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Research Section

The effect of paclitaxel on gene expression and activity of arylamine *N*-acetyltransferase and DNA-2-aminofluorene adduct formation in human leukemia HL-60 cells

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Abstract

N-Acetylation is recognized as the first step in arylamine metabolism. The enzyme responsible for *N*-acetylation is called arylamine *N*-acetyltransferase (NAT),which uses acetyl coenzyme A as the acetyl group donor. Paclitaxel has been shown to exhibit antineoplastic and anticancer activity. In this study, paclitaxel was selected to determine the inhibition of arylamine *N*-acetyltransferase activity, gene expression (NAT mRNA) and DNA-2-aminofluorene adduct formation in human leukemia HL-60 cell line. Paclitaxel (0.01–1 μM) did decrease the level of NAT mRNA in a dose-dependent manner. The results demonstrated that paclitaxel inhibited NAT activity and DNA-2-aminofluorene adduct formation in human leukemia HL-60 cells in a dose-dependent manner. Using standard steady-state kinetic analysis, it was demonstrated that paclitaxel was a possible uncompetitive inhibitor to NAT activity in cytosols based on the decrease in apparent values of K_m and V_{max}. This report is the first demonstration that paclitaxel affected human leukemia HL-60 cells NAT activity and DNA–2-aminofluorene adduct formation.

Keywords: *N*-acetyltransferase; Paclitaxel; Human leukemia cells; *N*-acetyl-2-ammofluorene; 2-aminofluorene; Gene expression

1. Introduction

Chronic exposure to environmental chemicals or carcinogens may lead to organ or tissue damage and the occurrence of cancer (Farber, 1984; Harries and Sun, 1984). Chemical carcinogens that cause organ or tissue lesions are initiated by the enzymatic formation of reactive metabolites that bind covalently to nucleic acids and initiate the neoplastic process (Miller and Miller, 1981; Gilette, 1982; Guengerich and Liebler, 1985). Arylamine carcinogens are recognized as being responsible for the initiation of human cancers such as bladder cancer (reviewed in Cartwright, 1983; Miller and Miller, 1983). *N*-Acetylation is thought to be the first step in arylamine metabolism. The enzyme responsible for *N*-acetylation is called arylamine *N*-acetyltransferase (NAT), which uses acetyl coenzyme A as a cofactor (Weber and Hein, 1985). Two isozymes that have NAT activity have been reported (Weber and Hein, 1985; Evans, 1989). These enzymes are designated NATI and

Abbreviations: AAF, *N*-acetyl-2-aminofluorene; AF, 2-aminofluorene; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; N-Ac-PABA, *N*-acetyl-*p*-aminobenzoic acid; NAT, arylamine *N*-acetyltransferase; PABA, *p*-aminobenzoic acid; PMSF, phenylmethylsulfonylfluoride.

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NAT2. Human and several other animal species exhibit a well-defined *N*-acetylation polymorphism. Individuals can be divided into rapid and slow acetylators based on the rate of *N*-acetylation of arylamine. In humans, epidemiological studies have suggested a predisposition of slow acetylators to arylamine-induced bladder cancer (Cartwright et al., 1982) and rapid acetylators to arylamine-induced colorectal cancer (Lang et al., 1986). Therefore, the genetic variation of NAT enzymes could affect the cancer occurrence among human populations.

Paclitaxel (Taxol) was purified from the bark of the Pacific yew *Taxus brevifolia* which exhibits cytotoxic and antitumor activity against some murine leukemia and solid tumors (Wani et al., 1971; Donebower and Rowinsky, 1994). Those solid tumors include ovarian cancers (Reed et al., 1995), breast cancer and lung cancer (Rowinsky et al., 1991), head and neck cancer (Donebower and Roinsky, 1994), metastatic melanoma (Wiernik et al., 1987) and leukemia (Lieu et al., 1997; Rowinsky et al., 1989). Although paclitaxel has been shown to affect leukemia cells, there is no available information that addresses the effects of paclitaxel on NAT gene expression and activity in human leukemia cells. Thus, the present study was undertaken to determine whether paclitaxel could affect NAT gene expression and activity and DNA-2-aminofluorene adduct formation in human leukemia HL-60 cells.

2. Materials and methods

2.1. Chemicals and reagents

Paclitaxel (Taxol) was obtained from Bristol Caribbean, Inc. (NJ, USA). 2-Aminofluorene (AF), *N*-acetyl-2-aminofluorene (AAF), *p*-aminobenzoic acid (PABA), *N*-acetyl-*p*-aminobenzoic acid (N-Ac-PABA), ethylenediaminetetraacetic acid (EDTA), leupeptin, Tris, dimethyl sulfoxide (DMSO), acetylcarnitine, dithiothreitol (DTT), bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF), micrococcal endonuclease, spleen exonuclease, acetyl-coenzyme A and carnitine acetyltransferase were obtained from Sigma Chemical Co. (St Louis, MO, USA). RPMI 1640 tissue culture medium and penicillin–streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). All of the chemicals used were reagent grade.

2.2. Culture of human leukemia cell line

The human promyelocytic leukemia cell line (HL-60) was obtained from the CellBank (Hsinchu, Taiwan, R.O.C.). The cells were placed into 75-cm^2 tissue culture flasks and incubated at 37 °C under a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2% penicillin–streptomycin (10,000 U/ml plus 10 mg/ml, respectively).

2.3. Preparation of human leukemia cell cytosols

About 5×10^7 HL-60 cells were placed in 2 ml of the lysis buffer (20 mM Tris–HCI, pH 7.5 at 4 °C, 1 mM DTT, 1 mM EDTA, 50 μM PMSF, and 10 μM leupeptin) as previously described (Chung et al., 1993, 2000). The suspensions were mixed well and were centrifuged at 9000 *g* for 10 min in a model 3200 Eppendorf/Brinkman centrifuge. The supernatant fraction was subsequently centrifuged at 10,000 *g* for 60 min. Finally the supernatant was kept on ice for NAT activity and protein determinations.

2.4. Determination of NAT activity in vitro

The determinations of acetyl-CoA-dependent *N*-acetylation of AF and PABA were performed as previously described by Chung et al. (2000).

2.5. Determination of intact cell NAT activity

HL-60 cells (in 1 ml RPMI 1640 medium with glutamine and 10% fetal bovine serum) were incubated with an arylamine substrate (AF or PABA) at 5×10^5 cells/ml in individual wells of a 24-well cell culture plate with or without dimethyl sulfoxide (DMSO) paclitaxel co-treatment for various times (6, 12, 18 and 24 h) at 37 °C in an atmosphere of 95% air:5% CO_2. Paclitaxel was dissolved in DMSO. The final concentration of DMSO was 1% in media. This concentration did not harm the cells in the media. At the end of incubation, the cells and medium were harvested by centrifugation. For experiments with AF, the supernatant was immediately extracted twice with ethyl acetate–methanol (95:5), the solvent evaporated, and the residue redissolved in methanol and assayed for AAF as described previously (Chung et al., 2000).

2.6. Protein determination

Protein concentrations in the HL-60 cell cytosols were determined by the method of Bradford (1976) with bovine serum albumin as the standard. All of the samples were assayed in triplicate.

2.7. Effects of various concentrations of paclitaxel on NAT activity in human leukemia cells in cytosols and intact cells

For cytosol studies, the reaction mixtures consisted of 50 μl cytosols, 20 μl of recycling mixture containing AF or PABA at selected concentrations as substrates, and 10 μl of various concentrations of paclitaxel. The reac-

tions were started by the addition of acetyl coenzyme A (AcCoA) to the reaction mixture. The control reactions had 20 μl distilled water in place of AcCoA. Following these steps, the effect of paclitaxel on NAT activity was determined as described in the NAT activity determination section in the HL-60 cells (Chung et al., 2000). For intact cells studies, HL-60 cells in the media as described above were incubated with various concentrations of AF at 5×10^5 cells/ml in individual wells of 24-well cell culture plates with or without paclitaxel co-treatment for the time of incubation. At the end of incubation, the cells and medium were harvested by centrifugation. 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 by using HPLC as described above (Chung et al., 2000). For experiments with PABA, aliquots of the supernatant were assayed directly for N-Ac-PABA by HPLC.

2.8. Time course effects of paclitaxel on NAT activity in human leukemia intact cells

HL-60 cells were incubated with various concentrations of AF at 5×10^5 cells/ml in individual wells of 24-well cell culture plates with or without DMSO paclitaxel co-treatment for the 6-, 12-, 18- and 24-h incubation. At the end of incubation, the acetylated AF or PABA and unacetylated AF or PABA were determined by using HPLC as described above (Chung et al., 2000).

2.9. Effects of paclitaxel on kinetic constants of NAT from human leukemia cell cytosols

Cytosols of HL-60 cells co-treated with or without DMSO 5 μM paclitaxel and selected concentrations of AF were determined for NAT activity as described above (Chung et al., 2000). All reactions were run in triplicate.

2.10. Detection and measurement of DNA adducts in human and mouse leukemia cell

Detection and measurement of DNA adducts were performed as described previously (Chung et al., 2000; Li et al., 2001).

2.11. Determination of NAT gene expression [reverse transcriptase polymerase chain reaction (RT-PCR)]

The total RNA was extracted from HL-60 cells by using Qiagen RNeasy Mini Kit 24 h after co-treatment with or without different concentrations of paclitaxel as described previously (Hsia et al., 2001). Briefly, total RNA (1.5 μg), 0.5 μg of oligo-dT primer and DEPC (diethyl pyrocarbonate)-treated water were combined into a microcentrifuge tube to a final volume of 12.5 μl. 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, the components in 50 μl of solution were as follows: 1.5 mM $MgC1_2$, 0.2 mM dNTP mix, 20 pmol of each primer (B-MDIEA-NAT1 and VPKHGD-X-NAT1 for NAT1, FP1-NAT2 and RP1-NAT2 for NAT2, Act b1 and Act b2 for β-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 as follows: B-MDIEA-NAT1, 5″-CACCCG-GATCCGGGATCATGGACATTGAAGC-3″, nt 435–454, GenBank accession number X17059; VPKHGD-X-NAT1, 5″-GGTCCTCGAGTCAATCACCATGTT-TGGGCAC-3″, nt 1295–1278, GENBANK accession number X17059; FP1-NAT2, 5″-CTAGTTCCTGGTT-GCTGGCC-3″, nt 79–98, GenBank accession number NM-000015; RP1-NAT2, 5″-TAACGTGAGGGTA-GAGAGGA-3″, nt 1073–1054, GenBank accession number NM-000015; Act b1, 5″-GCTCGTCGTCGA-CAACGGCTC-3″, nt 94–114, GenBank accession number NM-001101; Act2 b2, 5″-CAAACATGATC-TGGGTCATCTTCTC-3″, nt 446–422, GenBank accession number NM-001101; Ponte et al., 1984; Blum et al., 1990; Ebisawa and Deguchi, 1991).

2.12. Statistical analysis of data

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 (1967) that performs linear regression using a least-squares method.

3. Results

3.1. Effects of various concentrations of paclitaxel on human leukemia cells in intact cells

In intact cell study, AF or PABA was added to the cell culture and which subsequently absorbed into the cell. AF or PABA was *N*-acetylated inside the cells to form AAF or N-Ac-PABA. The AAF or N-Ac-PABA was finally excreted to the outside of the cells. Then the amounts of unacetylated AF or PABA and acetylated AF or PABA in the supernatant were determined by HPLC. The mean ±S.D. (standard deviation) of NAT activity co-treated with or without paclitaxel in the presence of AF or PABA in intact cells are given in Table 1.

Table 1
Effects of paclitaxel on *N*-acetyltransferase activity in human HL-60 intact cells

Paclitaxel	AAF	N-Ac-PABA
Control	6.09±0.67	5.87±0.60
0.01 mm	4.87±0.54	4.56±0.46[a]
0.1 mm	3.35±0.42[a,b]	3.04±0.31[a,b]
1 mm	2.89±0.35[a,b]	2.16±0.19[a,b]
5 mm	2.28±0.27[a,b]	1.74±0.15[a,b,c]
10 mm	1.83±0.16[a,b,c]	1.40±0.12[a,b,c]
25 m m	1.37±0.12[a,b,c,d]	0.90±0.09[a,b,cd]
50 mm	0.38±0.06[a,b,c,d,e,f]	0.24±0.06[a,b,c,d,e,f]
F value	72.605*	116.026*

[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.
Note: Values are mean±S.D. of activity (nmol/10^6 cells); $n=6$.
ANOVA analysis with Scheffe posteriori comparison was used. *$P<0.001$.

The data indicated that there was a decrease in AFF or N-Ac-PABA associated with increasing concentrations of paclitaxel in intact cells. For the intact cells examined, in the presence of 0.01, 0.1, 1, 5, 10, 25 and 50 μM paclitaxel, the *N*-acetylation of AF and PABA were inhibited from 20 to 94% and from 22 to 96% for AF and PABA acetylation, respectively (Table 1). Therefore, the IC_{50} of paclitaxel for NAT activity of human HL-60 cells was 1.25 μM.

3.2. Time course effects of paclitaxel on NAT activity in human leukemia intact cells

HL-60 cells that were incubated with AF produced AAF in the culture medium, but cells without AF and AF without cells did not show any detectable AAF in the medium in all examined time (Fig. 1). However, ncreased time of incubation led to increased AAF production up to 24 h (Fig. 1). The presence of 5 μM of paclitaxel decreased the amounts of AAF about 34–52% for HL-60 cells.

3.3. Effects of 5 μM paclitaxel on kinetic constants of NAT in human leukemia cells cytosols

In the presence or absence of paclitaxel, various concentrations of AF (0.087, 0.174, 0.348, 0.696 and 1.392 mM) were added to the recycling mixtures for determining cytosolic NAT kinetic constants. When 5 μM paclitaxel was added to the cytosol reaction mixtures, the apparent K_m and V_{max} values were decreased (Table 2 and Fig. 2). Clearly, both K_m and V_{max} values on the cytosolic NAT were decreased in the presence of paclitaxel in cytosol.

3.4. Effects of paclitaxel on DNA–AF adducts in human leukemia cells

After 18 h of incubation of HL-60 cells with AF in the presence or absence of paclitaxel, cells were harvested and DNA was isolated, hydrolyzed to nucleotides, adducted nucleotides were extracted into butanol and analyzed by HPLC (Levy et al., 1994; Chung et al., 2000). The results indicate that HL-60 cells activate AF to a metabolite able to bind covalently with DNA and increased AF concentration led to the increase of the DNA−AF adduct formation. Overall, the data indicated that paclitaxel decreased DNA−AF adduct formation in HL-60 cells in all examined AF doses (30 and 90 μM) (Fig. 3).

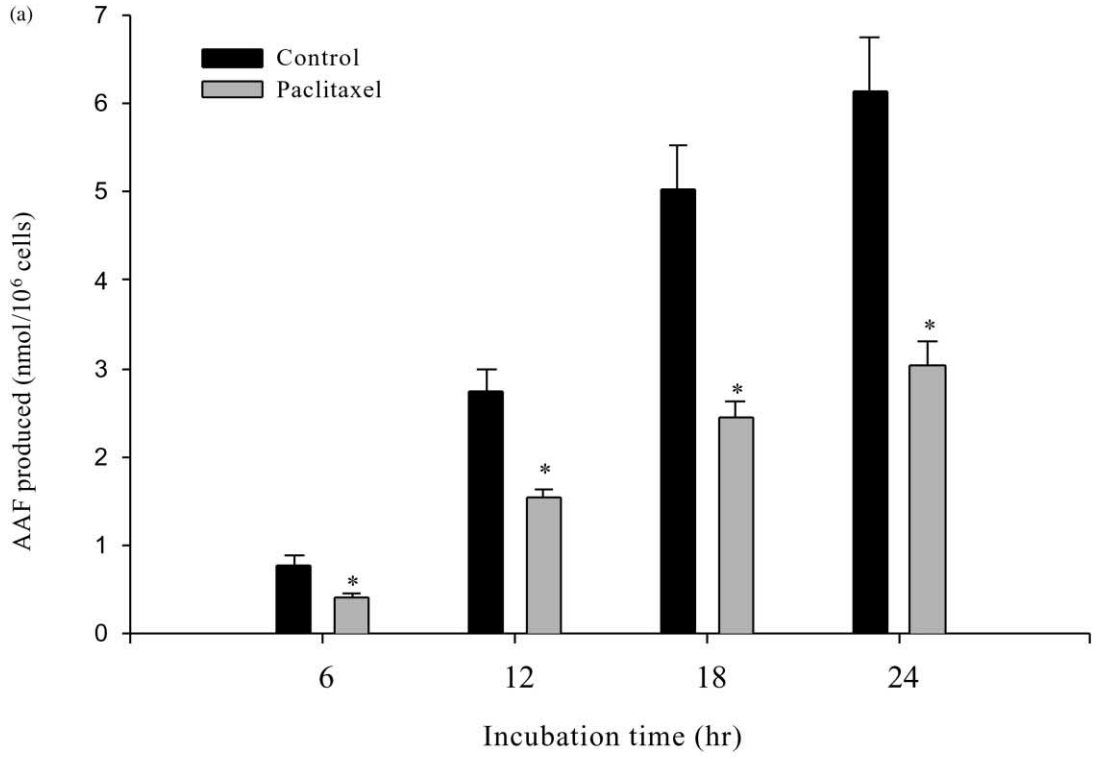
3.5. Dose-dependent effects of paclitaxel on NAT mRNA expression in HL-60 cells

Dose-dependent changes of NAT1 mRNA levels in response to various concentrations of paclitaxel were examined (Fig. 4a,b). Data presented in Fig. 4 (a,b) show that the NAT1 mRNA level increased as the paclitaxel levels decreased. β-Actin gene represents our using the same amounts of cDNA for NAT-PCR. NAT1 mRNA was present in human leukemia HL-60 cells, but NAT2 was not present in these examined cells. However, RT-PCR is not a quantitative assay and therefore the exact extent of the increase is uncertain. The results indicated that paclitaxel could affect the expression of NAT mRNA in HL-60 cells.

Table 2
Kinetic data for acetylation of substrates with or without paclitaxel co-treatment in human leukemia HL-60 cells

	AF		PABA	
	K_m (mm)	V_{max} (nmol/min/mg protein)	K_m (mm)	V_{max} (nmol/min/mg protein)
Control	5.26±0.42	33.38±5.29	2.70±0.32	16.17±2.89
Paclitaxel	2.70±0.18[a]	16.67±3.12[b]	2.22±0.21	12.50±1.06[b]

[a] Differs between 50 μM paclitaxel and control. $P<0.05$.
[b] Differs between 50 μM paclitaxel and control. $P<0.05$.
Note: Values are mean ±S.D. $n=3$. The Acetyl CoA and paclitaxel concentrations were 0.1 mM and 5 μM, and the kinetic constants were calculated from the modified HYPER Program of Cleland (1967).



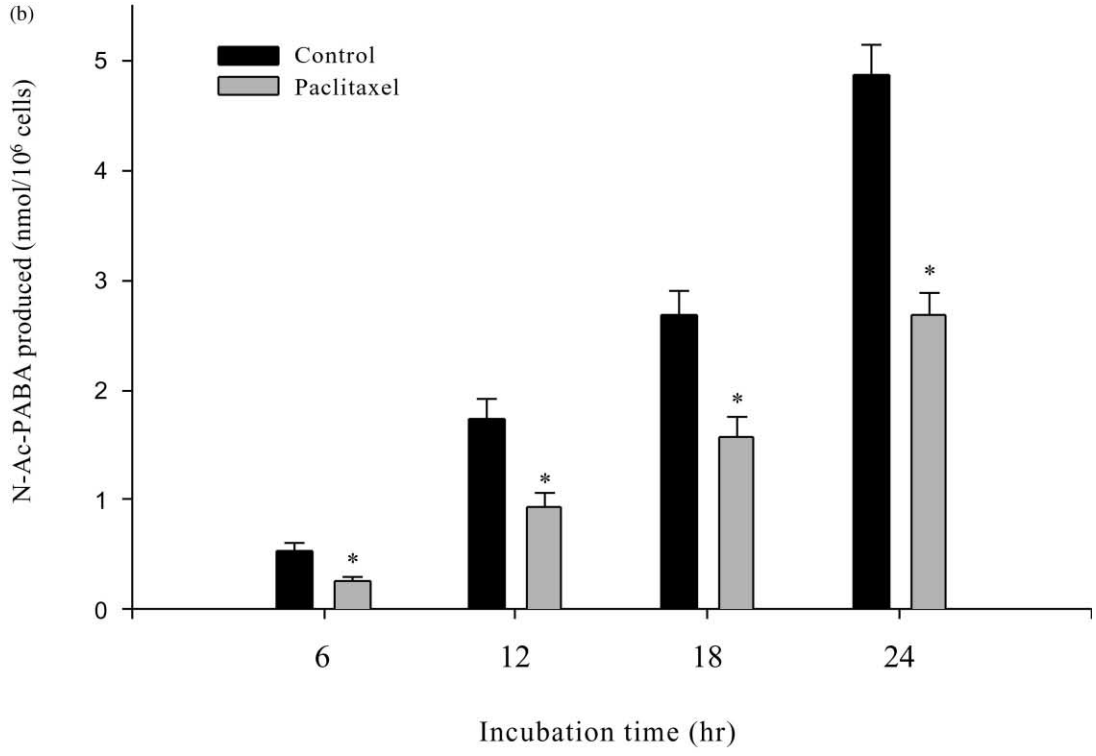
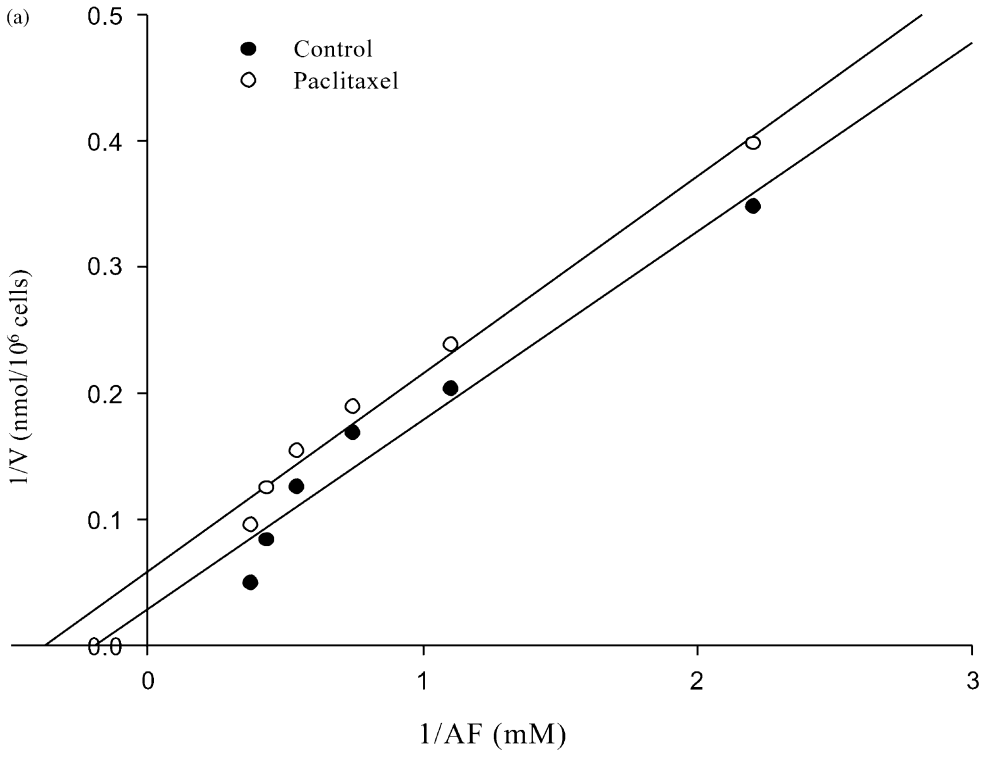


Fig. 1. Effects of paclitaxel and incubation time on *N*-acetyl-2-aminofluorene (AAF) production in intact human leukemia HL-60 intact cells. Human leukemia HL-60 cells were incubated with 2-aminofluorene (AF) or *p*-aminobenzoic acid (PABA) at 22.5 μM with or without paclitaxel (5 μM) co-treatment for the times shown (6, 12, 18 and 24 h). AF and AAF (Panel 1a), PABA and N-Ac-PABA (Panel 1b) were measured by HPLC assay. Each point represents the mean of triplicate assays. Values are mean ± S.D. ($n = 3$). *Differ between 5 μM paclitaxel and control, $P < 0.05$.



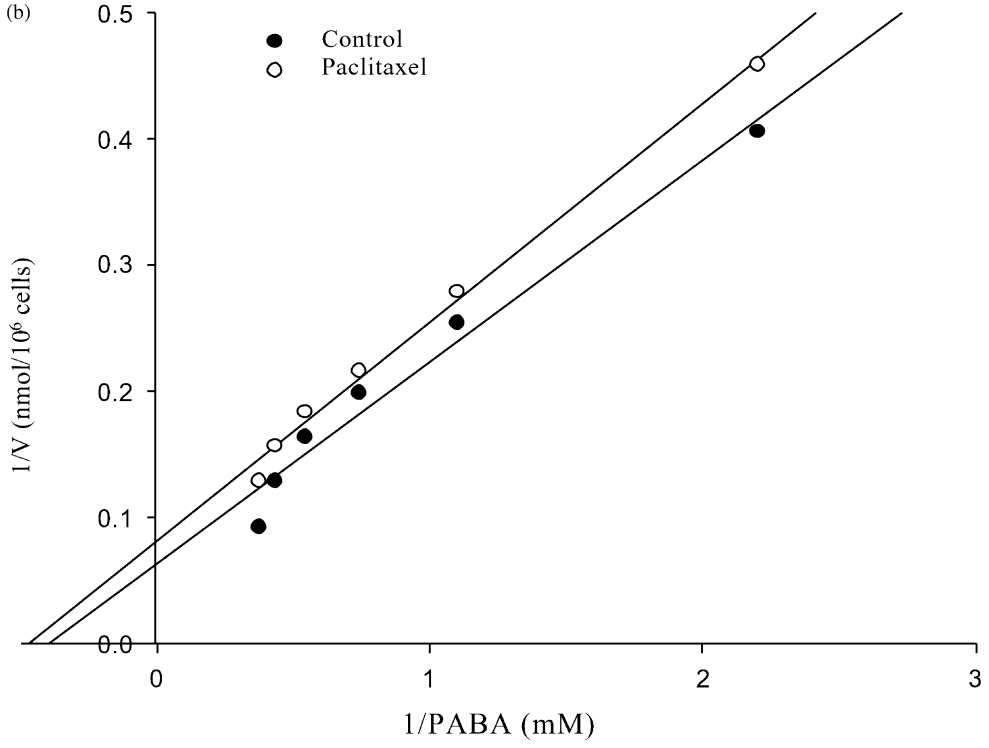


Fig. 2. Lineweaver–Burk double reciprocal plot of NAT in human leukemia HL-60 cells co-treatment with or without 5 μM paclitaxel. About 5×10^5 cells/ml containing 0.044, 0.088, 0.163, 0.303 and 0.709 mM AF were incubated for 18 h incubation. Then the amounts of AF and AAF (Panel 2a), PABA and N-Ac-PABA (Panel 2b) were determined as described in Materials and methods. Lineweaver–Burk double reciprocal plots were measured by using linear regression analysis of reciprocal substrate concentrations plotted against reciprocal initial velocities.

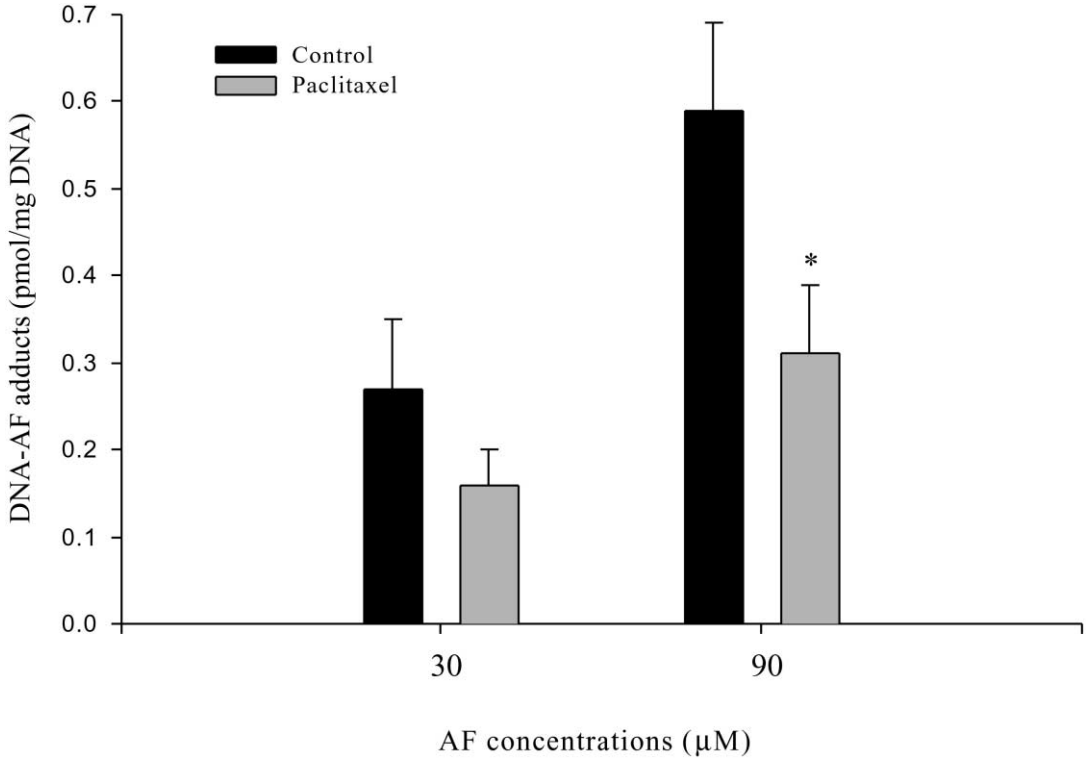


Fig. 3. Effects of paclitaxel on DNA–AF adduct formation by intact human leukemia HL-60 cells. Following the 18-h incubation of human leukemia HL-60 cells with AF (30 and 90 μM) in the presence of paclitaxel (5 μM), cells were harvested and DNA was prepared, hydrolyzed to nucleotides, adducted nucleotides were extracted into butanol and analyzed by HPLC. Values are means ±SD of six separate preparations. *Differ between 5 μM paclitaxel and control, $P<0.05$.

4. Discussion

Although cell-cycle arrest, apoptosis and cytotoxicity induced by paclitaxel (taxol) have been reported in many different mammalian cells, including human leukemia HL-60 cells, the effect of paclitaxel on NAT enzyme in human leukemia HL-60 cells has not been investigated. It is recognized that the cytotoxicity of paclitaxel is associated with its microtubule binding ability. Paclitaxel had been reported to affect the activation of protein kinase (Ponnathpur et al., 1995). Our previous studies already demonstrated that HL-60 cells did contain NAT activity (Li et al., 2001). Einisto et al. (1991) have demonstrated that increased levels of NAT activity are associated with increased sensitivity to the mutagenic effects of many arylamines. Other investigators also demonstrated that NAT enzymes play an important role in some aspects of chemical carcinogenesis (Grant et al., 1991; Minchin et al., 1992). More interesting is the fact that decreases in liver NAT activity are associated with breast and bladder cancer processes (Weber and Hein, 1985; Weber, 1987). Therefore, it is of interest to know whether paclitaxel could affect NAT activity in human leukemia HL-60 cells.

In the present study, we have examined the effect of paclitaxel on *N*-acetylation of AF in human leukemia HL-60 cells. The results indicate that paclitaxel inhibited the *N*-acetylation of AF in cytosols and intact cells based on the following observations: (i) the amounts of AAF in human leukemia HL-60 intact cells and cytosol were decreased in a dose-dependent manner; (ii) K_m and V_{max} values of NAT in human leukemia HL-60 cells cytosols were decreased; (iii) paclitaxel did affect NAT gene expression in this examined cell line. The result from the HL-60 cell cytosol and intact cells studies showed that there were significant differences in the *N*-acetylation of AF and PABA between the control and paclitaxel treatment groups based on the P values of Student's *t*-test and ANOVA analysis. The result also indicated that paclitaxel decreased the NAT kinetic constants (K_m and V_{max}) in the cytosols of HL-60 cells. Although the inhibition is uncompetitive, the nature of the interaction and the NAT protein domains from HL-60 cells involved in this interaction remain unclear. Further investigations are needed.

Thorgeirsson et al. (1983) have demonstrated that cytochrome P450 enzyme was also involved in the metabolism of acetylated AF (AAF) and that cytochrome P450-dependent formation of *N*-hydroxy-AAF is considered the initial rate-limiting step in the metabolism of AAF to mutagenic and potentially carcinogenic products such as AAF–DNA adduct formation. It was also reported that P4501A1 is particularly efficient for catalyzing conversion of AAF to 7-OH-AAF. The

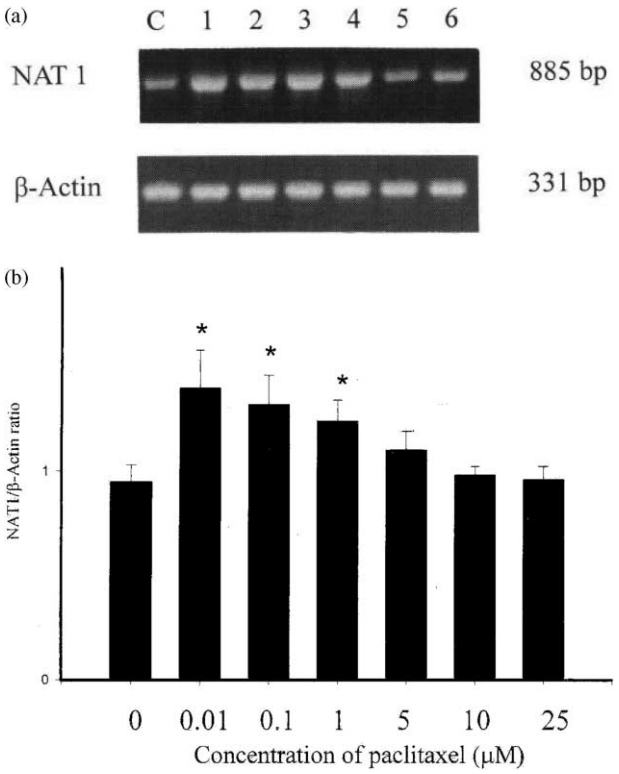


Fig. 4. Dose-dependent effect of paclitaxel on the expression of NAT mRNA in human leukemia HL-60 cells. The cells were incubated with various concentrations of paclitaxel for 18 h. The cells were collected to extract RNA. The extracted RNA was subjected to RT-PCR analysis using specific primers for NAT and β-actin, and then PCR-amplified cDNA derived from mRNA were applied to agarose gel-electrophoresis (a). The mRNA levels of NAT and β-actin on the gel-electrophoresis were quantified by densitometric analysis of gel-photograph and expressed as NAT/β-actin ratio (b). *Differ between 0.01 μM paclitaxel and control, $P<0.05$. *Differ between 0.1 μM paclitaxel and control, $P<0.05$. *Differ between 1 μM paclitaxel and control, $P<0.05$.

present study did not consider whether paclitaxel could affect cytochrome P450 activity in HL-60 cells. Apparently, further investigation is needed for future study. Although it was reported that paclitaxel could inhibit protein kinase activity, there was no information to show that paclitaxel could inhibit NAT enzyme activity in vitro and in intact cells. Consequently, our result offers some important information to show paclitaxel can affect NAT activity and NAT gene expression in human leukemia 60 cells. The reasons for choosing 50 μM paclitaxel for the time course, kinetic constants, NAT gene expression and AF–DNA adduct studies was that NAT activity in human leukemia cells cytosol was inhibited over 50% after co-treatment with 50 μM paclitaxel.

It is not known whether decreasing the *N*-acetylation of AF or even decreasing AF–DNA adduct formation and NAT gene expression in intact cells will lead to decreased leukemia formation in human, or whether paclitaxel would prevent the development of human leukemia. But the exact role of the arylamine NAT enzyme in leukemia and any other tissue malignances remains enigmatic. It has been proposed that the susceptibility to the carcinogenic effects of aromatic and heterocyclic amines may depend on: (a) the relative rates of *N*-acetylation and *N*-hydroxylation in the liver; (b) the route of excretion of arylamine compound metabolites; and (c) possibly the rates of glucuronide hydrolysis and NAT mediated activation in the target tissue (Smith et al., 1995). Conversion to mutagenic metabolites involves hydroxylation and acetylation, largely in the liver tissue, with additional acetylation of circulating hydroxyl metabolites occurring in other tissues or organs; it is still unknown. On the other hand, the metabolites of arylamine compounds from liver or other tissues may transfer to other target tissues via the circulation and then induce tissue malignancy. This point is still controversial since reports from liver tissues suggest that NAT enzyme is involved in the detoxification of exogenous amines (Weber and Hein, 1985; Evans, 1989). But other investigators found significant NAT enzyme could be involved in acetylation of ocular drugs (Campbell et al., 1991). Therefore, further investigation is needed, such as oral treatment of paclitaxel to rats in vivo.

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