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Berberine Decreased N-acetylation of 2-Aminofluorene through Inhibition of N-acetyltransferase Gene Expression in Human Leukemia HL-60 Cells

CHIN-CHUNG LIN[1,3], SHUNG-TE KAO[1], GUANG-WEI CHEN[1] and JING-GUNG CHUNG[2]

[1]*Graduate Institute of Chinese Medical Science, School of Chinese Medicine, Taiwan;*
[2]*Department of Microbiology, School of Biological Science and Biotechnology, China Medical University, Taiwan;*
[3]*Fong-Yuan Hospital, Fong Yuan, Taiwan, R.O.C.*

Abstract. *Previous studies have demonstrated that berberine decreases N-acetyltransferase (NAT) activity in human leukemia HL-60 cells, however, NAT gene expression has not been investigated. In this study, berberine was selected for testing the inhibition of N-acetylation of 2-aminofluorene (AF) and NAT gene expression in human HL-60 cells. The N-acetylation of AF was determined and quantitated by high performance liquid chromatography (HPLC). The data showed that a 24-hour berberine treatment decreased the amount of N-acetylation of AF in HL-60 cells. The NAT enzymes were stained and examined by Western blotting and flow cytometry. The results indicated that berberine decreased the levels of NAT protein in HL-60 cells. The expression of NAT gene (mRNAT NAT1) was determined by polymerase chain reaction (PCR), and it was found that berberine had inhibited the expression of mRNA NAT1 in human HL-60 cells.*

Berberine, a yellow benzylisoquinoline alkaloid, has a wide range of pharmacological effects, including anti-inflammation (1), antidiarrhetic, antimalaria (2) and liver tonic (3). Berberine displays antimicrobial activity to both Gram-positive and Gram-negative bacteria as well as to other micro-organisms (4, 5). Berberine has, subsequently, been examined for anticancer activity following evidence of antineoplastic properties (6-9). Berberine exhibits the ability to induce apoptosis in promyelocytic leukemia HL-60 cells, which formed berberine complexes with DNA (10).

Correspondence to: Dr. Jing-Gung Chung, Department of Microbiology, School of Biological Science and Biotechnology, China Medical University, No 91 Hsueh-Shih Road, Taichung 400, Taiwan, Republic of China. Tel: (4)22053366, Fax: (4)22053764, e-mail: jgchung@mail.cmu.edu.tw

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N-acetyltransferase (NAT) activities have been demonstrated in some human gastrointestinal cells (11). Many arylamine chemicals require N-acetylation before leading to carcinogenesis in target organs or tissues. N-acetylation is a first step for arylamine metabolism, and is catalyzed by cytosolic NAT with acetyl coenzyme A as an acetyl group donor (12). NAT activities can be divided into slow and rapid acetylator phenotypes, which are associated with the risks of individuals after exposure to arylamine carcinogens (13, 14). In our previous studies, we showed that berberine affects NAT activity in human cancer cell lines such as bladder T24 (15), colon Colo-205 (16), blood leukemia HL-60 (17), malignant astrocytoma (G9T/VGH) and brain glioblastoma multiforme (GBM 8401) (18). However, there are no reports concerning the effects of berberine on the NAT enzyme or its gene expression in human leukemia HL-60 cells. Thus, the present study was focused on the effects of berberine on N-acetylation of 2-aminofluorene (AF). NAT gene expression of human leukemia HL-60 cells was examined by Western blotting, PCR and flow cytometry.

Materials and Methods

Chemicals and reagents. Berberine, 2-aminofluorene (AF), Tris-HCl, dimethyl sulfoxide (DMSO), N-acetyl-2-aminofluorene (AAF), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), phenylmethylsulfonylfluoride (PMSF), acetylcarnitine, leupeptin, bovine serum albumin (BSA), acetyl-coenzyme A and carnitine acetyltransferase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640, fetal bovine serum, penicillin-streptomycin and L-glutamine were obtained from Gibco BRL (Grand Island, NY, USA). All of the chemicals used were reagent grade.

Human blood leukemia HL-60 cell line. HL-60 cells (human promyelocytic leukemia cell line) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). About $1x10^5$ cells/ml in RPMI 1640 medium

supplemented with 10% fetal bovine serum, penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 1% L-glutamine were placed into 75-cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 and 95% air atmosphere.

Effects of various concentrations of berberine on N-acetylation of AF in HL-60 intact cells. $5x10^5$ HL-60 cells/well of medium were incubated with 6.75 μM AF solution in individual wells of 24-well cell culture plate with or without berberine (0, 5, 15, 30 and 60 μM). After incubation for 24 h, the cells and media were removed and centrifuged. The supernatant was immediately extracted with ethyl acetate/ methanol (95:5). After the solvent had evaporated, the residue was redissolved in methanol and assayed for N-acetylation of AF (AAF) by HPLC, as described previously (17, 19-21).

Development of NAT antibody. A) *Preparation of recombinant proteins for immunization:* The preparation of recombinant protein for immunization was performed as previously described (22).

B) *Preparation of polyclonal antibody:* The preparation of polyclonal antibody was performed as previously described (22).

Effects of various concentrations of berberine on NAT protein of HL-60 cells examined and quantitated by flow cytometry. HL-60 cells ($2x10^5$/ml) were treated with 0, 5, 15, 30 and 60 μM of berberine for 24 h. The cells were harvested, stained by NAT-antibody and assayed by flow cytometry (Becton Dickinson FACS Calibur) using the prepared polyclonal antibody mentioned above. The intracellular NAT was determined as previously described (23, 24).

Effects of various concentrations of berberine on NAT protein of HL-60 cells examined and quantitated by Western blotting. Approximately $5x10^6$ HL-60 cells were placed in each well of a 6-well plate with 0, 5, 15, 30 and 60 μM of berberine for 24 h. The cells were then harvested, lysed, and Western blotting was preformed as described previously (24, 25).

Reverse transcriptase polymerase chain reaction (RT-PCR) for examining the effect of 30 μM berberine on NAT gene expression in HL-60 cells. The total RNA was extracted from HL-60 cells after exposure to 30 μM of berberine for 24 h, by using Qiagen RNeasy Mini Kit. The entire protocol has been described previously (25, 26). The sequence of primers was as follows: B-MDIEA-NAT1, 5'-CACCCGGATCCGGGATCATGGACATTGAAGC-3', nt 435-454, GenBank accession number X17059; VPKHGD-X-NAT1, 5'-GGTCCTCGAGTCAATCACCATGTTTGGGCAC-3', nt 1295-1278, GENBANK accession number X17059; FP1-NAT2, 5'-CTAGTTCCTGGTTGCTGGCC-3', nt 79-98, GenBank accession number NM-000015; RP1-NAT2, 5'-TAACGTGAGGGTAGAGA GGA-3', nt 1073-1054, GenBank accession number NM-000015; Act b1, 5'-GCTCGTCGTCGACAACGGCTC-3', nt 94-114, GenBank accession number NM-001101; Act2 b2, 5'-CAAACAT GATCTGGGTCATCTTCTC-3', nt 446-422, GenBank accession number NM-001101 (23, 24).

Statistical treatment of data. Statistical analysis of the data was performed with an unpaired Student's *t*-test and ANOVA analysis (25, 26).

Results

Inhibition of AF N-acetylation by berberine in intact human leukemia HL-60 cells. The representative profiles of AF and AF N-acetylation (AAF) from HPLC are presented in Figure 1, and the mean±SD values of AF and AAF in HL-60 intact cells treated with or without various concentrations of berberine are shown in Figure 2. In the presence of increasing berberine concentrations, AAF production was found to decrease.

Effects of berberine on the levels of NAT protein in human leukemia HL-60 cells. The percentage of NAT-antibody complex from HL-60 cells after treatment with or without various concentrations of berberine for 24 h was measured and quantitated by flow cytometry (Figure 3 and Table I) and Western blotting (Figure 4). NAT levels decreased in response to increasing berberine concentrations in both examined methods (Figure 4).

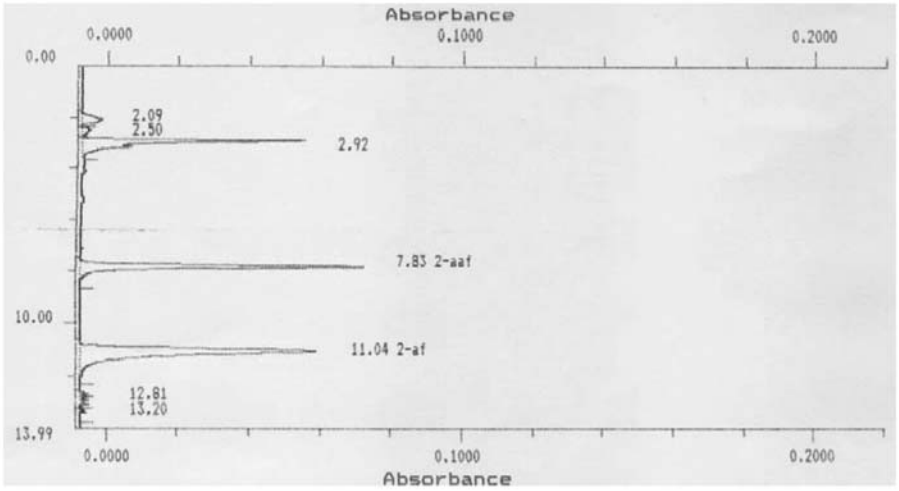
Effects of berberine on NAT1 mRNA expression in intact HL-60 cells as examined by polymerase chain reaction (PCR). The results presented in Figure 5 indicated that NAT1 mRNA levels decreased at 30 μM berberine and were significantly different from those in the control. The mRNA levels of NAT1 and β-actin on gel-electrophoresis were quantified by densitometric analysis of gel-photographs and expressed as NAT1/β-actin. The result do not indicate NAT2 expression.

Discussion

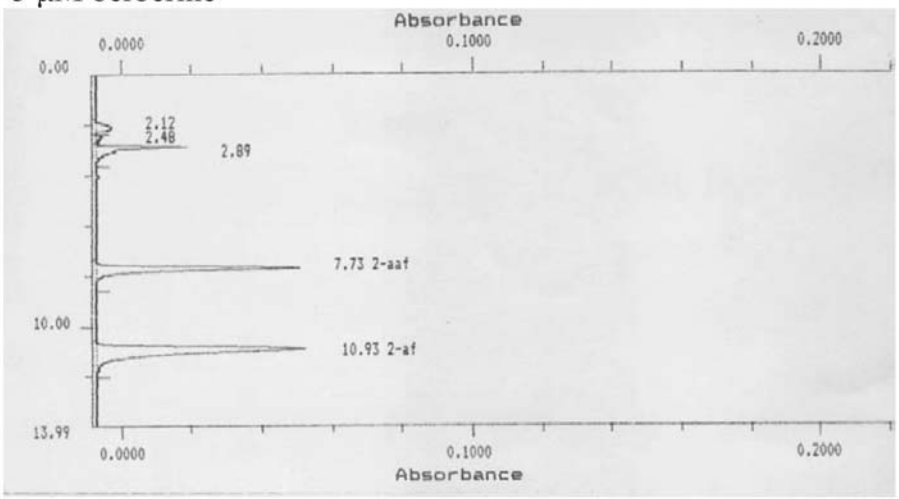
Many studies have demonstrated that AF N-acetylation can be induced by NAT in HL-60 cells. Our previous studies also showed that berberine affected the levels of N-acetylated AF (NAT activities) and increased incubation times, leading to an increase in AAF production, as determined by high performance liquid chromatography (HPLC) (19). However, there is no information addressing the effect of berberine on *NAT* gene expression in HL-60 cells. Therefore, in the present study, we first examined the effects of berberine on AF N-acetylation in intact HL-60 cells and then we examined whether the gene expression of *NAT* was affected by berberine. Berberine treatment attenuated the amounts of AF N-acetylation (AAF production) in HL-60 (Figures 1 and 2).

So far, the role of decreased NAT activity in cancer or leukemia development and/or prevention is still unclear. Based on the following three observations, it could be concluded that decreased NAT activity leads to decreased tumor production: (i) NAT enzyme has been shown to be involved in the process of chemical carcinogenesis (27, 28); (ii) the decrease of AF N-acetylation is significant in decreasing tumor development, as seen by the attenuation of liver NAT activity associated with

control



5 μM berberine



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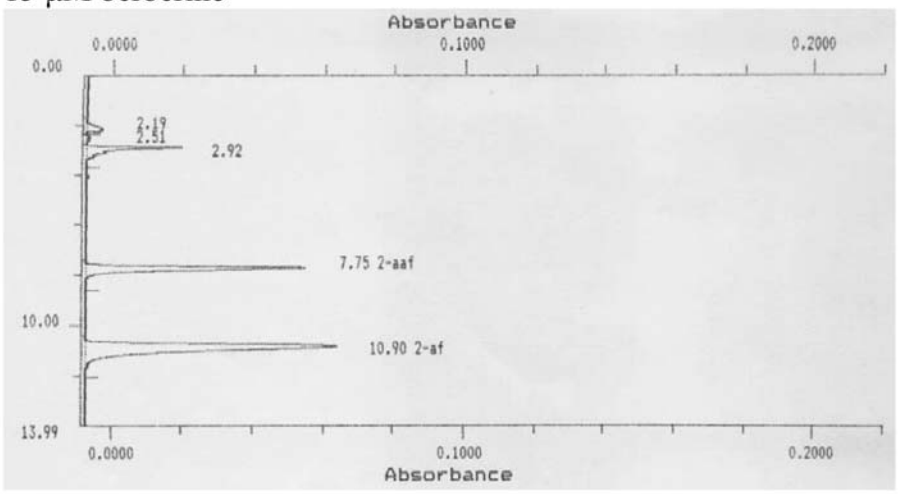

Figure 1.

Figure 1 *continued*

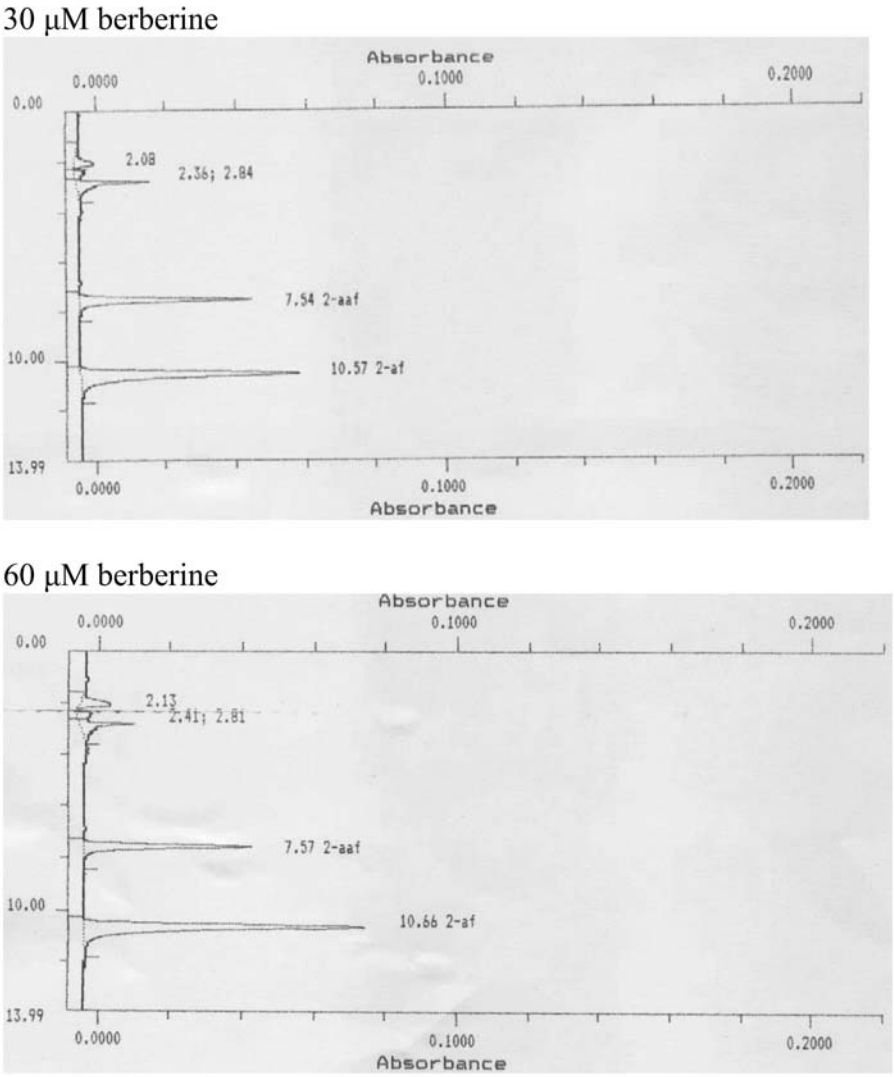

Figure 1. *Effect of berberine on the profiles of N-acetylation of AF in HL-60 cells. Approximately 2x10^5 cells/ml were incubated, as described in "Materials and Methods", with AF 6.75 μM, treated with or without, 0, 5, 15, 30 and 60 μM berberine. The supernatant was collected by centrifugation, and AF and AAF profiles were measured and determined by HPLC assay.*

breast and bladder cancer processes (29, 30); and (iii) increased NAT activity is associated with increased sensitivity to the mutagenic effects of many arylamines (31). Other factors may be associated with the susceptibility to carcinogenic effects within the human population, such as: (i) the levels of AF N-acetylation and N-hydroxylation in the liver, (ii) the route of excretion of AF metabolites from the organs, and (iii) the rates of glucuronide hydrolysis and NAT- mediated activation in the target organs (32).

Other studies have already pointed out that AF was N-acetylated to form AAF, which was further metabolized by means of a mechanism involving the cytochrome P450 enzyme. The cytochrome P450-dependent formation of N-hydroxyl-AAF is considered as the initial rate-limiting step in the metabolism of AAF to mutagenic and potentially carcinogenic products (33). Other investigators have also reported that cytochrome P4501A1 is particularly efficient in catalyzing the conversion of AAF to 7-OH-AAF (34). Two cytochrome P450 cDNAs in *Coptis japonica* cells involved in the biosynthesis of berberine have been reported (35). Liver microsomal cytochrome P450 is well known to exist in multiple forms and to be involved in the detoxification of xenobiotics (36). The effect of berebrine on cytochome P450 should be further investigated in future.

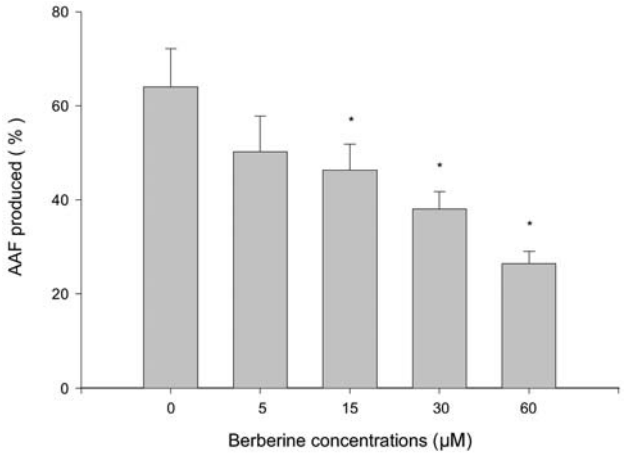

Figure 2. *The effect of various concentrations of berberine on NAT activity in HL-60 cells. The intact cells were incubated for 24 h in the presence of 0, 5, 15, 30 and 60 μM berberine. AF and AAF were measured by HPLC assay. Each point represents the mean of triplicate assays of three incubations of cells. *Mean significant differences between control and treated groups. $p<0.05$*

The results from this study also indicate that berberine decreases the amounts of AF N-acetylated in intact HL-60 cells in a dose-dependent manner. The results from PCR, Western blotting and flow cytometry also show that berberine affects *NAT1* mRNA gene expression and decreases the amounts of the NAT1 enzyme. The product of *NAT2* mRNA was not affected in the PCR experiments in HL-60 cells, since it is known that NAT2 is not present in these cells. The mechanism of the berberine-mediated decrease of N-acetylation of AF (AAF production) in HL-60 cells may involve inhibition of *NAT1* gene expression leading to decrease amounts of NAT.

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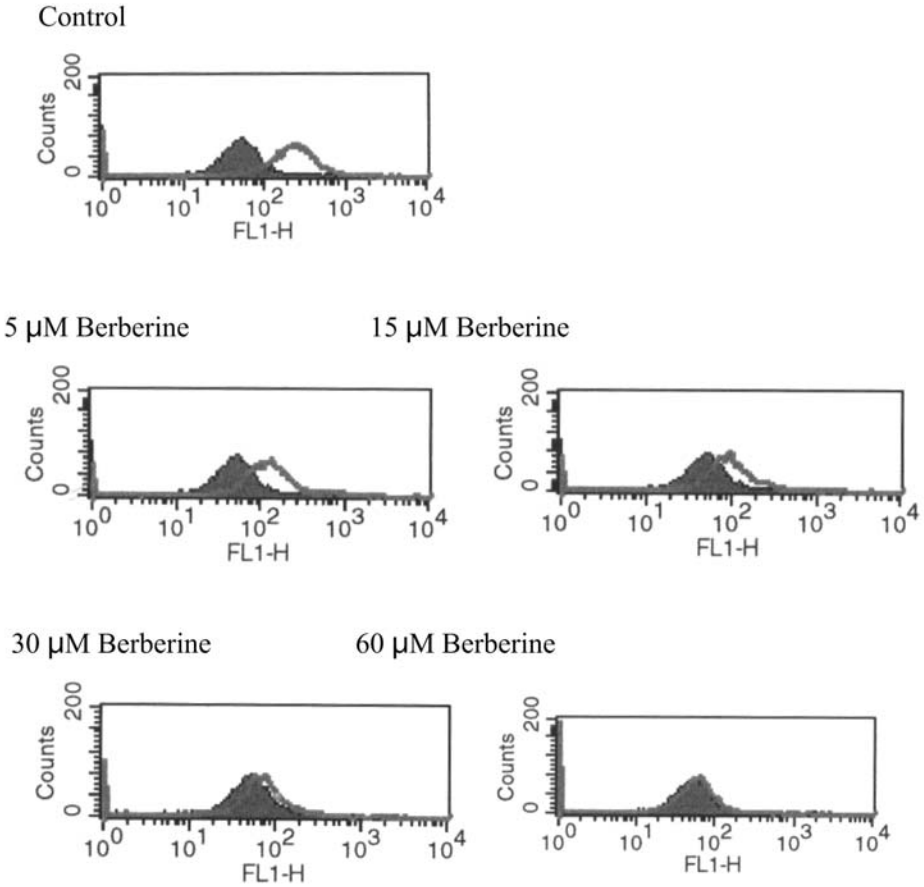

Figure 3. *Representative flow cytometry showing changes in NAT in human leukemia HL-60 cells in response to berberine. Approximately $5x10^6$/ml were treated with various concentrations of berberine for 24 h, followed by evaluation of NAT expression. NAT expression was examined and quantitated by flow cytometry, as described in Materials and Methods.*

Table I. *Flow cytometric analysis of NAT1 expression in human leukemia HL-60 cells treated with or without various concentrations of berberine for 24 h.*

Berberine (μM)	Percentage of cells stained by anti-NAT
0 (control)	82.9±8.6
5	72.8±8.2
15	54.4±6.9
30	41.6±4.9*
60	22.4±3.2*

Values are mean±S.D. n=3. HL-60 cells (1x10^6 cells/ml) were treated with various concentrations of berberine. The zero concentration was defined as control. The percentage of cells that were stained by anti-NAT antibody, and the stained cells were determined by flow cytometry, as described in the Materials and Methods section.
*differs between berberine and control. $p<0.05$.

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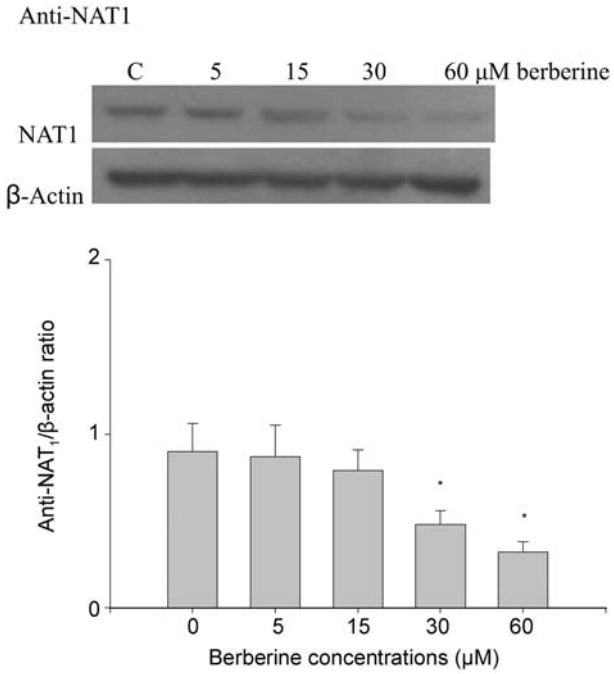

Figure 4. *Representative Western blot showing changes in NAT in human leukemia HL-60 cells in response to berberine. Approximately 5x10^6/ml cells were treated with various concentrations of berberine for 24 h, followed by evaluation of NAT expression. NAT expression was examined and quantitated by Western blotting, as described in Materials and Methods.*

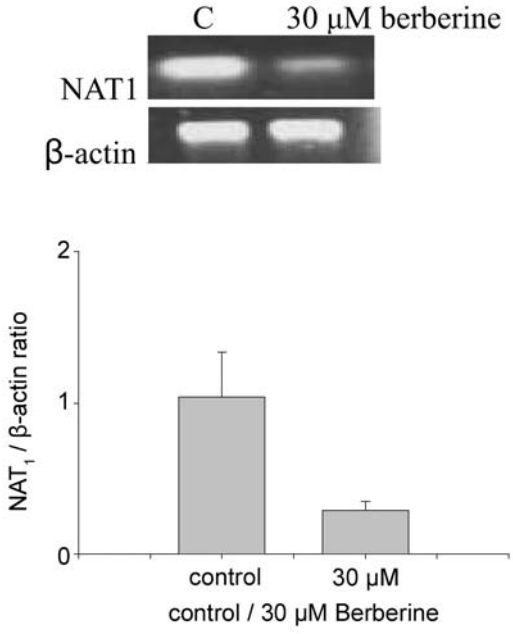

Figure 5. *The effect of berberine on NAT1 mRNA expression in human leukemia HL-60 cells. Approximately 5x10^6/ml cells were incubated with 30 μM berberine for 24 h. The cells were collected and RNA was extracted. The extracted RNA was subjected to RT-PCR analysis using specific primers for NAT1 and β-actin, and then PCR-amplified cDNA derived from mRNA were applied to agarose gel-electrophoresis. The mRNA NAT1 and the ratio of NAT1/β-actin are provided.*

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