Berberine induces double-strand DNA breaks in *Rev3* deficient cells

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Abstract. Berberine is a natural isoquinoline alkaloid, the majority of which is extracted from Huang Lian and other medicinal herbs. Numerous studies have revealed that berberine exhibits anticancer activity, however the mechanisms underlying this effect remain elusive. To examine these mechanisms, we analyzed the effects of berberine on a panel of DNA repair deficient chicken B lymphocyte (DT40) clones. Our results revealed that DT40 cells deficient in *Rev3* (*Rev3*$^{-/-}$), a translesion DNA synthesis (TLS) gene, were hypersensitive to berberine. Following berberine treatment, cell cycle analysis identified that G2/M arrest was increased in *Rev3*$^{-/-}$ cells. Furthermore, compared with wild-type cells (WT), berberine also induced a significant increase in double-strand breaks (DSBs) in *Rev3*$^{-/-}$ cells, as revealed by chromosomal aberration (CA) analysis. These results suggest that berberine is able to induce DNA damage, and that the *Rev3* associated DNA repair pathway participates in the processes that aid its repair.

Introduction

Berberine is the major active component of the Chinese herbal medicine Huang Lian (1), which has been widely used clinically as a natural microbial agent in the treatment of diseases like dysentery and gastroenteritis (2,3). In recent years, a number of studies have reported that berberine has antitumor effects on numerous cancer cell line types, including leukocyte, glioblastoma, esophageal, breast and osteosarcoma (4-8). The anticancer mechanisms of berberine appear to be complex: Certain studies have suggested that berberine may interfere with DNA replication, acting as an inhibitor of replication and repair enzymes (6), while others have implied it may induce DNA damage directly (9). In addition, it has been reported that berberine is capable of inducing apoptosis through DNA damage-independent signaling pathways, including fatty acid synthase (Fas) receptor and estrogen receptor (10,11).

As high doses of berberine provoke a wide variety of cellular responses, the mechanisms that explain its genotoxicity remain difficult to define. *Rev3*, a catalytic subunit of DNA polymerases ζ (Polζ), participates in translesion DNA synthesis (TLS) and has been identified as an important component in the maintenance of chromosomal DNA, in its ability to enhance tolerance to DNA damage (12). Previously, we generated *Rev3* deficient DNA repair deficient chicken B lymphocyte (DT40) cells (*Rev3*$^{-/-}$) and identified they were hypersensitive to various DNA damaging agents, including IR, cisplatin and UV (13,14). Besides their specific sensitivity, *Rev3*$^{-/-}$ cells also possess a number of advantages as DT40 clones. Firstly, the proliferation of DT40 clones with a cell cycle time of 8 h is more rapid than that of the majority of mammalian cell lines. Secondly, during logarithmic growth, 70% of DT40 cells are in S phase, in contrast to the 50% for mammalian cells, so that exogenous DNA damage may target DNA replication directly. Finally, due to the absence of functional p53, DT40 cells lack the G1/S checkpoint. Therefore, DNA repair activity contributes a greater extent to cell survival, which aids our observations of the chromosomal breaks in mitotic cells, as cells carrying double-strand breaks (DSBs) can enter the M phase without stimulating the apoptosis pathway (15). As a result, *Rev3*$^{-/-}$ cells have been characterized as an excellent tool for studying the DNA damage and repair mechanisms of different chemicals, including tamoxifen and nitric oxide (NO) (11,16).

In the present study, we used *Rev3*$^{-/-}$ and other DNA repair gene deficient cells to study the mechanisms of berberine. We identified that *Rev3*$^{-/-}$ and several other DNA repair gene deficient clones including *Rev1*$^{-/-}$, *Parp1*$^{-/-}$, *Rad18*$^{-/-}$, *Xrcc2*$^{-/-}$,

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Table I. DNA repair genes mutated in the analyzed DT40 clones.

Gene	Function	Reference
Rev3	TLS, catalytic subunit of Polζ	12
Rev1	TLS, deoxycytidyl transferase activity	19
Rad18	TLS	20
PARP1	Poly(ADP) ribosylation, related to single-strand and base excision repair	21
XPA	An initial step of nucleotide excision repair	20
DNA-PKcs	Nonhomologous end-joining-dependent double-strand break repair, the catalytic subunit of DNA-dependent protein kinase	22
Ku70	Initial step of nonhomologous end-joining-dependent double-strand break repair, associated with DNA-PK catalytic subunit	19
Rad52	Homologous recombination	23
Xrcc2	Rad51 paralog, homologous recombination, promotion of Rad51 assembly	24
Brca1	Homologous recombination	23
Brca2	Homologous recombination	25

Pol, polymerase; TLS, translesion DNA synthesis.

were hypersensitive to the naturally occurring alkaloid. Cell cycle and chromosomal break analysis demonstrated that berberine induced a significant increase in G2/M phase cells and chromosomal breaks in *Rev3*$^{-/-}$, compared with the wild-type (WT) cells. These results suggest that berberine is able to directly induce DNA damage, and that *Rev3* participates in the processes that aid its repair.

Materials and methods

Chemicals. The berberine chloride and alachlor was obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solution of berberine (20 mM) was prepared in DMSO and stored at -20˚C in aliquots until use.

Cell line and cell culture. The cells were cultured as described previously (17,18). The cell lines used in this study are listed in Table I. The phenotypes of these cells have been previously described (12,19-25). The WT cell line and the DNA repair-deficient cell lines differ only in the presence/absence of an endogenous DNA repair gene, other than this difference, their genetic backgrounds were identical (15). All cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% chicken serum, 0.1% β mercaptoethanol and 1% penicillin. The cell lines were maintained at 39˚C under a humidified atmosphere and 5% CO_2. The study was approved by the Ethics Committee of Sichuan University (Chengdu, China).

Measurement of cell proliferation by ATP assay. To assess cell numbers following treatment with berberine, we measured the amount of ATP in the whole cell lysate (26). Cells ($1.5x10^3$-$1.5x10^4$) were incubated in 1 ml culture medium per well, containing various concentrations of berberine. At 72 h, the ATP in the cellular lysates was measured to assess the number of live cells. At least three independent experiments were conducted. Sensitivity was calculated by dividing the number of drug-treated cells, by the number of untreated cells (13).

Cell-cycle analysis. Following treatment with 5 μM berberine for 16 h, cells were labeled for 10 min with 20 μM Bromodeoxyuridine (BrdU) and subsequently harvested. Harvested cells were fixed and analyzed as previously described (19): (i) In 4N HCl, 0.5% Triton X-100 for 30 min at room temperature; (ii) in FITC-conjugated anti-BrdU antibody for 1 h at room temperature; (iii) in 5 μg/ml propidium iodide (PI) in PBS. Following incubation, cells were washed with PBS containing 2% FCS and 0.1% sodium azide. Subsequent flow cytometric analysis was performed on a FACScan. Fluorescence data were displayed as dot plots using the Cell Quests software.

Chromosomal aberration (CA) analysis. Measurement of CA was performed as previously described (12). The chicken karyotype consisted of 80 chromosomes, including 11 major autosomal macrochromosomes, the ZW sex chromosomes, and 67 microchromosomes (15). In the present study, only the 11 major autosomal macrochromosomes were measured. For CA analysis, cells ($3.0x10^6$/3 ml) were treated with the test doses of 2.5 and 5 μM for berberine and 5 μM for alachlor for 16 h. Then cells were treated for 30 min with 0.1 μg/ml colcemid and went on harvesting for 2.5 h. We then suspended the cells with 75 mM KCL and incubated them for 15 min at room temperature, adding 5 ml of Carnoy's solution (mixture of acetic acid and methanol, 1:3) and left for at least 30 min at room temperature. The cells were then dropped onto ethanol-cleaned slides and dried by a flame. The dried slide was then dipped into 5% Giemsa solution, left for 8 min at room temperature and rinsed carefully with water and finally, they were air-dried. The cells were observed under a light microscope (magnification, x1,000).

Statistical analysis. Survival data were log-transformed giving approximate normality. Analysis of covariance (ANCOVA)

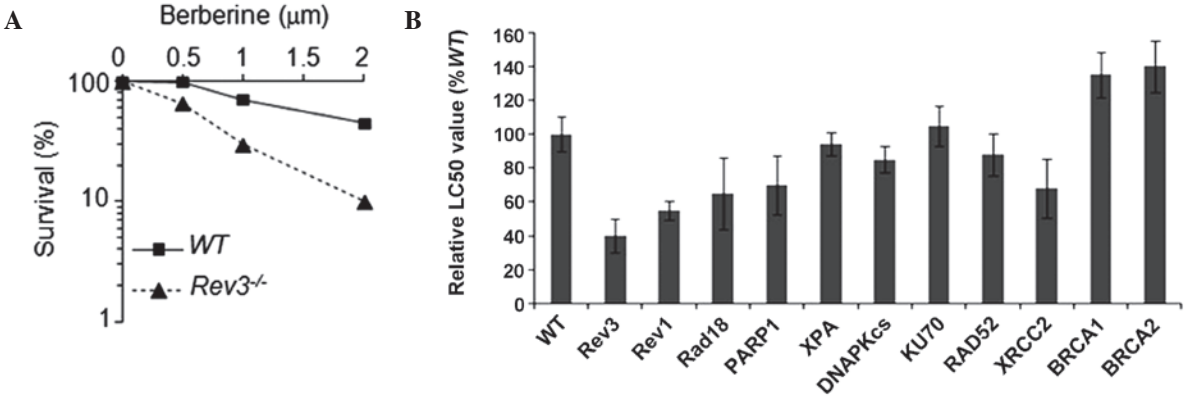

Figure 1. Cellular tolerance to berberine. (A) Cells of the indicated genotype were exposed to berberine for 72 h, a period during which WT cells were able to divide nine times in the absence of exogenous DNA damage. The x-axis represents the concentration of berberine and the y-axis represents the relative number of surviving cells at 72 h. (B) Relative LC50 values of berberine. Values shown are the mean ± SD. WT, wild-type; LC50, the concentration of berberine that reduces cellular survival to 50% relative to cellular survival without berberine treatment.

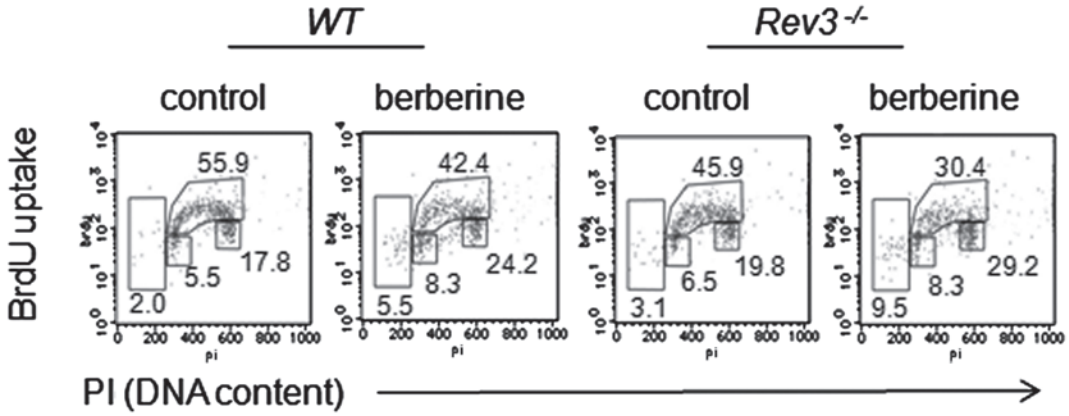

Figure 2. Representative cell-cycle distribution of the indicated cell cultures as measured by pulse BrdU incorporation and DNA content in flow cytometric analysis. Cell-cycle distribution of berberine treated cells that were pulse labeled with BrdU for 10 min and subsequently stained with FITC-conjugated anti-BrdU antibody (y-axis, log scale) and PI (x-axis, linear scale). The upper gate indicates cells incorporating BrdU (S phase), the lower middle gate indicates G1 cells and the lower-right gate indicates G2/M cells. The sub G1 fraction (lower-left gate) indicates dead cells. The number in each gate indicates the percentage of gated events. The berberine pretreatment time is 16 h. PI, propidium iodide; WT, wild-type; *Rev3*$^{-/-}$, *Rev3* deficient DT40; BrdU, bromodeoxyuridine.

was used to test for differences in the linear dose-response curves between WT and a series of mutant cells. Viability of the DT40 cells was estimated using regression curves. Regression-curve equations were used to calculate LC50 (lethal concentration, 50%) values. Relative LC50 values were normalized according to the LC50 value of the parental WT cells.

Results

Rev3$^{-/-}$ *cells are sensitive to berberine*. As *Rev3*$^{-/-}$ are specifically sensitive to a variety of DNA damaging agents, in the present study we assessed whether *Rev3*$^{-/-}$ cells exhibit a higher sensitivity to berberine. We treated WT and *Rev3*$^{-/-}$ cells with varying concentrations of berberine for 72 h, and measured the viability of cells using an ATP assay. As summarized in Fig. 1, *Rev3*$^{-/-}$ cells exhibited a significant increase in sensitivity to berberine when compared with WT cells. This observation indicated that berberine may induce DNA damage, and that *Rev3* associated DNA repair is an important regulatory process in cellular tolerance to berberine-induced genotoxicity. To study whether other DNA repair pathways were also involved in tolerance to berberine, we assessed the sensitivity of berberine in a panel of DT40 clones, which were separately deficient in different DNA repair pathways (Table I). The DNA repair pathway mutants demonstrated sensitivity to berberine in the following order: Rev3 > Rev1 > Parp1 = Xrcc2 = Rad18 > WT (parental DT40 cells)

Berberine induces enhanced arrest of cell cycle in G2/M phase. As DT40 cells are deficient in p53, and agents causing DNA damage would lead to cell arrest in G2/M phase, we assessed if the increased sensitivity of *Rev3*$^{-/-}$ to berberine was also correlated with G2/M arrest. Data from the pulse labeled with BrdU indicated that berberine induced an increase in the accumulation of *Rev3*$^{-/-}$ cells in the G2/M phase compared with that in the WT cells (Fig. 2).

Berberine induces an increase of chromosome breaks in Rev3$^{-/-}$ *cells*. To examine if the higher sensitivity of *Rev3*$^{-/-}$ to berberine was specifically associated with DNA damage, we measured the number of chromosome breaks in mitotic cells.

Table II. Frequencies of chromosomal aberrations in WT and *Rev3* deficient DT40 cells.

Cell	Treatment	Chromatid		Isochromatid		Exchange	Total
		Gaps	Breaks	Gaps	Breaks		
WT	-	0	2	0	0	0	2
WT	Berberine (2.5 μm)	0	2	0	1	1	4
WT	Berberine (5 μm)	1	3	1	1	1	7
WT	Alachlor (5 μm)	0	13	1	2	2	18
Rev3$^{-/-}$	-	1	1	1	1	0	4
Rev3$^{-/-}$	Berberine (2.5 μm)	1	9	1	2	2	15
Rev3$^{-/-}$	Berberine (5 μm)	1	17	2	2	1	23
Rev3$^{-/-}$	Alachlor (5 μm)	0	35	4	2	5	46

The data represent the numbers of aberrations per 50 cells. At least 50 mitotic cells were analyzed for each genotype. The pretreatment time is 16 h. WT, wild-type; *Rev3*$^{-/-}$, *Rev3* deficient DT40.

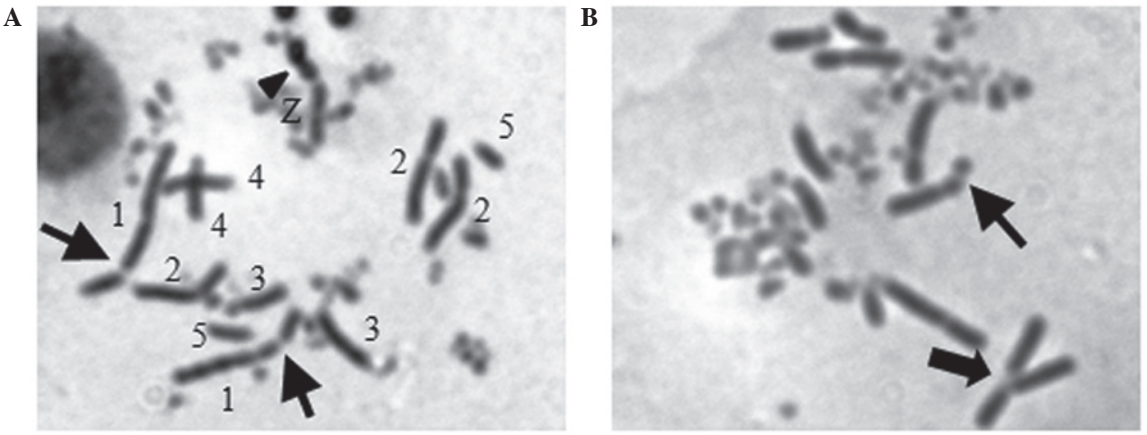

Figure 3. Representative karyotype analysis of berberine and alachlor pretreated *Rev3*-deficient cells. (A) CAs in *Rev3*$^{-/-}$ cells following 5 μM berberine pretreatment for 16 h. (B) CAs in *Rev3*$^{-/-}$ cells following 5 μM alachlor pretreatment for 16 h. Macrochromosomes 1-5 and Z are identified. Chromatid breaks are shown by arrows and gaps are shown by arrowheads, respectively. Exchanges are shown by crude arrows. CA, chromosome aberration; *Rev3*$^{-/-}$, *Rev3* deficient DT40.

Fifteen CAs were detectable in 50 mitotic *Rev3*$^{-/-}$ cells when the concentration of berberine was 2.5 μM, while only 4 CAs were identified in WT cells. As the concentration elevated to 5 μM, the CAs in *Rev3*$^{-/-}$ cells markedly increased to 23, by contrast, WT cells showed fewer aberrations of only 7 (Table II and Fig. 3). This result was consistent with the positive control, alachlor. The data suggest that the hypersensitivity of *Rev3*$^{-/-}$ to berberine is correlated with a significant increase of berberine-induced CAs.

Discussion

In the present study, we utilized a panel of DNA repair deficient DT40 cells to study the genotoxicity mechanisms of the natural alkaloid, berberine. Our results demonstrated that cells deficient in *Rev3* exhibited the highest sensitivity to berberine. The cell cycle and chromosome breaks assay provided evidence that more G2/M arrest and chromosome breaks occurred in *Rev3*$^{-/-}$ cells than in WT cells. While the *Rev*$^{-/-}$ cells were the most sensitive, the other DNA repair gene deficient cells, including *Rev1*$^{-/-}$, *Parp1*$^{-/-}$, *Rad18*$^{-/-}$ and *Xrcc2*$^{-/-}$ cells were also sensitive to berberine, but *polβ*$^{-/-}$, *XPA*$^{-/-}$, *DNA-PK*$^{-/-}$ cells were not. Two important homologous recombination (HR) enzymes, Brca1 and Brca2, were slightly resistant to berberine. These results provided clear evidence that berberine is able to induce DNA damages, and that *Rev3* as well as *Rev1*, *Rad18*, *Parp1* and *Xrcc2* are important in processes that repair these damages.

Rev3 is a catalytic subunit of Polζ. Studies in yeast have revealed that Polζ cells are able to efficiently bypass abasic sites, through extending from nucleotides inserted opposite the lesion, by other TLS DNA polymerases. These data suggest Polζ may be involved in the extension process of TLS in higher eukaryotes (27). One reverse genetics study in DT40 cells identified that Rev3 participated in not only TLS but also HR (12). Rev1 and Rad18 appear to have a similar function to Rev3 in maintaining genomic stability by TLS and HR (19). The high sensitivity of *Rev3*$^{-/-}$, *Rev1*$^{-/-}$ and *Rad18*$^{-/-}$ to berberine implies that TLS or HR may participate in berberine-induced DNA damage. TLS and HR are two major pathways that promote survival following post-replication DNA damage (28,29).

HR accurately repairs DSBs that arise during the mitotic cell cycle or those induced by radiotherapy (30,31). This process also releases replication forks that stall at damaged template DNA strands, by using intact sister chromatids as a template (32,33). The function of TLS is to release stalled replication forks by filling gaps on daughter strands that remain, following DNA replication caused by damage on the mother strand (34). Although cells deficient in *Brca1* or *Brca2*, were not sensitive but slightly resistant to berberine, cells deficient in *Xrcc2*, a RAD51 paralog in HR (35), did exhibit sensitivity to berberine. *Parp1* regulates several processes of DNA repair and also participates in HR. The sensitivity of *Parp1*$^{-/-}$ and *Xrcc2*$^{-/-}$ cells to berberine suggested that HR may participate in berberine-induced DNA repair. The importance of HR in repairing berberine-induced DNA damage has also been proved in yeast (9). Earlier studies have demonstrated that *Brca1*$^{-/-}$ and *Brca2*$^{-/-}$ cells were highly sensitive to several chemotherapeutic agents including cisplatin, camptothecin and olaparib, which separately stalled DNA replicative polymerases, via the formation of intra- and inter-strand crosslinks, inhibition of topoisomerase I and inhibition of *Parp1* (36). In addition, previous studies have shown that *Rad18*$^{-/-}$ rather than *Rev3*$^{-/-}$ was more sensitive to camptothecin (37). These differences imply that the anticancer mechanisms of berberine are different from the aforementioned chemotherapeutic agents, although all of them are able to induce DSBs and HR repair. These results present that there are different DNA damage mechanisms between berberine and these agents, although certain studies considered that berberine may function as a topoisomerase I inhibitor like camptothecin (38).

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