Ketoprofen-inhibited *N*-Acetyltransferase Activity and Gene Expression in Human Colon Tumor Cells

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Abstract. *The activation of ketoprofen, which inhibits the outgrowth of azoxymethane-induced aberrant crypt foci in the rat colon, on the inhibition of arylamine N-acetyltransferase (NAT) activity (N-acetylation of substrates), gene expression (mRNA NAT) and 2-aminofluorene (AF)-DNA adduct formation was studied in a human colon tumor (adenocarcinoma) cell line (colo 205). Cellular cytosols (9000 xg supernatant) and intact colon tumor cells were used. The NAT activity in colo 205 cells was inhibited by ketoprofen in a dose- and time -dependent manner in both examined systems. The data also indicated that ketoprofen decreased the apparent value of Vmax of NAT enzymes, being a competitive inhibitor of NAT enzymes. The AF-DNA adduct formation in colo 205 cells was also decreased by ketoprofen. Based on the results from PCR, it was shown that ketoprofen affected mRNA NAT expression in human colon colo 205 cells. The cells were stained with anti-NAT antibody, then analyzed by flow cytometry. The results showed that ketoprofen decreased the percentage of cells stained by anti-NAT. This report is the first to demonstrate that ketoprofen inhibits human colon tumor cell NAT activity, gene expression and DNA adduct formation.*

Some of the arylamine carcinogens have been known to induce tumors in humans (1). *N*-acetylation is the primary step for the metabolism of arylamine carcinogens, which is catalyzed by *N*-acetyltransferase (NAT). NAT localizes in the cytosol fraction of the organs or tissues and requires Acetyl-Coenzyme A as a cofactor for its activity (2). The two NAT isozymes (NAT1 and NAT2) differ significantly in their intrinsic stabilities and acceptor substrate selectivities (3). In humans, the *NAT* genes are located on chromosome 8 and both have been identified and sequenced, and encode for 2 different human liver arylamine NATs (5, 6). NAT1 and NAT2 represent the monomorphic and polymorphic proteins, respectively (3, 5). Humans can be classified as rapid, intermediate or slow acetylators. In statistical and epidemiological studies, slow acetylation was shown to be linked to increased drug toxicity from sulfonamides (6) and isoniazide (2) and to increased susceptibility to occupational bladder cancer in human populations (7). Rapid acetylation has been linked to increased risk of colorectal cancer (8, 9). Human colon (10) and bladder (11) tissues have been reported to display NAT activity (*N*-acetylation of substrates).

Ketoprofen, 2 - (3 - benzoylphenyl) propionic acid, a non-steroidal anti-inflammatory drug, has been reported to block nitric oxide-induced apoptosis and the dedifferentiation of articular chondrocytes by the modulation of extracellular signal-regulated kinase, p38 kinase and protein kinase C alpha and zeta, and to be independent of the regulation of cyclooxygenase activity (12). Ketoprofen can induce intestinal permeability changes by reducing the prostaglandin tight junction (13). It was also reported that 0.25 mg/kg ketoprofen, administered daily for 30 days, reduced joint pain in dogs and no gastrointestinal or other side-effects were observed (14). Wargovich *et al.* reported that ketoprofen could inhibit the outgrowth of azoxymethane-induced aberrant crypt foci in the colon of F344 rats (15).

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The present studies were focused on the effects of ketoprofen on the *N*-acetylation and DNA adducts of 2-aminofluorene in a human colon tumor cell line.

Materials and Methods

Chemicals and reagents. Ketoprofen, ethylenediaminetetraacetic acid (EDTA), 2-aminofluorene (AF), *N*-acetyl-2-aminofluorene (AAF), *p*-aminobenzoic acid (PABA), *N*-acetyl-p-aminobenzoic acid (*N*-Ac-PABA), Acetyl-Coenzyme A (AcCoA), acetylcarnitine, leupeptin, bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF), Tris, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), micrococcal endonuclease spleen exonuclease and carnitine acetyltransferase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the chemicals used were of reagent grade.

Human colon tumor cell line. The human colon tumor (adenocarcinoma) colo 205 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were placed into 75-cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 atmosphere in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and 2% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin).

Preparation of human colon tumor cell cytosols. Approximately $5x10^7$ cells were dispersed in 2 ml of the lysis buffer (20 mM Tris/HCl, pH 7.5 (at 4˚C, 1 mM DTT, 1 mM EDTA, 50 μM PMSF, and 10 μM leupeptin), as previously described (16). The suspensions were centrifuged at 9000 xg for 1 min in a model 3200 Eppendorf/Brinkman centrifuge and the supernatant fraction was subsequently centrifuged at 10,000 xg for 60 min. The supernatant (S9) was kept on ice for NAT activity and protein determinations.

NAT activity determinations. The NAT activity was determined using an AcCoA recycling assay and high pressure liquid chromatography. The determinations of the AcCoA-dependent *N*-acetylation of PABA and AF were performed as described by Chung *et al.* (16). The reaction mixtures consisted of 50 μl cytosols, 20 μl of recycling mixture containing AF or PABA at 6.75 μM as substrates, and 10 μl of selected concentrations of ketoprofen. The reactions were initiated by the addition of AcCoA. The control reactions had 20 μl distilled water in place of AcCoA. The NAT activity was determined as described previously (16). All the compounds were quantitated by comparison of the integrated area of the elution peak with that of known amounts of standards. The NAT activity was expressed as nmol acetylated substrate per min per mg of cytosolic protein. Protein concentrations in the human colon tumor cell cytosols were determined by the method of Bradford (17) with BSA as the standard. All the samples were assayed in triplicate.

Intact cell NAT activity determination. Human colon tumor cells (in 1 ml RPMI 1640 medium with glutamine and 10% fetal calf serum) were incubated with arylamine substrate (6.75 μM AF or PABA) ($1x10^6$ cells/ml) in individual wells of 24-well cell culture plates with or without ketoprofen (0.05, 0.5, 5, 50 and 500 μM) for the time (6, 12, 18 and 24 h) indicated, at 37˚C in 95% air 5% CO_2. Then, the cells and media were removed and centrifuged. For experiments with AF, the supernatant was immediately extracted with ethyl acetate/ methanol (95:5), the solvent evaporated and the residue redissolved in methanol and assayed for AAF, as described above. For experiments with PABA, aliquots of the supernatant were assayed directly for *N*-Ac-PABA. The NAT activity was determined as described above.

Effects of ketoprofen on the kinetic constants of NAT from human colon tumor cells. Cytosols treated with or without 50 μM ketoprofen and concentrations (0.373, 0.435, 0.543, 0.745, 1.102 and 2.245 mM) of AF or PABA were assayed for NAT activity, as described above.

Preparation of polyclonal antibody. Six-week-old female BALB/c mice were immunized with the prepared recombinant protein (NAT). Each mouse was initially injected with 0.5 ml of pristane. About 100 μg antigen, mixed with an equal volume of complete Freund's adjuvant, were applied *s.c.* for 10-15 days. The antigen was emulsified with incomplete Freund's adjuvant and injected *i.p.* for 10-20 days. The serum-free myeloma cells ($1x10^6$/ml) in PBS were then injected *i.p.* into the mouse. The ascites fluids, which normally accumulated after 1 week, were collected daily for 5-8 days (18).

Detection of NAT protein levels by flow cytometry. The level of intracellular NAT in the colo 205 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using the prepared polyclonal antibody mentioned above. The cells were treated with various concentrations (0, 0.05, 0.5, 5, 50 and 500 μM) of ketoprofen for 24 h to detect the intracellular NAT. The cells were washed twice, re-suspended in 100 μl of ice-cold 1% formaldehyde for 5 min and mixed with 100 μl of ice-cold 99% methanol for 30 min. The cells were then washed 3 times with 0.1% BSA in PBS and mixed with 100 μl of 0.1% Triton X-100 in PBS with 0.1% sodium citrate on ice for 45 min. After being washed 3 times with the same buffer, the cells were incubated with the polyclonal antibody at 4˚C for 2.5 h, then washed 3 times with 0.1% BSA in PBS. The cells were subsequently stained with FITC-labelled secondary antibody (goat anti-mouse IgG; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 4˚C for 35 min. Again, the cells were washed 3 times, re-suspended in PBS and analyzed by flow cytometry (18).

Detection and measurements of DNA adducts from colo 205 cells after ketoprofen treatment. The detection of DNA adducts has been described previously (19). Briefly, colo 205 cells were incubated with AF and/or ketoprofen and DNA was prepared as described previously (16). DNA-adduct levels were examined and quantited as described previously (16, 19, 20).

Reverse transcriptase polymerase chain reaction (RT-PCR) for examining mRNA NAT expression from colo 205 cells after ketoprofen treatment. The total RNA was extracted from colo 205 cells after or without cotreatment of different concentrations of ketoprofen, as described previously (21). RT-PCR was used to examine the mRNA NAT expression, as described previously (4, 21-24).

Statistical treatment of data. The statistical analysis of the data was performed with an unpaired Student's *t*-test, ANOVA with Scheffe and the SAS program. The kinetic constants were calculated with the Cleland HYPER Program (25) that performs linear regression using a least-squares method.

Table I. *Effects of ketoprofen on N-acetyltransferase activity in human colo 205 cell cytosol.*

Ketoprofen concentrations	AAF	*N*-Ac-PABA
Control	1.08±0.27	0.98±0.20
0.05 μM	0.97±0.24	0.86±0.18
0.5 μM	0.80±0.18	0.76±0.16
5 μM	0.54±0.12[a]	0.48±0.11[a]
50 μM	0.33±0.10[a,b]	0.30±0.0[a,b]
500 μM	0.12±0.04[a,b,c]	0.10±0.04[a,b,c]
F value	68.402*	104.022*

Note. Values are mean±S.D. of activity (nmol/min/mg protein cells); n=3. ANOVA analysis with Scheffe *post hoc* comparisons was used. *$p<0.001$.
[a]Significantly different, at p level of 0.05, when compared to control.
[b]Significantly different, at p level of 0.05, when compared to 5 μM.
[c]Significantly different, at p level of 0.05, when compared to 50 μM.

Table II. *Effects of ketoprofen on N-acetyltransferase activity in intact human colo 205 cells.*

Ketoprofen concentrations	AAF	*N*-Ac-PABA
Control	5.14±0.58	4.82±0.50
0.05 μM	4.74±0.50	4.66±0.48
0.5 μM	4.12±0.39	4.02±0.32
5 μM	3.68±0.24[a]	3.38±0.19[a]
50 μM	2.85±0.19[a,b]	2.40±0.14[a,b]
500 μM	1.59±0.12[a,b,c]	1.26±0.09[a,b,c]
F value	76.842*	122.248*

Note. Values are mean±S.D. of activity (nmol/10^6 cells); n=6. ANOVA analysis with Scheffe posteriori comparison was used. *$p<0.001$.
[a]Significantly different, at p level of 0.05, when compared to control.
[b]Significantly different, at p level of 0.05, when compared to 5 μM.
[c]Significantly different, at p level of 0.05, when compared to 50 μM.

Results

Effects of various concentrations of ketoprofen on the NAT activity in cytosol of human colon tumor cells. The possible effects of ketoprofen on the NAT activity in the cytosol of human colon tumor cells were examined by HPLC through assessing the percentage of acetylation of AF and PABA. The mean values ± SD (standard deviation) of NAT activity after treatment with (or without) ketoprofen with both substrates in the cytosols are given in Table I. The data indicated that there was decreased NAT activity in cytosols associated with increased ketoprofen concentrations.

Effects of various concentrations of ketoprofen on NAT activity in intact human colon tumor cells. The data for the *N*-acetylation of AF and PABA measured from intact colo 205 cells treated with (or without) 50 μM ketoprofen for various time-periods are presented in the Table II. In the presence of 50 μM ketoprofen, the *N*-acetylation of AF and PABA was decreased by about 45% and 54% for AF and PABA, respectively.

Effects of incubation time on AAF and N-Ac-PABA production by human colon tumor cells. To determine the time-course effect of 50 μM ketoprofen on AF and PABA acetylation, the cells were incubated with (or without) ketoprofen at 37˚C and harvested at 6, 12, 18 and 24 h. An increased time of incubation led to increased AAF and *N*-Ac-PABA production up to 24 h (Figure 1A and B). The figures also demonstrate that ketoprofen decreased the production of AAF and *N*-Ac-PABA in the examined time-periods.

Effects of 50 μM ketoprofen on kinetic constants of NAT in human colon tumor cells. In the presence or absence of ketoprofen, specific concentrations of AF and PABA (0.373,

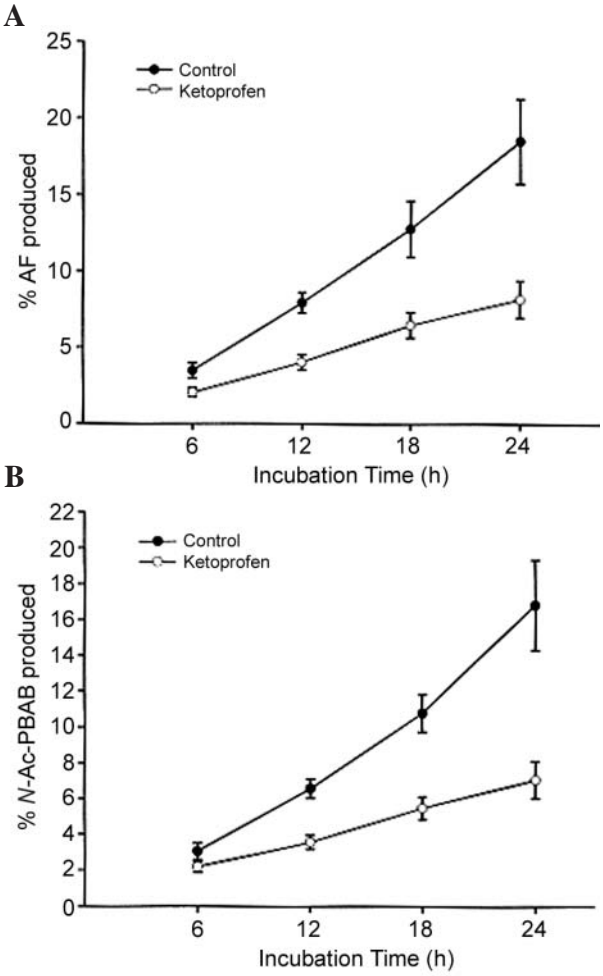

Figure 1. *Effects of incubation time on N-acetylation of AF in human colon tumor cells. Colo 205 cells were incubated with AF or PABA at 6.75 μM and with 50 μM ketoprofen treatment for 6, 12, 18 and 24 h. N-acetylated AF (panel A) and PABA (panel B) production was measured by the HPLC assay. Each point represents the mean of triplicate assays of 3 incubations of cells. *mean differs between ketoprofen and control. $p<0.05$*

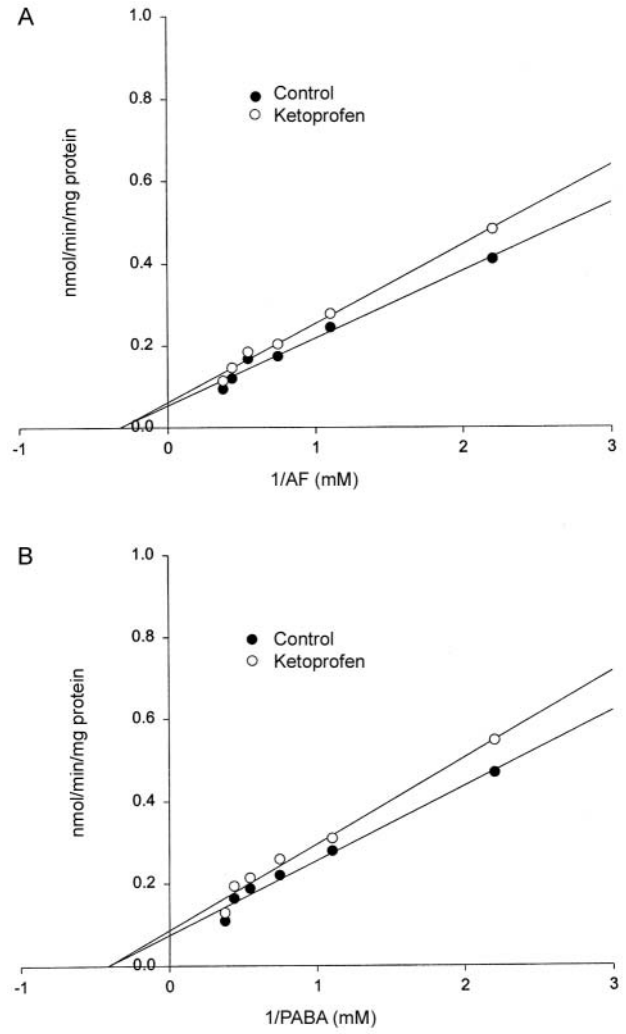

Figure 2. *Lineweaver-Burk double reciprocal plot of NAT activity in human colon tumor cells by cytosol examination. Recycling mixtures containing 0.087, 0.174, 0.384, 0.696 and 1.392 mM AF (panel A) and PABA (panel B) were used. Cytosols were prepared as described in the "Materials and Methods". The Lineweaver-Burk double reciprocal plots were measured by using linear regression analysis of reciprocal substrate concentrations plotted against reciprocal initial velocities. Full circle = control. Empty circle = ketoprofen.*

0.435, 0.543, 0.745, 1.102 and 2.205 mM, respectively) were added to the recycling mixtures in order to determine the human colon tumor cell NAT kinetic constants. For the cytosol examinations, 50 μM of ketoprofen were added to the reaction mixtures and the apparent values of Vmax were decreased by 21.3% and 26.1% for the acetylation of AF and PABA, respectively (Figure 2A and B and Table III).

Effects of ketoprofen on the NAT protein in colo 205 cells. NAT protein was measured by the formation of the NAT antigen-antibody complex. The percentage of NAT antibody complex from colo 205 cells treated with (or without) various concentrations of ketoprofen for 24 h was measured by flow cytometry. The data showed that ketoprofen decreased the percentage of cells stained with NAT antibody (Table IV).

Table III. *Kinetic data for acetylation of AF and PABA in human colo 205 cells.*

	AF		PABA	
	Km (mM)	Vmax(nmol/min/mg)	Km (mM)	Vmax(nmol/min/mg)
Control	3.12±0.34	14.02±2.69	2.74±0.87	13.48±2.84
Ketoprofen	3.04±0.22	[a]11.36±2.04	2.82±0.56	[a]10.02±2.17

Values are mean±S.D. n=3. The AcCoA and ketoprofen concentrations were 0.1 mM and 50 μM, respectively and the kinetic constants were calculated from the modified HYPER Program of Cleland. All experiments and controls were run in triplicate.
[a]Differs between 50 μM ketoprofen and control. $p<0.05$.

Table IV. *The percentage of ketoprofen-treated colo 205 cells stained by the anti-NAT antibody*

Ketoprofen (μM)	Percentage of cells stained by anti-NAT
0 (control)	48.40±5.21
0.05	46.28±4.86
0.5	41.16±4.42
5	34.49±5.69
50	*24.14±3.19
500	*16.09±2.38

Values are mean±S.D. n=3. The colo 205 cells ($1x10^6$ cells/ml) were cotreated with various concentrations of ketoprofen. The zero concentration was defined as control. The percentage of cells stained by anti-NAT antibody was determined by flow cytometry as described in the Materials and Methods section.
*Differs between ketoprofen and control. $p<0.05$.

Efects of 50 μM ketoprofen on DNA-AF adduct formation in human colon tumor cells. Following 24-h incubation of human colon tumor cells with AF with (or without) ketoprofen treatment, the cells were recovered and DNA was prepared, hydroylzed to nucleotide, and the adducted nucleotides were extracted into butanol and analyzed by HPLC. The results indicated that the colon tumor cells activate AF to a metabolite which was able to bind covalently with DNA, and also induced a dose-dependent effect (Table V). In the presence of 50 μM ketoprofen, the AF-DNA adduct formation decreased for various concentrations of AF.

Table V. *AF-DNA adduct formation (pmol adduct/mg DNA) following 2-h incubation of human colon tumor cells with or without 50 μM ketoprofen.*

	Adducts (pmol/mg DNA)		
Cancer cell	30 μM 2-AF	60 μM 2-AF	90 μM 2-AF
Colo 205 cells	0.69±0.21	0.96±0.28	1.21±0.34
Colo 205 cells + ketoprofen	[a]0.29±0.11	[a]0.42±0.18	[a]0.54±0.21

[a]Differ between control and ketoprofen cotreatment groups using SAS program for analysis. F value is 12.54. Pr >F is 0.0071.
Values are means±SE of 6 separate preparations (colo 205 cells, incubation with AF, DNA preparation, postlabelling and HPLC).

Dose-dependent effects of ketoprofen on NAT mRNA expression in colo 205 cells. The dose-dependent changes of NAT1 mRNA levels in response to ketoprofen were studied. The data presented in Figure 3 indicate that the NAT1 mRNA level decreased with increasing levels of ketoprofen (50-500 μM).

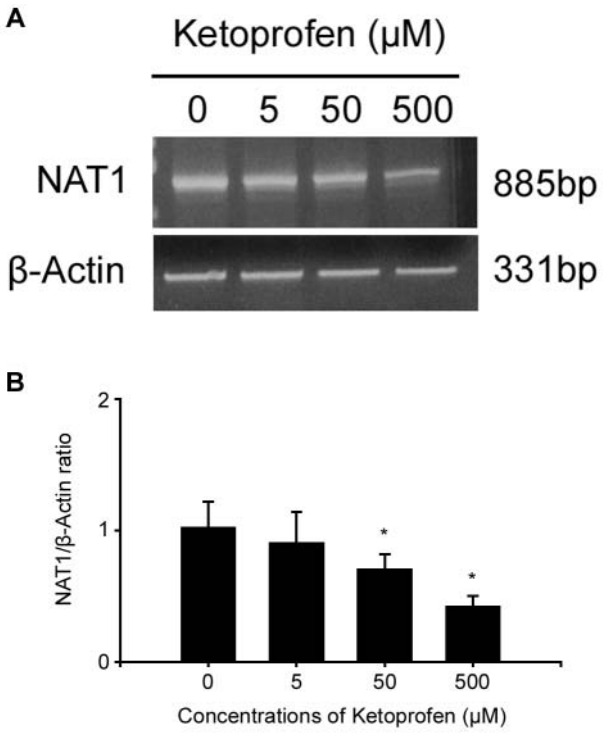

Figure 3. *Dose-dependent effect of ketoprofen on the expression of NAT mRNA in human colon tumor cells (colo 205). The cells were incubated with various concentrations of ketoprofen for 24 h, then collected to extract RNA. The extracted RNA was subjected to RT-PCR analysis using specific primers for NAT1 and β-actin, and then PCR-amplified cDNA derived from mRNA was applied to agarose gel-electrophoresis (A). The mRNA levels of NAT1 and β-actin on the gel-electrophoresis were quantified by densitometric analysis of the gel-photograph and expressed as the NAT1/β-actin ratio (B).*

Discussion

The purpose of the present study was to examine whether ketoprofen could inhibit the NAT activity and gene expression, as well as the AF-DNA adduct formation, in human colon tumor colo 205 cells. The data indicated that ketoprofen inhibited NAT activitiy in colo 205 cells, based on the following observations: (i) NAT activity in colo 205 cells (cytosol and intact cells) was inhibited; (ii) the apparent values of Vmax of NAT in colo 205 cells were decreased; (iii) ketoprofen inhibited mRNA NAT1 expression; (iv) the percentage of NAT from examined cells treated with ketoprofen decreased; (v) AF-DNA adduct formation was decreased by ketoprofen.

The cytochrome P450 enzyme was shown to be involved in the metabolism of *N*-acetylated AF and the cytochrome P450-dependent formation of *N*-hydroxy-AAF was considered to be involved in the initial rate-limiting step in the metabolism of AAF to mutagenic and/or carcinogenic products (26). Particularly, cytochrome P4501A1 is efficient in catalyzing the conversion of AAF to 7-OH-AAF (27, 28). In the present study, it was not found whether or not ketoprofen could inhibit cP450 activity in colo 205 cells. Other factors should be considered regarding an individual's susceptibility to the carcinogenic effects of aromatic and heterocyclic amine carcinogens such as the relative rates of *N*-acetylation and *N*-hydroxylation in the liver, the route of excretion of metabolites, possibly the rates of glucuronide hydrolysis and NAT-mediated activation in the target tissue (29).

The data from the cytosol and intact human colon tumor cell studies show that there were significant differences in NAT activity between the control and ketoprofen treatment groups. The results indicated that ketoprofen inhibited the NAT activity in colo 205 cells in both examined systems, in a dose-dependent manner. Increased concentrations of AF and PABA resulted in increased production of both AAF and *N*-Ac-PABA. The kinetic studies also indicated that ketoprofen lowered the Vmax values of NAT in the cytosols of colo 205 cells. There were several reasons for choosing 50 μM for the reported examinations. First, NAT activity in human colon tumor cells was inhibited by over 50% after co-treatment with 50 μM ketoprofen. Second, the higher concentration of ketoprofen was more difficult to dissolve in DMSO. Third, the concentration of ketoprofen (50 μM) was much lower than that which led to significantly increased intestinal permeability (5 mM) (14). However, the exact amount of ketoprofen that finally

reaches the colon may be lower. It was also reported that ketoprofen attained high concentrations in subcutaneous tissues after multiple topical applications, since these tissues appeared to act as a reservior of ketoprofen (30). It is possible that the intact cells take up less ketoprofen than that used in the cytosol experiments. Of significance is the observation that, after the cells had been treated with ketoprofen, the percent of NAT in the cells decreased, as determined by the flow cytometric and Western blotting analyses with the anti-NAT antibody. Ketoprofen also decreased AF-DNA adduct formation in the colo 205 cells. The PCR experiment further demonstrated that ketoprofen inhibited mRNA NAT1 gene expression, which may lead to decreased *N*-acetylation of AF and PABA because the total amount of NAT was decreased. Carcinogen-DNA adduct formation has been recognized as the first step in chemical carcinogenesis, and the levels of AF-DNA adduct formation were reported to be related to the initiation of carcinogenesis (31). Ketoprofen may affect the AF metabolites in the cell culture, a hypothesis which requires further investigation.

A metabolic characteristic, or drug metabolism (9), possibly related to NAT-involved colorectal carcinogenesis as well as a genotype (32) for rapid or slow acetylators, have been reported. The rapid acetylators have a higher risk of inducing colorectal cancers (33). Many studies have attempted to relate the acetylation phenotype to cancer susceptibility. Recently, it has been demonstrated that the combination of rapid-acetylator phenotypes with a high consumption of meat would increase the risk of colorectal cancer (34). The relationship between meat intake, acetylator status and colorectal carcinogenesis may involve exposure to heterocyclic amines generated by cooking meat for long periods at high temperatures. Our findings indicated that ketoprotein inhibited NAT gene expression and protein levels may then lead to decreased the *N*-acetylation of AF or PABA.

Acknowledgements

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