Abstract. Human epidemiological studies suggest an association between N-acetyltransferase (NAT) activity and the incidence of bladder and colorectal cancers. In this study, paclitaxel was selected to examine the inhibition of arylamine NAT activity, gene expression and 2-aminofluorene-DNA adduct formation in a human osteogenic sarcoma cell line (U-2 OS). The activity of NAT was determined by high performance liquid chromatography (HPLC) assay for the amounts of acetylated 2-aminofluorene (AF) and p-aminobenzoic acid (PABA) and nonacetylated AF and PABA. Human osteogenic sarcoma cell cytosols and intact cells were used to examine the NAT activity, gene expression and AF-DNA adduct formation. The results demonstrated that NAT activity, percent of NAT in examined cells, gene expression (NAT1 mRNA) and AF-DNA adduct formation in human osteogenic sarcoma cells were inhibited and decreased by paclitaxel in a dose-dependent manner. The results also demonstrated that paclitaxel decreased the apparent values of Km and Vmax from intact human osteogenic sarcoma cells (U-2 OS). Thus, paclitaxel is an uncompetitive inhibitor of the NAT enzyme.

Acetyl CoA-dependent N-acetyltransferase (NAT) is involved in the metabolic activation of arylamine carcinogens that leads to carcinogenesis in specific target tissues (1-4). The acetylated arylamine can be further activated by other enzymes such as glucuronyl transferase, deacetylase, sulfotransferase and/or acetyltransferase and then forms an ultimate carcinogenic species (1-3). The enzymatic acetylation of the N-hydroxy compound produces extremely reactive N-acetoxyarylamine derivatives that spontaneously hydrolyze to form DNA adducts (5). DNA-carcinogen adduct formation is an important step in the initiation of arylamine-induced carcinogenesis (6). It has been reported that arylamine-DNA adducts formation and persistence were correlated with the cytotoxic, mutagenic, carcinogenic and teratogenic effects of these agents (7,8).

Human and other mammals exhibit a well defined genetic polymorphism in NAT activity resulting in rapid, intermediate and slow acetylator phenotypes (9,10). The rapid acetylator phenotype has been shown to predispose humans to colorectal cancer, whereas the slow acetylator phenotype is related to arylamine-induced bladder cancer (9,11-13). Two NATs (NAT1 and NAT2) were found in human (14), encoded as NAT1 and NAT2 genes, and both located on chromosome 8, but separated by at least 25 kb (15,16). In human, NAT exhibits different substrate specificities; p-aminobenzoic acid is a good substrate for NAT1 while isoniazid and sulfamethazine are efficiently metabolized by NAT2 (9,15,17) and both NATs can acetylate 2-aminofluorene (18). Therefore, the role of NAT in chemical carcinogenesis is worth examining.

Paclitaxel (taxol) was isolated from the bark of the Pacific yew Taxus brevifolia and was reported to possess cytotoxic activity against a broad range of murine leukemia (19) and solid tumors such as chemotherapy-resistant epithelial ovarian cancer, advanced breast cancer, small cell and non-small cell lung cancer and head and neck cancer (20). In our previous studies we had demonstrated that paclitaxel affects the NAT activity of human lung cancer cells and leukemia cells (21,22). The present study is focused on the effects of paclitaxel on NAT activity, gene expression and 2-aminofluorene-DNA adduct formation in a human osteogenic sarcoma cell line (U-2 OS).
Materials and Methods

Chemicals and reagents. Paclitaxel (Taxol) was obtained from Bristol Caribbean, Inc (NJ, USA), P-aminobenzoic acid (PABA), N-acetyl-p-aminobenzoic acid (N-Ac-PABA), 2-aminofluorene (AF), N-acetyl-2-aminofluorene (AAF), ethylenediaminetetraacetic acid (EDTA), acetyl carnitine, Tris, leupeptin, bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF), dimethyl sulfoxide (DMSO), diisothiocetic acid (DTT), Acetyl-CoA and carnitine acetyltransferase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals used were reagent grade.

Human osteogenic sarcoma cell line. Human osteogenic sarcoma cell line (U-2 OS: human; female; 15 years old) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells have been cultured for four generations in our laboratory and continuously checked for viability. The cells were cultured in Pristane treated ascites fluids, which normally accumulated after 1 week, were placed in a 15-ml tube which contained 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 by Chung et al. (23). The suspensions were centrifuged at 9000 g for 1 min in a model 3200 Eppendorf/Brinkman centrifuge, the supernatant fraction harvested and subsequently centrifuged at 10,000 g for 60 min. Finally the supernatant was kept on ice for NAT activity and protein determinations.

NAT activity determination. The determination of Acetyl-CoA-dependent N-acetylation of AF in U-2 OS cytosols was performed as described by Chung et al. (23). Protein concentration in the U-2 OS cell cytosols was determined by the method of Bradford, with bovine serum albumin as the standard (24). All samples were assayed in triplicate.

Effects of various concentrations of paclitaxel on NAT activity in cytosols of U-2 OS cells. Paclitaxel was dissolved in DMSO at various concentrations (0.01-50 μM). The reaction mixtures consisted of 50 μl cytosols diluted as required, 20 μl of recycling mixture containing AF or PABA (0.22 μM) for substrate and 10 μl of selected paclitaxel. The reaction was started by the addition of 20 μl AcCoA. The control (DMSO treated only) reaction had 20 μl distilled water in place of AcCoA. Following these steps, the reaction was stopped by the addition of 10 ml of 0.5 M EDTA. The unincorporated and incorporated isotopes were separated by sephadex G-50 chromatography. Postlabeled adducted nucleotides were separated by Beckman HPLC (pump 168 and detector 126) using Ultrasphere C18 reversed phase ion-pairing column 4.6x25 cm eluted at a flow rate of 1.5 ml/min with 30 mM KH₂PO₄, pH 6.0, containing 10% CH₃CN for 10 min followed by a linear gradient of 90% 30 mM KH₂PO₄, pH 6.0, 5 mM tetrabutylammonium phosphate and 50% CH₃CN for 65 min. UV absorbance was followed at 254 nm. Samples (1 ml = 1.5 ml) were collected and quantitated by scintillation spectrometry (4,21). Calculation of adduct formation was made by dividing the radioactivity in the adduct peak (after correction for recovery and efficiency of counting) by the specific activity of the ATP used in labeling. Adduct levels are reported as the pmol adduct/mg DNA analyzed (23,25,26).

Preparation of polyclonal antibody. Six 6-week-old female BALB/c mice were immunized with the prepared recombinant protein (NAT). At first each mouse was injected with 0.5 ml of pristane. Then about 100 μg of 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, then boosted again. Myeloma cells (1x10⁶/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 (27).

Detection of NAT protein by flow cytometry. The level of intracellular NAT of the examined cells was determined by flow cytometry (Becton Dickinson FACSS Calibur), using the prepared polyclonal antibody mentioned above. Cells were cotreated with various concentrations (0.01, 0.1, 1, 5, 10, 25 and 50 μM) of paclitaxel for 24 h to detect the intracellular NAT. The cells were washed twice, re-suspended in 100 μl of ice-cold 1% formaldehyde for acetyl-AAF as described above. For PABA and N-Ac-PABA assay, the aliquot sample was immediately injected into HPLC and the amounts of PABA and N-Ac-PABA assayed.

Effects of paclitaxel on the kinetic constants of NAT from U-2 OS cells. For the intact cell studies, 1 x 10⁶ cells were incubated with selected concentrations (0.373, 0.435, 0.543, 0.745, 1.102 and 2.245 mM) of AF or PABA and with and without paclitaxel for 18 h in a 37°C incubator. Following incubation, the cells and media suspensions were removed and centrifuged. In the experiment with AF, the supernatant was immediately extracted with ethyl acetate/methanol (95:5), the solvent evaporated and the residue redissolved in methanol and assayed by HPLC (23).
for 5 min and mixed with 100 \( \mu \)l of ice-cold 99% methanol for 30 min. Then the cells were washed three times with 0.1% BSA in PBS and mixed with 100 \( \mu \)l of 0.1% Triton X-100 in PBS with 0.1% sodium citrate on ice for 45 min. After being washed three times with the same buffer, the cells were incubated with polyonal antibody at 4°C for 2.5 h and then washed three times with 0.1% BSA in PBS. The cells were then stained with FITC-labeled secondary antibody (goat anti-mouse IgG; Jackson ImmunoResearch; Laboratories, West Grove, PA, USA) at 4°C for 35 min. Again, the cells were washed three times, re-suspended in PBS and analyzed by flow cytometry (27).

Reverse transcriptase polymerase chain reaction (RT-PCR). The total RNA was extracted from U-2 OS cells by using Qiagen RNeasy Mini Kit at the indicated time, after treatment with different concentrations of paclitaxel. Total RNA (1.5 \( \mu \)g), 0.5 \( \mu \)g of oligo-dT primer and DEPC (diethyl pyrocarbonate)-treated water was combined into a microcentrifuge tube to a final volume of 12.5 \( \mu \)l. The entire mixture was heated at 70°C for 10 min and chilled on ice for at least 1 min. The subsequent procedures for conducting reverse transcription were exactly the same as those in the instruction manual (First-strand cDNA synthesis kit, Novagen). The reverse transcription products from total RNA served as a template for PCR. When amplifying target cDNA, components in 50 \( \mu \)l of solution were as follows: 1.5 mM MgCl\(_2\), 0.2 mM dNTP mix, 20 pmoles of each primer (B-MDIEA-NAT1 & VPKHGD-X-NAT1 for NAT1, FP1-NAT2 & RP1-NAT2 for NAT2, Act b1 & Act b2 for beta-actin), cDNA template corresponding to the amount synthesized from 50 ng of total RNA and 2 units of DyNAzyme DNA polymerase. The sequence of primers were as follows: B-MDIEA-NAT1, 5'-(CGGCCGATCCGATCACCAGGATATGGAACGC-3', nt 435-454, GenBank accession number X17059; VPKHGD-X-NAT1, 5'-(GGTCTCTGACTGATCACTCACCAGGATATGGAACGC-3', nt 1295-1278, GenBank accession number X17059; FP1-NAT2, 5'-CTAGTTCTCCTGTCGTCGGCC-3', nt 79-98, GenBank accession number NM-000015; RP1-NAT2, 5'-TAACGTGAGGGTAGAGAGGA-3', nt 1073-1054, GenBank accession number NM-000015; Act b1, 5'-GCTCGTCGTCGACAACGGCTC-3', nt 94-114, GenBank accession number NM-001101; Act b2, 5'-CAAACA TGATCTGGTGTCATCTTCT-3', nt 446-422, GenBank accession number NM-001101.

Statistical analysis. Statistical analysis of the data was performed with an unpaired Student's t-test and ANOVA analysis. The kinetic constants were calculated with the Cleland HYPER Program (29) that performs linear regression using a least-squares method.

Results

Effects of various concentrations of paclitaxel on NAT activity in U-2 OS cell cytosols. The possible effects of paclitaxel on NAT activity in U-2 OS cell cytosols were examined by HPLC assessing the amounts of acetylated AF or PABA and nonacetylated AF or PABA. The means±SD (standard deviation) of acetylated AF or PABA treated with or without paclitaxel are given in Table I. The data indicated that the NAT activity decreased with increased concentrations of paclitaxel in cytosols.

Effects of 50 \( \mu \)M paclitaxel on the acetylation of various concentrations of AF and PABA in U-2 OS cells. The NAT activity for the acetylation of AF or PABA (15, 30, 60 and 100 \( \mu \)M) measured in intact cells was decreased after treatment with paclitaxel as presented in Figure 1A. In the presence of 50 \( \mu \)M paclitaxel, the acetylated AF and PABA were decreased.

Effects of incubation time with paclitaxel on AAF and N-Ac-PABA production by intact U-2 OS cells. In order to determine the time course effect of 50 \( \mu \)M paclitaxel on the acetylation of AF and PABA in U-2 OS cells, the cells were incubated at 37°C with or without paclitaxel and harvested at 6, 12, 18, 24 and 48 h, respectively. An increased time of incubation led to increasing AAF and N-Ac-PABA production up to 48 h (Figure 1B). The figure also demonstrated that paclitaxel did decrease the production of AAF and N-Ac-PABA in the examined time periods.

Effects of paclitaxel on the kinetic constants of NAT in U-2 OS cells. In the presence or absence of 50 \( \mu \)M paclitaxel, specific concentrations of AF and PABA (0.373, 0.435, 0.543, 0.745, 1.102 and 2.205 mM, respectively) were added to the recycling mixtures for determining U-2 OS cell NAT kinetic constants. The effects of paclitaxel on the Km and Vmax values in U-2 OS cells are presented in Figure 2 and Table II. Clearly, the apparent values of Km and Vmax for the U-2 OS cell NAT were decreased in the presence of paclitaxel in intact cell examinations.

Effects of paclitaxel on the NAT protein in human cancer cells. NAT protein was measured by the NAT antibody. The percentage of NAT-antibody complex from various examined cancer cells treated with or without various concentrations of paclitaxel for 24 h were measured by flow cytometry. The data indicated that paclitaxel decreased the percentage of NAT-antibody complex in the examined cells (Figure 3). Thus, the NAT levels were decreased in response to paclitaxel concentrations.

Dose-dependent effects of paclitaxel on NAT mRNA expression in U-2 OS cells. The data presented in Figure 4 show that the NAT1 mRNA level was decreased at increased levels of paclitaxel (> 5 \( \mu \)M). NAT1 mRNA was detected but NAT2 was not detected in the U-2 OS cells. The results indicated that paclitaxel could decrease the expression of NAT mRNA in a dose-dependent manner in U-2 OS cells.

Discussion

The etiology of human bone cancer is not well known; nevertheless, it is generally accepted that bone cancer has multifactorial, including environmental, determinants
Arylamine carcinogens require metabolic activation by host enzymes as a prerequisite for the initiation of carcinogenesis in human tissues. Arylamine N-acetylation is catalyzed by cytosolic acetyl coenzyme A-dependent N-acetyltransferase (NAT) which is expressed in liver and extrahepatic tissues (9,10,31,32). NAT enzymes exist in laboratory animals and humans and the NAT activity has been demonstrated to be involved in chemical carcinogenesis. Furthermore, an individual’s sensitivity to the toxicity after exposure to many arylamines is related to the acetylator phenotypes of NAT (9). It is well known that N-formylation and N-acetylation occur as a common reaction in the in vivo metabolism of arylamines in mammalian species. NAT activity has not been reported in the human osteogenic sarcoma cells. Since paclitaxel had been shown to possess anticancer capacity (19,20), it was of interest to study whether or not paclitaxel would affect the NAT activity of human bone tumor cells. The results indicated that paclitaxel did decrease the NAT activity, gene expression and AF-DNA adduct formation in human U-2 OS cells.

Paclitaxel could markedly inhibit the N-acetylation of AF (NAT activity) in cytosols and intact human osteogenic sarcoma cells in a time- and dose-dependent manner. The apparent values of Km and Vmax from AF acetylation by NAT with or without paclitaxel treatment were also examined. Both kinetic constants (Km and Vmax) were decreased; thus paclitaxel acted as an uncompetitive inhibitor in this reaction. Enzyme inhibitors can be divided into 3 groups: the first is a competitive inhibitor which competes with the substrate for binding at the same site on the enzyme; the second is a noncompetitive inhibitor which binds to a different site but blocks the conversion of the substrate to products; and the third is an uncompetitive inhibitor that binds only to the enzyme-substrate complex (33). Paclitaxel inhibited NAT gene expression (NAT mRNA). Paclitaxel also decreased AF-DNA adduct formation in human osteogenic sarcoma cells (data not shown). Carcinogen-DNA adduct formation plays an important role in the initiation of chemically-induced carcinogenesis in the target organs (6,34).

Table I. Effects of paclitaxel on N-acetylation of AF in cytosols of human U-2 OS cells.

<table>
<thead>
<tr>
<th>Paclitaxel treatment (µM)</th>
<th>Acetylated substrates (nmol/min/mg protein)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAF</td>
<td>N-Ac-PABA</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.01±0.22</td>
<td>2.20±0.18</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>3.06±0.19</td>
<td>2.22±0.16</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>3.14±0.24</td>
<td>2.26±0.17</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.80±0.18</td>
<td>2.01±0.14</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.66±0.12</td>
<td>1.70±0.12</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.19±0.15 abcde</td>
<td>1.51±0.11 abcde</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>1.87±0.10 abcde</td>
<td>1.32±0.09 abcde</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.04±0.09 abcdefgh</td>
<td>0.87±0.06 abcdefgh</td>
<td></td>
</tr>
<tr>
<td>F value</td>
<td>55.094*</td>
<td>41.927*</td>
<td></td>
</tr>
</tbody>
</table>

Note. Values are mean±SD of AF N-acetylation (nmol/min/mg protein); n=6. Anova analysis with Scheffe posteriori comparison was used. * p<0.001. a Significantly different, p<0.05, when compared to control. b Significantly different, p<0.05, when compared to 0.01 µM. c Significantly different, p<0.05, when compared to 0.1 µM. d Significantly different, p<0.05, when compared to 1 µM. e Significantly different, p<0.05, when compared to 5 µM. f Significantly different, p<0.05, when compared to 10 µM. g Significantly different, p<0.05, when compared to 25 µM.
The susceptibility of individuals to the carcinogenic effects of aromatic and heterocyclic amines may depend on (i) the relative rates of \(N\)-acetylation and \(N\)-hydroxylation in the liver, (ii) the route of excretion of metabolites, (iii) the rates of glucuronide hydrolysis, and (iv) NAT activation in the target tissue. For example, the administration of nonacetylated arylamines (AF) to dogs has resulted in the formation of only urinary bladder tumors (35,36). In contrast, administration of acetylated arylacetamides (AAF) to dogs has resulted in the formation of both urinary bladder tumors and hepatomas (9). Thus, the NAT enzyme plays an important role in the induction of arylamine carcinogenesis in target organs of individuals.

From the present study it is difficult to extrapolate the quantity of paclitaxel that would be needed to be consumed by humans to potentially reduce the NAT activity and AF-DNA adduct formation in target cells \textit{in vivo}, although paclitaxel dosology has been thoroughly studied in human patients (37-39). The concentration (50 \(\mu\)M) of paclitaxel selected for our studies induced an over 50% decrease in NAT activity \textit{in vitro} in the examined human osteogenic sarcoma cells (U-2 OS).

Two NATs (NAT1 and NAT2) have been described for humans (4,14). The substrate specificities of both NATs are different, e.g., NAT2 acetylates sulfamethazine, whereas NAT1 does not. In contrast NAT1 acetylates \(p\)-aminobenzoic acid and \(p\)-aminosalicylic acid, whereas NAT2 does not. However, both enzymes (NAT1 and NAT2) show significant activities to acetylate AF (18). The results of Figure 3 also show that paclitaxel decreased gene expression (NAT1 mRNA) in U-2 OS cells. NAT2 gene product was not found in U-2 OS cells since no NAT2 mRNA was detected by RT-PCR and NAT PCR. This finding is in agreement with our earlier studies that U-2 OS cells did not acetylate sulfamethazine which is a NAT2 substrate (data not shown).

The present study demonstrated that paclitaxel could decrease NAT1 enzyme activity (inhibited AF acetylation) and gene expression in U-2 OS cells that may lead to decreasing the \(N\)-acetylation of AF and AF-DNA adduct formation. However, it is not known whether paclitaxel could decrease or prevent the development of human bone cancer. Other investigators have already demonstrated that promoted NAT activities are associated with an increased sensitivity to the mutagenic effects of many arylamines (40). Furthermore, the attenuation of liver NAT activity has been shown to be associated with breast and bladder cancer (9). Apparently, changes of NAT activity are related to several diseases.

\textbf{Table II. Kinetic data for acetylation of AF and PABA in human U-2 OS cells.}

<table>
<thead>
<tr>
<th></th>
<th>AF</th>
<th>PABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_m) (mM)</td>
<td>(V_{max}) (nmol/min/(mg) protein)</td>
<td>(K_m) (mM)</td>
</tr>
<tr>
<td>Control</td>
<td>2.50±0.71</td>
<td>4.54±0.62</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>1.78±0.43 *</td>
<td>2.71±0.46 b</td>
</tr>
</tbody>
</table>

Values are mean±SD, \(n=3\).

* 50 \(\mu\)M paclitaxel and control differ with \(p<0.05\).

b 50 \(\mu\)M paclitaxel and control differ with \(p<0.01\).
Figure 3. Effects of paclitaxel treatment on NAT expression in U-2 OS cells. Cells (1x10^6/ml) were treated with 25 µM paclitaxel for 24 h followed by evaluation of NAT expression. NAT expression was estimated by flow cytometry. Data were acquired and analyzed using flow cytometry, as described in Materials and Methods. A: colo 205; B: colo 320 DM; C: colo 320 HSR.
Acknowledgements

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Figure 4. Dose-dependent effect of paclitaxel on the expression of NAT mRNA in human osteogenic sarcoma cells (U-2 OS). The cells were incubated with various concentrations of paclitaxel for 24 h, and then they were collected to extract total RNA. The extracted RNA was subjected to RT-PCR analysis using specific primers for NAT and β-actin, and then PCR-amplified cDNA derived from mRNA was applied to agarose gel-electrophoresis (panel A). The mRNA levels of NAT and β-actin were quantified by densitometric analysis of gel-photograph and expressed as NAT/β-actin ratio (panel B).


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