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# N-Acetyltransferase Activity is Involved in Paclitaxel-induced N-Acetylation of 2-Aminofluorene in Human Bladder Cancer Cells (T24)

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**Abstract.** *Arylamine N-acetyltransferase (NAT) plays an important role in the metabolism of 2-aminofluorene (AF) and some types of arylamine drugs and carcinogens. Our previous studies have demonstrated that paclitaxel decreases NAT activity in human bladder, blood, colon and lung cancer cells. In this study, paclitaxel was selected to test the inhibition of NAT activity (N-acetylation of AF) and NAT gene expression in a human bladder cancer cell line (T24). The NAT activity was determined by high performance liquid chromatography for measuring the levels of N-acetylation of AF. The data showed that a 24-hour paclitaxel treatment decreased the amount of N-acetylation of AF in T24 cells. The NAT enzymes were stained and analyzed by Western blotting and flow cytometry. The tests indicated that paclitaxel decreased the levels of NAT in T24 cells. The expression of the NAT gene (mRNAT NAT) was determined by polymerase chain reaction (PCR) and cDNA microarray and it was found that paclitaxel induced the down-regulation of mRNA NAT expression in T24 cells.*

It is well documented that arylamine compounds are carcinogenic for humans and laboratory animals (1,2). N-acetylation, a major metabolic step for arylamine carcinogens, is catalyzed by cytosolic arylamine N-acetyltransferase (NAT), using acetyl-coenzyme A as an acetyl group donor for its activity (2). Individual sensitivity to toxicity and carcinogenicity after exposure to some arylamine chemicals is related to the acetylator phenotypes of NAT (3). The NAT activity (N-acetylation of substrates) of individuals can be divided into rapid and slow acetylation (2). The rapid acetylator phenotype is associated with an increased risk of colorectal cancer (4,5) and the slow acetylator phenotype with increased drug toxicity from sulfonamides (6) and increased susceptibility to bladder cancer (7). Therefore, the NAT activities in genetical variations of target organs or tissues may indicate different risks for arylamine-induced neoplasms among the human population.

Paclitaxel (taxol) ($C_{47}H_{51}NO_{14}$), a poly-oxygenated naturally occurring diterpenoid, was first isolated from the bark of the Pacific yew tree (*Taxus brevifolia*) and is considered to be one of the most important drugs in human cancer chemotherapy (8,9). Paclitaxel is used as an antitumor agent for chemotherapy-resistant epithelial ovarian cancer, advanced breast cancer, small cell and non-small cell lung cancer and head and neck cancer (10). Paclitaxel is a cell cycle-specific agent which blocks the cells in the G2/M-phase and displays toxicity *via* apoptosis (11,12). Paclitaxel induces apoptosis in human osteoblastic cells Saos-2 with CD95L up-regulation and Bcl-2 phosphorylation (13). In our previous studies, we showed that paclitaxel affects NAT activity and gene expression in human cancer cell lines such as bladder T24 (14), A549 lung cancer cells (15) and human HL-60 leukemia cells (16). However there are no reports concerning the effects of paclitaxel on human bladder cancer cell NAT enzyme and its gene expression. Thus, the present studies were focused on the effects of paclitaxel on N-acetylation and the NAT gene expression of human bladder T24 cancer cells were examined by Western blotting, PCR, flow cytometry and cDNA microarray.

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*Key Words:* N-acetyltransferase, paclitaxel, bladder cancer cell line (T24), 2-aminofluorene, flow cytometry, cDNA microarray.

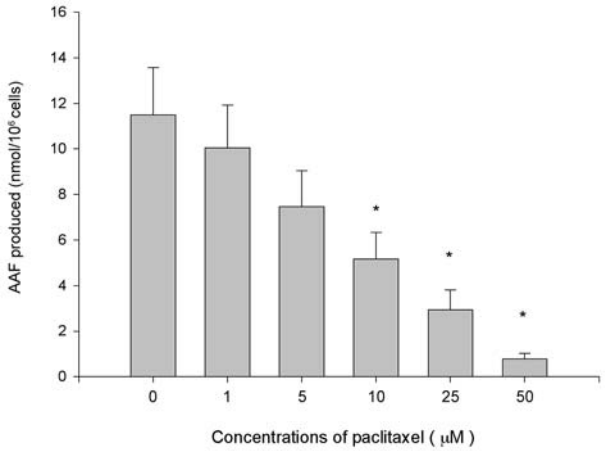


Figure 1. *Effects of various concentrations of paclitaxel on N-acetylation of AF by human bladder T24 cancer cells. T24 cells (1x10$^6$/ml) were incubated with 6.75 μM AF and with 0, 1, 5, 10, 25 and 50 μM paclitaxel co-treatment for 24 hours. N-acetylated AF was measured by HPLC assay. Each point represents the mean of triplicate assays of three incubations of cells. * mean differs between paclitaxel and control. $p<0.05$.*

Table I. *The percentage of human bladder T24 cancer cells treated by paclitaxel which were stained by the NAT antibody.*

| Paclitaxel (μM) | Percentage of cells stained by anti-NAT |
|---|---|
| 0 (control) | 64.24 ± 6.37 |
| 1 | 56.68 ± 6.38 |
| 5 | 48.46 ± 5.96 |
| 10 | *36.44 ± 4.69 |
| 25 | *16.44 ± 3.02 |
| 50 | *5.12 ± 1.05 |

Values are mean ± S.D. n=6. The T24 cancer cells (1x10$^6$ cells/ml) were co-treated with various concentrations of paclitaxel. The zero concentration was defined as control. The cells were stained by NAT antibody and the percentage of stained cells were determined by flow cytometry as described in the Materials and Methods section.
*differs between paclitaxel and control. $p<0.05$.

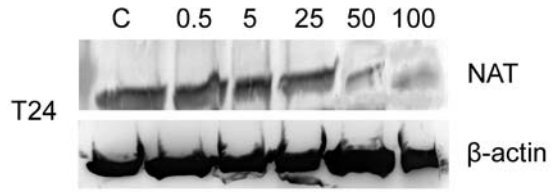


Figure 2. *Representative Western blot showed changes in NAT in human bladder T24 cancer cells in response to paclitaxel. T24 cells (5x10$^6$/ml) were treated with 0, 1, 5, 10, 25 and 50 μM paclitaxel for 24 hours followed by evaluation of NAT expression. NAT expression was estimated by Western blotting as described in Materials and Methods.*

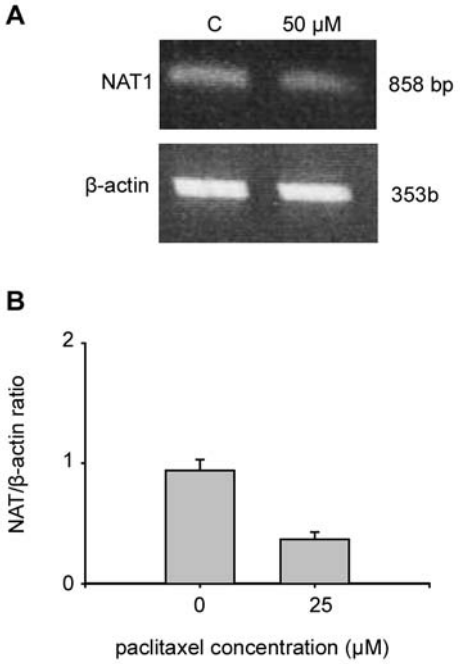


Figure 3. *Effect of paclitaxel on the expression of NAT mRNA in human bladder T24 cancer cells. T24 cells were incubated with 25 μM paclitaxel for 24 hours. The cells were collected to extract RNA. The extracted RNA was subjected to RT-PCR analysis using specific primers for NAT and β-actin, and then PCR-amplified cDNA derived from mRNA were applied to agarose gel-electrophoresis (panel 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 (panel B).*

## Materials and Methods

*Chemicals and reagents.* Paclitaxel (Taxol), ethylenediaminetetraacetic acid (EDTA), 2-aminofluorene (AF) and N-acetyl-2-aminofluorene (AAF), TrisHCl, dithiothreitol (DTT), Acetyl-CoA (AcCoA), acetylcarnitine, leupeptin, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF), complete Freund's adjuvant and carnitine acetyltransferase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All of the chemicals used were reagent grade.

*Human bladder cancer cell line (T24).* Human bladder carcinoma cell line (T24: human; female; 81 years old) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The T24 cells were placed into 75-cm$^3$ tissue culture flasks and grown at 37˚C under a humidified 5% $CO_2$ atmosphere in McCoy's 5a medium (Sigma) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 5% L-glutamine (200 mM) and 2% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin), as described previously (14).

*Determination of intact cell NAT activity.* T24 cells (in 1 ml McCoy's 5a medium with L-glutamine and 10% fetal calf serum) were individually incubated with 6.75 μM 2-aminofluorene (AF) (arylamine substrate) at 1x10$^6$ cells/ml in individual wells of a 24-well cell culture plate, with or without paclitaxel (1, 5, 10, 25 and 50 μM) co-treatment for 24 h, at 37˚C in 95% air 5% $CO_2$. At the end of

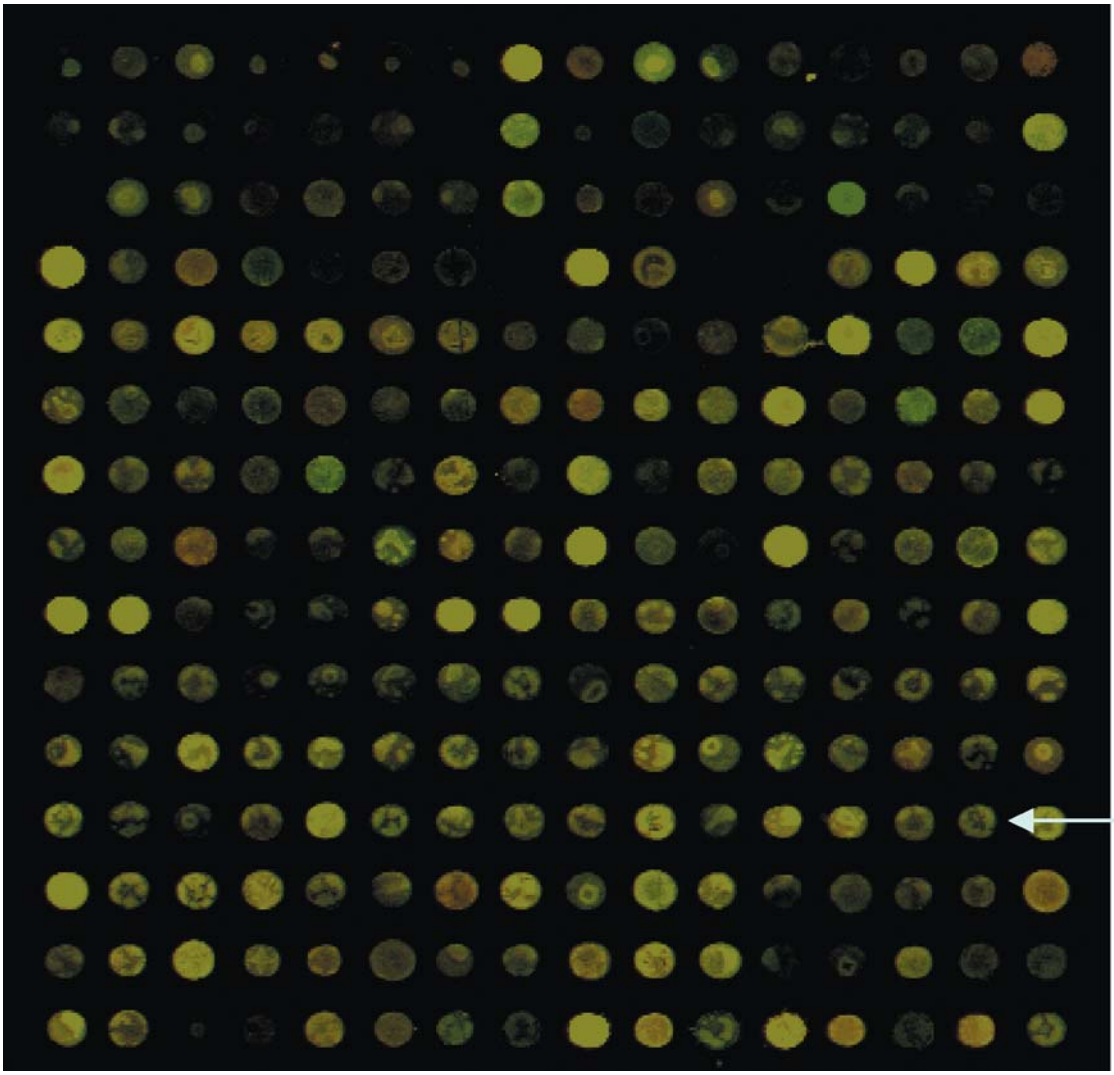

Figure 4. *Down-regulation of NAT gene in human bladder T24 cancer cells treated with 25 μM paclitaxel, assayed by cDNA microarray. T24 cancer cells (5x10$^6$ cells/ml) in 6-well plate treated with or without 25 μM paclitaxel for 24 hours. Red color spot represents up-regulation and green color spot represents down-regulation. The arrow marks the NAT gene down-regulation.*

incubation, the cells and media were removed and centrifuged. The supernatant was immediately extracted with ethyl acetate/methanol (95:5), the solvent evaporated and the residue redissolved in methanol and assayed for N-acetylation of 2-aminofluorene (AAF), as described previously (14-16).

*Preparation of polyclonal antibody of NAT.* 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, was applied *s.c.* for 10-15 days. The antigen was emulsified with incomplete Freund's adjuvant, injected *i.p.* for 10-20 days and then boosted again. Myeloma cells (1x10$^6$/ml) in PBS were then injected *i.p.* into the mouse. The ascites fluids, which normally accumulate after 1 week, were collected daily for 5-8 days (17).

*Detection of NAT protein in human bladder T24 cells by flow cytometry.* The level of intracellular NAT of the human bladder T24 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using the prepared polyclonal antibody mentioned above. Cells were cotreated with various concentrations (1, 5, 10 25 and 50 μM) of paclitaxel for 24 h to detect the intracellular NAT. The cells were harvested and 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. Then the cells were washed three 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 three times with the same buffer, the cells were incubated with polyclonal 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 (17).

*Detection of NAT protein in human bladder T24 cells by Western blotting.* About $1x10^7$ cells were placed in a 6-well plate cotreated with or without various concentrations of paclitaxel for 24 h. Cells from individual cells lines (T24) were then harvested and centrifuged to remove and discard the medium, while the cells were washed with PBS. The cells were lysed in 100 μl of triple detergent buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.02% $NaN_3$, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, 1μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin A). The samples were then sonicated, incubated on ice for 20 min and centrifuged at 12000g for 10 min at 4˚C. The supernatants were collected and the Bradford assay was performed to determine the protein concentration. Proteins (50 μg/lane) were separated on 12% SDS-polyacrylamide gels and blotted onto polyvinyliene difluoride membranes. The membrane was incubated with 5% bovine serum albumin and primary antibody (anti-NAT) overnight at 4˚C. The blots were washed three times in PBS with 0.04% Tween-20 (PBST) for 5 min then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at 1:1000 dilution in PBST containing 5% milk for 2 h at room temperature. The membranes were washed with PBST and visualized using the ECL detection system (Amersham, Piscataway, NJ, USA) and quantitated by densitometry using ImageQuant image analysis.

*Reverse transcriptase polymerase chain reaction (RT-PCR).* The total RNA was extracted from the examined cells (T24) by using the Qiagen RNeasy Mini Kit at 24 h, with or without cotreatment of 20 μM paclitaxel as described previously (15). 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 template for PCR. When amplifying the target cDNA, the components in 50 μl 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 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'-TAACGTGAGGGTAGA GAGGA-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'-CAAACATGATCTGGG TCATCTTCTC-3', nt 446-422, GenBank accession number NM-001101 (17-19)

*cDNA Microarray.* The total RNA was extracted from human bladder cancer cells (T24) co-treated with or without 25μM paclitaxel by using the Qiagen RNeasy Mini Kit for the indicated time. The total RNA was used for cDNA synthesis and then cDNA was labeled for microarray hybridization. The fluorescence-labeled cDNA hybridized to its complement on the chip and the resulting localized concentrations of fluorescent molecules were detected and quantitated (Asia BioInnovations Corporation, Taipei, Taiwan) (20,21).

*Statistical treatment of data.* Statistical analysis of the data was performed with an unpaired Student's *t*-test and SAS program.

## Results

*Effects of various concentrations of paclitaxel on human intact bladder T24 cancer cells NAT activity.* The data of N-acetylation of AF measured from intact cancer cells co-treated with or without 1, 5, 10, 25 and 50 μM paclitaxel for 24 h are presented in Figure 1. In the presence of various concentrations of paclitaxel, the levels of N-acetylation of AF were decreased by about 4-92% in comparison to control.

*Effects of paclitaxel on the levels of NAT in human bladder T24 cancer cells were examined by flow cytometry.* NAT protein was measured by the NAT antibody formation of an antigen-antibody complex. The percentage of NAT-antibody complex from examined T24 cells that were cotreated with or without various concentrations of paclitaxel for 24 h was determined by flow cytometry. The data indicated that paclitaxel decreased the percentage of NAT-antibody complex in examined cells (Table I).

*Effects of paclitaxel on the levels of NAT in human bladder T24 cancer cells were examined by Western blotting.* The effects of various concentrations of paclitaxel on the total levels of NAT from T24 cells were also confirmed by Western blotting as shown in Figure 2. The NAT levels were decreased in response to paclitaxel.

*Effects of paclitaxel on NAT mRNA expression in human bladderT24 cancer cell line.* The changes of NAT1 mRNA levels in response to the effect of paclitaxel were examined and are presented in Figure 3 A and B. Data presented in Figure 3B show that NAT1 mRNA levels decreased following treatment of T24 cells with 25 μM paclitaxel.

*Effects of paclitaxel on NAT mRNA expression in intact T24 cancer cells were examined by cDNA microarray.* The results from cDNA microarray are presented in Figure 4. The arrow mark on the block of cDNA microarray of Figure 4 indicates that the NAT gene is down-regulated (green color spot) in the paclitaxel-treated T24 cells. Apparently paclitaxel did inhibit the NAT gene expression.

## Discussion

Arylamine NAT enzyme has been found in many laboratory animals and also in human tissues (2), and it has been implicated in chemical carcinogenesis (22). NAT affects the DNA-adduct formation, distribution and metabolism of AF in various organs and tissues of rats (23, 24). Bladder cancer is the 11th most common cancer in the world, accounting for 3-4% of all malignancies (25).

Bladder cancer is the 13th most common cause of mortality for males in Taiwan (People Health Bureau, Taiwan, 2001). Our previous studies have shown that paclitaxel affected NAT activity and gene expression in the human lung A549 cell line (15) and affected NAT activity in human bladder T24 and TSGH cancer cell lines (26).

The present study focuses on how pacitaxel affects NAT activity, using Western blotting, flow cytometry and cDNA microarray in the human bladder T24 cancer cell line. At first, the data from HPLC analysis demonstrated that paclitaxel decreasesd NAT activity (N-acetylation of AF) (Figure 1). This is in agreement with our earlier studies (26). Then the data from Western blot and flow cytometric analysis demonstrated that paclitaxel decreased the amount of NAT protein. Finally, the gene expression data from PCR and cDNA microarray clearly demonstrated that paclitaxel did in fact inhibit gene expression of NAT. Therefore, paclitaxel may affect NAT gene expression, leading to a decrease in the amounts of NAT, then finally leading to decreased N-acetylation of AF in T24 cells.

The enzymatic mechanisms for the ring-hydroxylation of certain primary arylamines have been examined in rats, and the data suggested that the rat hepatic cytochromes P-450 monooxygenases (CYPs) and flavin-containing monooxygenase catalyzed several ring-hydroxylations (27). Except NAT, there are no other factors that are important for AF metabolism such as cytochrome P450 enzymes (CYPs). AF is N-acetylated by NAT, then metabolized by CYP enzymes to form reactive metabolites which bind to DNA to form DNA-AF metabolite adducts and it may finally lead to cancer development (28). The cytochrome P450-dependent formation of N-hydroxy-AAF is considered to play an important role in the initial rate-limiting step for the metabolism of AAF to mutagenic and potentially carcinogenic products (29).

From our present study, we were unable to draw the firm conclusion that paclitaxel could decrease or prevent the production of bladder cancer cells in human after exposure to AF. The important point is that other investigators had also found that increased levels of NAT activity are associated with increased sensitivity to the mutagenic effects of many arylamines (30) and the attenuation of NAT activity in liver is associated with several disease processes, such as breast and bladder cancer (2,3). However, whether decreasing the NAT level and gene expression (NAT mRNA) of NAT could decrease sensitivity to the mutagenic effects of many arylamines is still unclear. Our previous studies have demonstrated that decreased NAT activity is associated with the decreasing of AF-DNA adduct formation in human bladder T24 cancer cells (31). Other investigators have demonstrated that the activity of NAT was related to bladder cancer occurrence (15, 32) and, when mice were continuously fed AAF, they developed bladder tumor (33). Furthermore, the data showed that paclitaxel decreased the NAT gene expression and protein levels *in vitro* .

Thus, for the prevention of bladder cancer occurrence, we should examine the effects of paclitaxel on CYPs activity, enzyme levels and gene expression, since CYPs and NAT expression are biomarkers of carcinogen-DNA adduct levels as well as of human cancer susceptibility (34). Cytochrome P450 enzyme is also involved in the metabolism of N-acetylated AF (AAF). Other investigators reported that cytochrome P4501A1 is particularly efficient in catalyzing the conversion of AAF to 7-OH-AAF (35,36). Smith *et al.* (37) reported that an individual's susceptibility to the carcinogenic effects of aromatic and heterocyclic amine carcinogens may depend on: a) the relative rates of N-acetylation and N-hydroxylation in the liver; b) the route of excretion of metabolites from the tissues and organ; and c) possibly, the rates of glucuronide hydrolysis and NAT-mediated activation in the target tissue (37).

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