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Arylamine *N*-acetyltransferases: a new inhibitor of apoptosis in HepG2 cells*

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Abstract: Objective: To explore how arylamine *N*-acetyltransferases (NATs) is related to cell apoptosis. Methods: NAT activity in apoptotic HepG2 cells was measured using high performance liquid chromatography (HPLC); the apoptosis rate of HepG2 cells acted upon by an NAT inhibitor was measured using flow cytometry. Results: NAT activity was lowered in apoptotic HepG2 cells; apoptosis rate induced by camptothecin (CAM) increased after inhibition of NAT activity in HepG2 cells. Conclusion: NAT can inhibit apoptosis in HepG2 cells.

Key words: Arylamine *N*-acetyltransferases (NATs), Apoptosis, HepG2, Camptothecin (CAM)
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INTRODUCTION

Arylamine *N*-acetyltransferases (NATs), phase II-drug metabolizing enzymes, are a group of enzymes that catalyze the addition of an acetyl group from Acetyl coenzyme A (AcCoA) to a terminal nitrogen on suitable substrates such as arylamines, hydrazines (Weber and Hein, 1985), arylhydroxylamines (Hein *et al*., 1993) or arylhydrazines (Fig.1). They play an important role in the metabolism of drugs and toxins as well as in detoxification (Hein *et al*., 1992; 1994; King *et al*., 1997). The active center of NATs is a Cys^{69}-His^{107}-Asp^{122} triad. In other words, the triplet of cysteine residue (Cys^{69}), the histidine residue (His^{107}), and the aspartic acid residue (Asp^{122}) have the same active center as cysteine proteases (Turk *et al*., 1998). This structural similarity between NATs and cysteine proteases suggests that they share a common evolutionary origin. Their similar crystal structures also suggest that NATs exert their effects through a mechanism strategy partially common to cysteine proteases (Rodrigues-Lima *et al*., 2001). Since cysteine proteases are an important kind of enzymes associated with cell apoptosis (Gao *et al*., 2005; Ho and Hawkins, 2005), we speculated that NATs may also be associated with cell apoptosis, and then designed the following experiments to test this speculation.

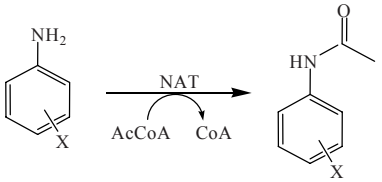

Fig.1 Arylamine *N*-acetyltransferases (NATs) transfer the acetyl group

MATERIALS AND METHODS

Cell line

Human hepatocarcinoma cell line HepG2 was

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originally purchased from American Type Culture Collection (ATCC), USA and was maintained by the Institute of Cancer Studies of Heilongjiang Cancer Hospital, China.

Drugs and reagents

Acridine orange (AO) and ethidium bromide (EB) were purchased from Amresco, USA; Triton X-100, RNase A, propidium iodide (PI), 2-AAF, Tris, leupeptin, AcCoA and bovine serum albumin (BSA) were purchased from Sigma, USA; phenylmethylsulfonyl fluoride (PMSF) was purchased from Amresco, USA; dithiothreitol (DTT) was purchased from Sinopharm Group Chemical Reagent Co., Ltd., Shanghai, China; acetylcarnitine, 2-AF and carnitine acetyltransferase were purchased from Fluka, USA; RPMI 1640 culture medium was purchased from Hyclone, USA; pancreatin was purchased from Gibco, USA; fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., China.

Apparatuses

Waters HPLC (2695 pump, 2487 ultraviolet (UV) detector, automatic sample-injector, and Empower Workstation) and SymmetryShield C_{18} inverted chromatographic column (4.6 mm×250 mm, 5 μm) were from Waters Co., Ltd., USA; super clean table SW-CJ-2F was from Suzhou Purification Equipment Co., Ltd., China; carbon dioxide incubator CO-150 was from New Brunswick Scientific Co., Inc., USA; ultrasonic extractor AS-2060B was from Tianjin Autoscience Instruments Co., Ltd., China; invert microscope CKX-41-32 Olympus was from Japan; flow cytometer EPICS XL-MCL was from Beckman-Coulter, USA.

Preparation of the reagents

Cell lysate (20 ml): 20 μl of 1 mol/L DTT, 5.845 ml of 10 mg/ml ethylenediaminetetraacetic acid (EDTA), 10 μl of 100 mmol/L PMSF, 23.280 μl of 2 mg/ml leupeptin, 14.356 ml of 20 mmol/L Tris-HCl.

AcCoA cycle mixture (3 ml): 6 μl of 1 mol/L DTT, 17.535 μl of 10 mg/ml EDTA, 10.787 mg of acetylcarnitine, 6 U of carnitine acetyltransferase, 2.9177 ml of 50 mmol/L Tris-HCl.

PI staining solution (50 ml): 0.2916 g of NaCl, 2.5 mg of PI, 0.5 mg of RNase A, 1 ml of Triton X-100, 50 mg of sodium citrate. These were dissolved with double-distilled water in a 50 ml volumetric flask. With its volume brought to scale, the solution was kept away from light at 4 °C.

Changes of NAT activity in apoptotic HepG2 cells

1. Cell incubation

HepG2 cells of a suitable concentration were inoculated in a culture flask containing RPMI 1640 solution with 10% (v/v) of fetal bovine serum. The culture was then incubated at 37 °C, with 5% (v/v) CO_2 and saturated humidity. The cells were made to adhere to the inner wall of the flask as they grew, and culture transfer was performed once every 2~3 d.

2. Observation of apoptosis in HepG2 cells induced by CAM

(1) Grouping and drug administration

HepG2 cells in the phase of logarithmic growth were taken and digested with pancreatin, and RPMI 1640 culture medium containing 10% (v/v) of fetal bovine serum was added to adjust the cells to a concentration of 5×10^5 cells/ml. The cell solution was then divided and put in two 100-ml culture flasks as the negative control and the CAM group, respectively. The culture flasks were set in a CO_2 incubator and incubated for 24 h at 37 °C and 5% (v/v) CO_2, after which CAM was added to the CAM group to reach a final concentration of 0.4 μg/ml, while an equal volume of RPMI 1640 culture medium was added to the negative control. The samples were then incubated in a CO_2 incubator for 24 h at 37 °C and 5% (v/v) CO_2.

(2) Apoptosis observed using flow cytometry (Wang *et al*., 2007)

After digested with pancreatin, HepG2 cells were rinsed 3 times with phosphate buffered solution (PBS). The supernatant was discarded, and the residue was blown in a small amount of PBS and mixed well, after which 2 ml of cold ethanol (70%, v/v) was added to fixate the cells, and the sample was left at 4 °C for the night (the fixation needs more than 12 h). The sample was centrifuged the next day, the supernatant was discarded, and the residue was rinsed 3 times with PBS. About 0.1 ml of liquid was retained, and 800 μl of PI staining solution was added to and thoroughly mixed with it. The sample was then left to be stained in dark for 30 min. After it was filtered through a nylon net, the sample was measured with a flow cytometer, with an activation wavelength of 488

nm (Zhang *et al*., 2004).

3. Measurement of NAT activity

HepG2 cells in the phase of logarithmic growth were taken and digested with pancreatin, and RPMI 1640 culture medium containing 10% (v/v) of fetal bovine serum was added to adjust the cells to a suitable concentration. The cell solution was then divided into 7 equal portions, with each in a 100-ml culture flask. The concentration of the cell solution was 5×10^5 cells/ml. After the culture flasks were incubated in a CO_2 incubator for 24 h at 37 °C and 5% (v/v) CO_2, 2-AF as substrates and CAM were added, so that the final concentrations were 45 μmol/L and 0.4 μg/ml, respectively, but no CAM was added to the negative control group. After the samples were incubated in a CO_2 incubator for 24 h at 37 °C and 5% (v/v) CO_2, the culture medium was drawn and centrifuged for 10 min at 3500×*g*, after which the supernatants were immediately extracted with equal volumes of the mixture of ethyl acetate:methanol (95:5, v/v). The supernatants were drawn and evaporation was allowed to proceed until the samples were dry, after which methanol was added to dissolve the residue thoroughly and to bring volume of the solution up to 2 ml. The sample was then measured using Waters HPLC with an automatic sample injection of 20 μl. Conditions used for the HPLC measurement were: SymmetryShield C_{18} inverted chromatographic column (4.6 mm×250 mm, 5 μm); mobile phase (20 mmol/L KH_2PO_4 (pH 4.5):CH_3CN= 53:47, v/v); UV detector with a detecting wavelength of 288 nm and a column temperature of (25±5) °C. The retention time was about 13.5 min for 2-AAF, and about 17 min for AF. NAT activity was studied through HPLC analysis of the yield of 2-AAF and expressed in nmol acetylated substrate/10^6 cells.

Interference with apoptosis through the inhibition of NAT activity

1. Cell incubation

The procedure followed is the same as abovementioned in "Changes of NAT activity in apoptotic HepG2 cells".

2. Grouping and drug administration

The experiment was performed on 5 groups, namely, the negative control, the CAM group, the CAM+100 μmol/L iodoacetamide group, the CAM+ 500 μmol/L iodoacetamide group, and the iodoacetamide group. After the cells had adhered to the flask wall, the negative control was treated with an equal volume of RPMI 1640 culture medium; the CAM group was treated with CAM (final concentration 0.08 μg/ml); the CAM+100 μmol/L iodoacetamide group was treated with CAM (final concentration 0.08 μg/ml) and iodoacetamide (final concentration 100 μmol/L); the CAM+500 μmol/L iodoacetamide group was treated with CAM (final concentration 0.08 μg/ml) and iodoacetamide (final concentration 500 μmol/L); and the iodoacetamide group was treated with iodoacetamide (final concentration 500 μmol/L). The samples were incubated at 5% (v/v) CO_2 and 37 °C for 48 h.

3. Apoptosis observed using flow cytometry

The procedure followed is the same as abovementioned in "Changes of NAT activity in apoptotic HepG2 cells".

Data analysis

Data were presented as mean±*SD*. Statistical significance was evaluated with Student's *t*-test.

RESULTS

Observation of apoptosis in HepG2 cells induced by CAM

Fig.2 shows that cells in negative control had a round shape and their DNA structures kept intact, but the morphology of cells in 0.4 μg/ml CAM group was significantly different. A typical sign for apoptosis appeared in the 0.4 μg/ml CAM group. Fig.3 shows that in 0.4 μg/ml CAM group, the G_2/M peak disappeared, and a sub-G_0 peak of apoptosis appeared prior to the G_0/G_1 phase. All these results show that 0.4 μg/ml CAM could induce the apoptosis of $HepG_2$ cells.

Changes of NAT activity in apoptotic HepG2 cells

The results are shown in Fig.4 and Table 1. After 0.08 μg/ml of CAM was applied to HepG2 cells for 48 h, the amount of 2-AAF produced was lowered, which means that the activity of NAT was decreased.

Interference with apoptosis through the inhibition of NAT activity

As shown in Fig.5, no apoptosis peak appeared

for the negative control; CAM in the concentration of 0.08 μg/ml induced apoptosis in HepG2 cells, with an apoptosis rate of 10.4%; the apoptosis rate for the 0.08 μg/ml CAM+100 μmol/L iodoacetamide group was 17.4%; the apoptosis rate for the 0.08 μg/ml CAM+500 μmol/L iodoacetamide group was 30.6%; but iodoacetamide alone failed to induce apoptosis in HepG2 cells. So iodoacetamide is an inhibitor of NAT activity. Our results show that when NAT activity was inhibited, the apoptosis rate induced by CAM was increased, which implies that NAT can act to inhibit apoptosis of HepG2 cells, and that the lowering or loss of NAT activity can promote apoptosis in tumor cells.

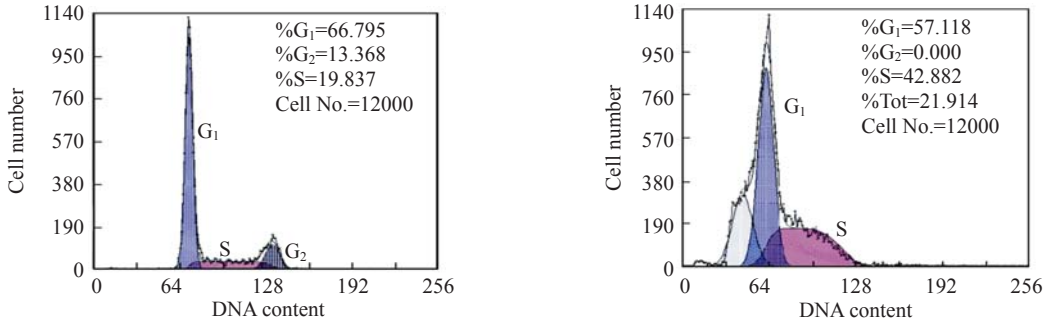
Table 1 Changes of NAT activity in apoptotic HepG2 cells induced by CAM

Group	Concentration of drug (μg/ml)	2-AAF peak areas ($\times10^3$ AU·s)
Control		773±1.89
CAM	0.08	489±1.61**

**P<0.01 compared with control

(a) (b)

Fig.2 Effect of 0.4 μg/ml camptothecin (CAM) on the morphology of HepG2 cells observed using confocal microscopy with acridine orange (AO)/ethidium bromide (EB) double staining. (a) Control; (b) 0.4 μg/ml CAM group



(a) (b)

Fig.3 Effect of 0.4 μg/ml camptothecin (CAM) on the apoptosis rate of HepG2 cells observed using flow cytometry with PI single staining. (a) Control; (b) 0.4 μg/ml CAM group

%G_1: Percent of G_1 phase; %S: Percent of S phase; %G_2: Percent of G_2 phase; %Tot: Apoptosis rate, i.e., percent of sub-G_0 phase; Cell No.: Cell numbers determined

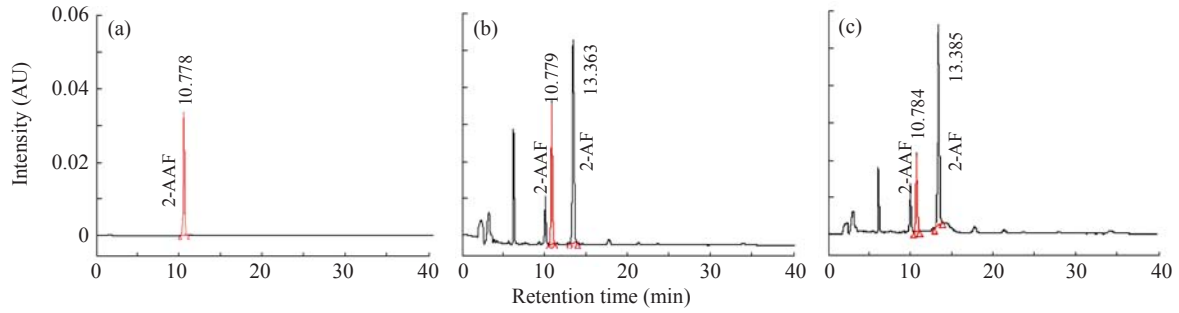


Fig.4 The effect of CAM on NAT activity in HepG2 cells observed using HPLC. (a) 2-AAF standard; (b) Control; (c) 0.4 μg/ml CAM group

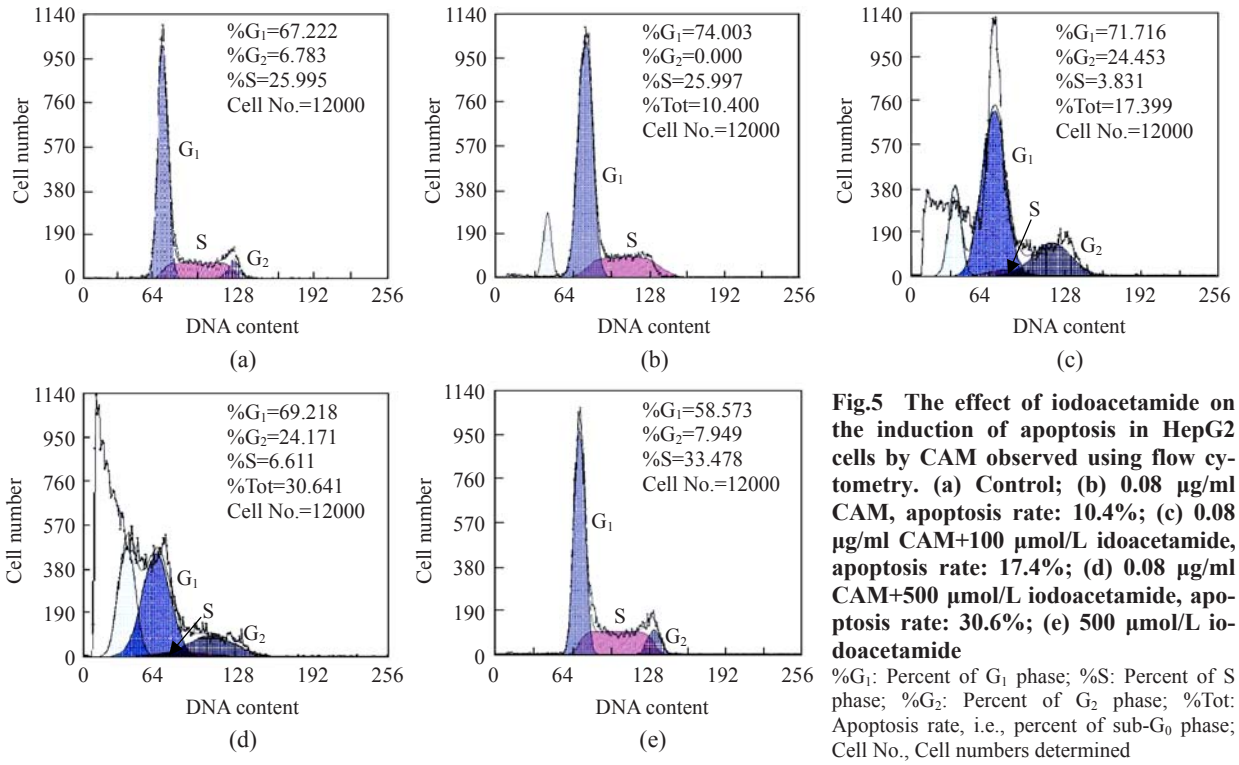


Fig.5 The effect of iodoacetamide on the induction of apoptosis in HepG2 cells by CAM observed using flow cytometry. (a) Control; (b) 0.08 μg/ml CAM, apoptosis rate: 10.4%; (c) 0.08 μg/ml CAM+100 μmol/L idoacetamide, apoptosis rate: 17.4%; (d) 0.08 μg/ml CAM+500 μmol/L iodoacetamide, apoptosis rate: 30.6%; (e) 500 μmol/L iodoacetamide

$\%G_1$: Percent of G_1 phase; %S: Percent of S phase; $\%G_2$: Percent of G_2 phase; %Tot: Apoptosis rate, i.e., percent of sub-G_0 phase; Cell No., Cell numbers determined

DISCUSSION

The structural similarity between NATs and cysteine proteases suggests that they may be derived from the same source. Their similar crystal structures also reveal that NATs exert their effect through the same mechanism as cysteine proteases (Sinclair*et al*., 2000). Since cysteine proteases are closely associated to apoptosis (Fan *et al*., 2005; Harwood *et al*., 2005; Bozhkov *et al*., 2005), NATs may also be suggested to be associated with apoptosis.

To test this speculation, the AcCoA-HPLC assay was conducted, with 2-AF as the substrate, to observe the effect of CAM on the amount of 2-AF metabolized into 2-AAF by NATs in the cytoplasm of HepG2 cells, and hence indirectly to observe the activity of NATs. We designed two experiments: (1) CAM was applied to HepG2 cells over a range of concentrations and incubation times, and the change in NAT activity was observed; (2) observation was made on the change in the apoptosis rate in HepG2 cells induced by CAM after NAT inhibitor was added. The results show that in Experiment (1) NAT activity was lowered in the CAM group relative to the negative control, i.e., NAT activity was lowered in apoptotic HepG2 cells, and that in Experiment (2) after NAT activity was inhibited by iodoacetamide, an NAT inhibitor, the apoptosis rate in HepG2 cells induced by CAM was increased. Thus we conclude that NATs are associated with apoptosis to some extent, and that lowering NAT activity can promote apoptosis in HepG2 cells.

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