay was done in triplicate. The results were expressed as mean ± SD of more than three independent experiments.

Alkaline Comet Assay

The effect of berberine on cellular DNA was evaluated by the comet assay. The cells were incubated in different concentrations (0-200 µM) of berberine at 37 °C for 1 h. After incubation, the treated cells were washed with PBS and the cells were then mixed with 85 µl of 1% low melting point agarose gel (Type VII, Sigma) in the microcentrifuge tube. The mixture was applied to a microscope slide precoated with 85 µl of 1% standard agarose (Sigma) in PBS. Cell membrane was lysed with a cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris,

pH 10, 1% Triton X-100). Lysis was performed by submerging the slides in a staining jar in the absence of light for 1 h at 4 °C. Subsequently, slides were transferred to an electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA (4 °C) for 40 min to allow for DNA unwinding and expression of alkali-labile sites. Electrophoresis was performed for 30 min at 25 V (0.83 V/cm). The current was maintained at 0.3 A by adjusting the electrophoresis solution level. After electrophoresis, slides were neutralized by three changes (3 x 5 min) of 0.4 M Tris with pH 7.5. Slides were dried and stained with 20 µg/mL ethidium bromide for image analysis (23). The results were expressed as mean ± SD of more than three independent experiments. The changes in tail DNA content were statistically evaluated using Dunnett's t-test.

Effect of antioxidants on berberine induced cytotoxicity and DNA damage

Two antioxidants and one antioxidant enzyme, namely D-mannitol, Trolox and catalase, were used to explore the molecular mechanism of berberine on NPC/HK1. The cells were co-incubated with various concentrations of berberine (0-200 µM) and a fixed concentration of antioxidant (200 µM D-mannitol, or 200 µM Trolox, or 233 IU per ml catalase) at 37 °C. The trypan blue and comet assay were performed 1 h after incubation. The results were expressed as mean ± SD of three independent experiments.

Effect of berberine on DNA repair capacity

The DNA repair capacity of NPC cells after berberine treatment was determined by the comet assay. The DNA damage of the cells, with or without a 1.5 h recovery period, was measured and compared. The DNA damage, mediated by hydrogen peroxide, was used as a positive control of repair ability. The NPC cells were incubated with various concentrations with either 1 ml berberine solution (25, 50 and 100 µM) at 37 °C for 30 min or 1 ml of H₂O₂ (50, 75 and 100 µM) at 4 °C for 5 min. After incubation, the mixture was centrifuged and the supernatant was removed. The cell pellets were mixed with low melting point agarose gel for the comet assay. The control for the recovery period using 1.0 ml of RPMI with 10% FBS was added and the cells were incubated for an additional 1.5 h at 37 °C. At the end of the recovery period, the cells were centrifuged, washed with PBS twice, and mixed with the low melting point agarose gel for the comet assay measurement. The results were

expressed as mean \pm SD of more than three independent experiments.

Detection of cell morphological changes by the DAPI nuclear stain

Apoptosis was evaluated by the DAPI (4,6-diamidine-2,phenylindole dihydrochloride) nuclear staining method (22). Berberine was dissolved in the culture medium (RPMI 1640 with 10% fetal calf serum). The cells were treated in 100 and 200 μ M of berberine from 2 h to 24 h. The cells were then washed in PBS and fixed with 4% paraformaldehyde in PBS for 15 min. The cells were washed again and stained with 1 ml of 1% DAPI stain at 37 °C for 15 min. The nuclear regions of the cells were examined by fluorescent microscope using a 330-380 nm excitation and a 420 nm emission filter. Apoptotic nuclei showed the standard characteristics of a condensed chromatin pattern. The cells were identified, and the percentage of apoptotic cells was determined by counting 100 cells per field for more than 10 fields; two individual experiments were performed (22-24).

Cellular localization of berberine

The berberine uptake and localization in NPC cells were determined by employing its own fluorescent properties (14). Berberine, dissolved in the culture medium (with final concentration of 200 and 400 μ M), was incubated with the cells for 2 to 46 h. Afterwards, the cells were washed twice in PBS, followed by examination under a fluorescent microscope (excitation 420 nm; emission 520 nm).

Results

Cytotoxicity of berberine on NPC/HK1 cell

The cytotoxic activity was expressed in percentage of cell viability. The duration of the treatment was 1 to 5 h. The results showed that the cytotoxic effect was positively related to berberine concentration (Figure 1). After 1 h incubation, the cell viability was generally decreased, while the concentration of berberine increased. At a berberine concentration of 5 μ M, cell viability was reduced to 15% (after 1 h treatment) when compared to the untreated cell. At 200 μ M of berberine, cell viability dropped to 60%.

Berberine-induced DNA damage

After 30 min of berberine treatment (5–200 μ M), DNA damage was evidenced in the comet assay. The mean

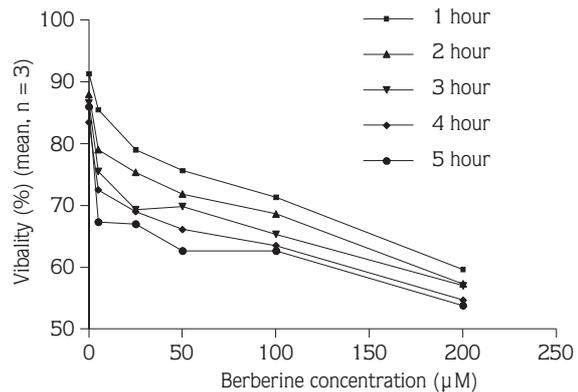


Figure 1. Viability of NPC/HK1 cells after berberine treatment. Cells were incubated in different concentrations (5–200 μ M) of berberine for 1 to 5 h. Each data point represents the mean of three individual experiments.

tail DNA content increased as berberine concentration increased (Figure 2). However, the results indicated that a statistically significant increase in DNA damage was observed only at 200 μ M (Dunnett's t-test, $p < 0.01$).

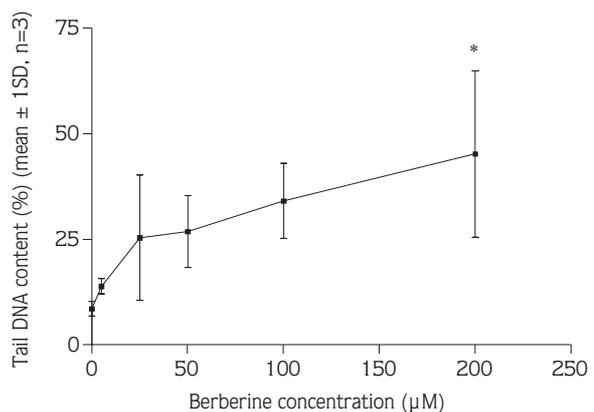


Figure 2. DNA damage of NPC/HK1 cells after 30 min treatment of berberine at different concentrations (5–200 μ M). DNA damage was expressed in percentage of DNA content in the comet tails. The results are mean \pm 1 SD of three individual experiments.

Effect of antioxidants on berberine-induced cytotoxicity and DNA damage

The cell viability and DNA damage of NPC/HK1 cells, after berberine treatment with or without co-incubation of antioxidants, was compared. The results showed that co-incubation of berberine with D-mannitol at 200 μ M was unable to increase the cell viability or reduce DNA damage (unpaired t-test, $p > 0.05$, data not shown). Furthermore, no protection but adverse effects of Trolox treatment was observed on DNA damage (unpaired t-test,

$p < 0.05$) (Figure 3). In contrast, for the co-incubation of berberine and catalase, cell viability was increased when compared to those incubated with berberine alone (unpaired t-test, $p < 0.05$) (Figure 4). This indicated that the berberine-induced cell death may be partly mediated by hydrogen peroxide.

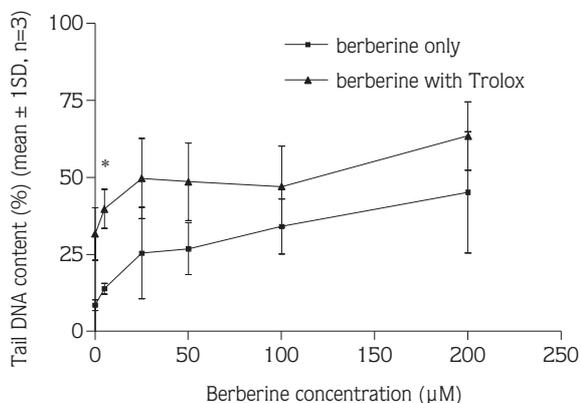


Figure 3. DNA damage of NPC/HK1 cells after 1 h treatment of berberine at different concentrations (5-200 µM) with or without co-incubation with Trolox. A statistically significant increase in tail DNA content was found with the presence of Trolox (Unpaired t-test, * = $p < 0.05$).

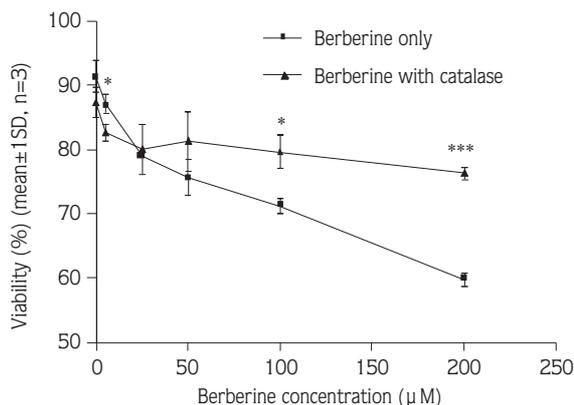
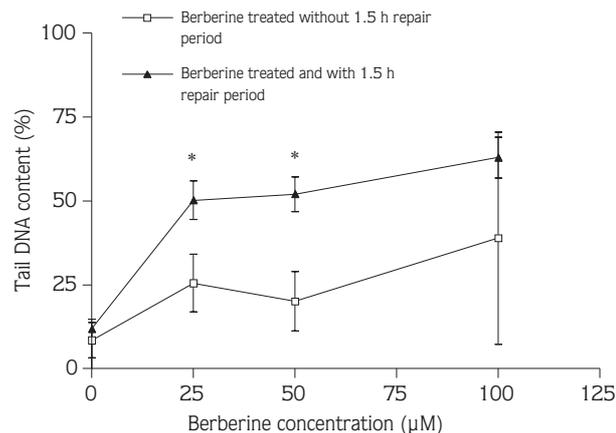


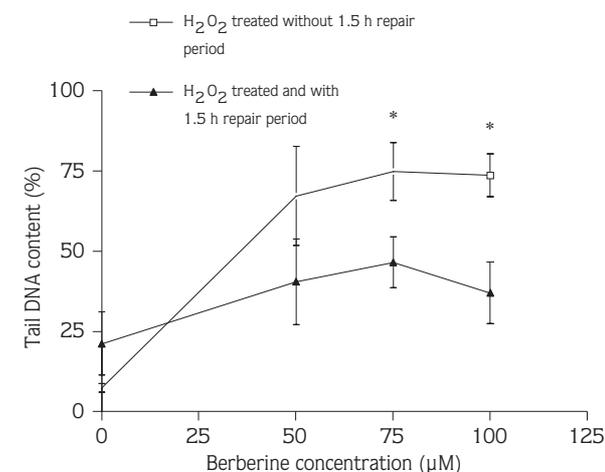
Figure 4. Viability of NPC/HK1 cells after 1 h treatment of berberine at different concentrations (5-200 µM) with or without co-incubation with catalase. A statistically significant increase in viability was found with the presence of catalase (Unpaired t-test, * = $p < 0.05$, *** = $p < 0.001$).

Effect of berberine on DNA repairing capacity

These results showed that NPC/HK1 cells were not able to repair the damaged DNA after berberine treatment. The tail DNA content was increased further after 1.5 h, when compared with that of the matched concentration, without allowing the cells to recover (Figure 5a).



(a)



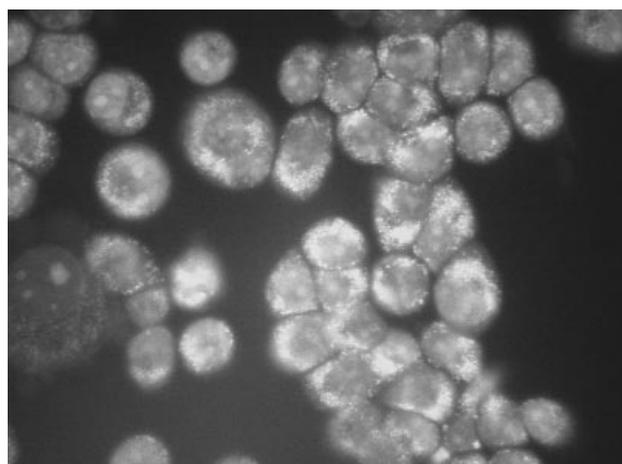
(b)

Figure 5. DNA damage of NPC/HK1 cells immediately after treatment or with 1.5 h of recovery period. a) NPC/HK1 cells treated with 25, 50 and 100 µM berberine for 30 min at 37 °C. b) NPC/HK1 cells treated with 50, 75 and 100 µM hydrogen peroxide for 5 min at 4 °C. Results are mean ± 1 SD of three individual experiments.

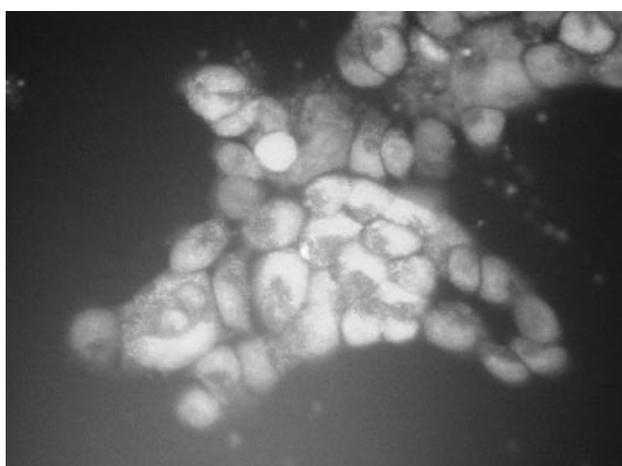
H₂O₂ treatment was used as a positive control of DNA repair ability. NPC/HK1 cells were able to repair H₂O₂ mediated DNA damage. The results showed a statistically significant decrease in DNA damage after the 1.5 h recovery period at 75 and 100 µM (Figure 5b).

Apoptosis by DAPI

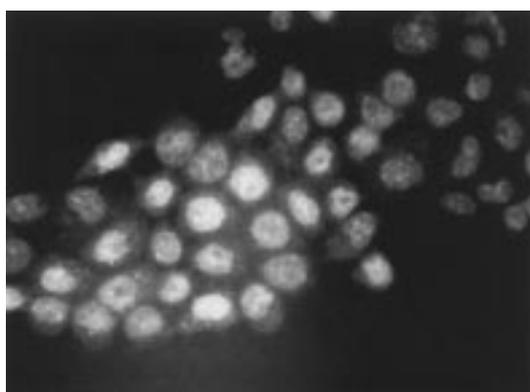
No apoptotic cells were observed at 100 µM and 200 µM berberine treatment from 2 to 19 h of incubation. Only a few apoptotic cells were present after 24 h with 200 µM berberine treatment (200 µM); this was non-significant.



(a)



(b)



(c)

Figure 6. NPC/HK1 cells cultured on culture dish were incubated with 200 μM of berberine for 2-46 h. a) Cells treated for 2 h; b) Cells treated for 21 h; c) Cells treated for 46 h. Fluorescence indicates the presence of berberine.

Drug uptake, and intracellular localization of berberine

Berberine was observed mainly in the cytoplasmic region at 2 h of incubation at 200 μM . At higher concentrations and longer incubation times (200 μM , 8 h), berberine was observed near the nuclear regions of the cells (Figures 6).

Discussion

In this study, the cancer cell killing effect was assessed and an effective cytotoxicity (LD_{50}) was achieved within a reasonable length of treatment (5 h) and concentration (200 μM). In addition to cell killing, DNA was found to be damaged after treatment. It was indicated that berberine toxicity on NPC/HK1 cells may be partly mediated through hydrogen peroxide (Figure 4), because catalase alleviated the adverse effect on the DNA and the viability of NPC cells contributed by berberine. The DNA damage caused by hydrogen peroxide was repaired (Figure 5b). Therefore, inhibition of DNA repair activity did not occur via the presence of hydrogen peroxide. Furthermore, berberine was observed to be near the nuclear region. It has been noted that berberine was able to bind with DNA (18) and may inhibit DNA topoisomerase II. Therefore, inhibition of DNA repair ability and stability may also contribute to the cytotoxic effect on the NPC cells. However, no significant amount of apoptotic cells was triggered in the cells after berberine treatment, even though this has been observed in other cell lines (18,25). This may be due to the different thresholds of drug treatment that are required in order to see a biological effect.

A large variation in tail DNA content was observed in the comet assay data. Since the comet assay is a highly sensitive tool for DNA damage detection, a large amount of damaged DNA may lead to difficulty in defining the comet head and tail and hence give variations in tail DNA content among individual experiments.

In conclusion, berberine treatment on NPC cells demonstrated a high killing efficacy. The DNA damaging effect and the inhibition of DNA repair contributed to the cytotoxic activity in the cancer cells; this may be part of the mechanisms of action of berberine on cancer cells.

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