in vivo *18*: 21-32 (2004)

The Inhibition of N-Acetyltransferase Activity and Gene Expression in Human Bladder Cancer Cells (T24) by Shikonin

CHIN-CHUNG YEH[1,2], LII-TZU WU[3], SHUW-YUAN LIN[4] ,TE-MAO LI[2] and JING-GUNG CHUNG[3]

[1]*Department of Urology, China Medical University Hospital and*
[2]*Chinese School of Medicine, China Medical University, Taichung City;*
[4]*Department of Food and Nutrition, Hung-Kuang University, 34 Chung-Chie Road, Sha Lu, Taichung Hsien 433;*
[3]*Department of Microbiology, China Medical University, Taichung City, Taiwan, R.O.C.*

Abstract. *Shikonin has the potential to prevent, or be used in the treatment of, bladder transitional cell carcinoma induced by arylamines. We evaluated its effectiveness by measuring the amount of acetylated 2-aminofluorene (AF), AF-DNA adducts, changes of NAT mRNA and the amount of NAT enzyme. T24 human bladder cancer cells were incubated with 30 μM AF with different concentrations of shikonin for various times. T24 cells treated with shikonin (16 μM) were then harvested and used in 2 experiments: 1). T24 cells were incubated with 22.5 μM AF and shikonin (0, 16 μM) (co-treatment) for 6, 12, 18, 24 and 48 h. 2). T24 cells were incubated with various concentrations of AF and shikonin (0, 16 μM) for 24 h. AF and AAF were measured by HPLC. Then in the prepared human T24 cell cytosols different concentrations of AF and shikonin were added to measure the kinetic constants of NAT. Next, AF-DNA adducts in human T24 cells with or without treatment with shikonin were detected and measured. The final two steps included measuring the NAT Ag-Ab complex after treatment with and without shikonin and evaluating the effect of shikonin on the NAT genes. Higher concentrations of shikonin induced decreasing AF acetylation. We found that the longer the culture period, the greater the difference in AF acetylation in the same shikonin concentrations. It was also noted that increase in AAF was proportional to incubation time. In the presence of 16 μM of shikonin, N-acetylation of AF decreased by up to 72-84%. Shikonin decreased the amount of AAF production in human T24 cells in all examined AF doses. Both Km and Vmax values in the cytosolic NAT decreased after the addition of shikonin to the cytosol. Finally, shikonin decreased the amount of AAF production and AF-DNA adducts formation in human T24 cells in all examined AF doses. The percentage of cells stained by antibody was significantly different after treatment with shikonin, especially with the higher shikonin concentrations. The NAT1 mRNA level and the NAT1/β-actin ratio decreased significantly with higher concentrations (16-24 μM) of shikonin. Shikonin affected NAT activity, gene expression (NAT1 mRNA), AF-DNA adducts formation and formation of NAT Ag-Ab in human bladder tumor T24 cells. Therefore, shikonin should be considered as a candidate agent for the prevention or treatment of transitional cell carcinoma.*

Correspondence to: Dr. Jing-Gung Chung, Department of Microbiology, China Medical University, 91 Hsueh-Shih Road, Taichung 400, Taiwan, Republic of China. Tel: (04) 2235-7748, Fax: (04) 2235-7748, e-mail: jgchung@mail.cmu.edu.tw

Key Words: Arylamine (N-acetyl-2-aminofluorene and 2-aminofluorene), AF-DNA adducts, AF (aminofluorene), AAF (acetylated aminofluorene), bladder cancer cells.

Zicao [purple gromwell, the dried root of *Lithospermum Erythrohizon* Sieb. et Zucc, *Arnebia euchroma* (Royle) Johnst, *Arnebia guttata* Bunge and others], referred to as "shikon" in Japan, is a commonly used herbal medicine in China. It is believed that shikonin possesses detoxification properties and it has been used for thousands of years to treat macular eruptions, measles, sore-throat, carbuncles and burns (1). Based on the long history of its use, shikonin has been extensively characterized (2). Shikonin and its derivatives are the primary active components isolated from Zicao plants (3-6) and their multiple pharmacological actions have been documented (2). Zicao is a commonly used anticancer herbal medicine in China (7) and medicinal mixtures containing purified shikonin are reported to be safe and effective in the treatment of late stage lung cancer patients (8).

About 347 males per 100,000 of the male population in Taiwan annually die of bladder transitional cell carcinoma, perhaps related to high local levels of industrial pollution. Arylamines are an important component of industrial pollution. Genetic variations in xenobiotic metabolism affect cancer occurrence the in human population (9) and different species have different risk in the bioactivation and detoxification of

many arylamine drugs and carcinogens (10). Occupational exposure to aromatic amines explains up to 25 percent of bladder cancers in some areas of Western countries; possibly higher estimates apply in limited areas of developing countries. Aromatic amines as a component of environmental tobacco smoke contaminate the ambient air (11). The carcinogenic action of arylamine carcinogens requires N-acetylation followed by metabolic activation to form electrophiles that can bind to DNA (12). N-acetylation is an important step in arylamine metabolism and is performed by arylamine N-acetyltransferase (NAT) (3), which usually uses acetyl coenzyme A as an acetyl group donation. The two isoenzymes of NATs, NAT1 and NAT2 (13), catalyze the N-acetylation of arylamine carcinogens (4-aminobiphenyl and 2-aminofluorene) (14). Individuals can be categorized as having either rapid or slow acetylator phenotype. The susceptibility to the arylamine carcinogens may modulate the risk of colorectal and bladder carcinogenesis (15-17).

Until now, there is no available information to address the effects of shikonin on NAT activity and gene expression in a human bladder cancer cell line. We designed this study to determine whether or not shikonin affects NAT activity, gene expression and DNA-2-aminofluorene adduct formation in human T24 bladder transitional cell carcinoma cells.

Materials and Methods

Chemicals and reagents. Shikonin was obtained from Ichimaru Pharcos Co.Ltd (GIFU-PREF, Japan). RNeasy® Mini Kit (50) and QIAamp DNA Mini Kit (50) were obtained from Qiagen Inc. Ethylene-diamine-tetra-acetic acid (EDTA), 2-aminofluorene (AF), N-acetyl-2-aminofluorene (AAF), acetylcarnitine, leupeptin, bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF), Tris-HCl, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), micrococcal endonuclease, spleen exonuclease, acetyl-coenzyme A 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. The human bladder cancer cell line (T24: human; female; 81 years old) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were cultured for several generations in our laboratory and their viability was checked periodically. The cells were placed into 75-cm^3 tissue culture flasks and grown at 37˚C under humidified 5% CO_2 and 95% air in 90% McCoy's 5a medium (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and 2% penicillin (100U/ml penicillin) with streptomycin (10 mg/ml streptomycin).

AF acetylation by NAT in T24 intact cells under various concentrations of shikonin and for various time periods. T24 cells (in 1 ml RPMI 1640 media with glutamine and 10% fetal calf serum) were incubated with arylamine substrate (AF, 30 μM) at $1x10^6$ cells/ml in individual wells of a 24-well cell culture plate with different concentrations (0, 1.6, 3.2, 4, 8, 16, 24 μM) of shikonin for the time periods (6, 12, 18, 24, 48 h) indicated at 37˚C in 95% air and 5% CO_2. A shikonin concentration of zero was used as control. The experiment was divided into 5 groups according to the timing: A, B, C, D and E. At the end of 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 AAF by high performance liquid chromography (HPLC, Beckman: Detector module 168, Programmable Solvent module 126) (18).

After 24-h incubation of T24 cells with AF in the presence of shikonin (16 μM), the media and cells were harvested for 2 experiments: 1). Time course effects of shikonin on NAT activity T24 cells were incubated with 22.5 μM AF at $1x10^6$ cells/ml in individual wells of a 24-well cell culture plate with shikonin (0, 16 μM) for 6, 12, 18, 24 and 48 h. A shikonin concentration of zero was used as control. At the end of incubation, the acetylated AF (AAF) and non-acetylated AF were determined by HPLC as described above (18). 2). Shikonin effects on N-acetylation of various AF concentrations. T24 cells were incubated for 24 h with various concentrations of AF (15, 30, 60, 90 μM) at $1x10^6$ cells/ml in individual wells of 24-well cell culture plate with shikonin (0, 16 μM). A shikonin concentration of zero was used as the control. At the end of incubation, the acetylated AF (AAF) and non-acetylated AF were determined by HPLC as described above (18).

Shikonin effects on the kinetic constants of NAT in the T24 cell cytosols

a. Preparation of human T24 cell cytosol. 5 x 10^7 cells were placed in 2 ml of 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 (18). The suspensions were centrifuged at 9000 g for 1 min in a HERMLE Z200 M/H centrifuge, and the supernatant fraction was subsequently centrifuged at 10,000 g for 60 min. The supernatant was kept on ice for the NAT activity and protein determination. For cytosol examinations, the reaction mixtures consisted of 50 μl of cytosol, 20 μl of recycling mixture containing AF at selected concentrations (0.087, 0.174, 0.348, 0.696 and 1.392 mM) as substrate, and 10 μl 16 μM shikonin. The reactions were started by the addition of acetyl coenzyme A (AcCoA). The control reactions had 20 μl distilled water in place of Ac-CoA. NAT activity was determined as previously described (18). All reactive procedures were run in triplicate.

b. NAT activity determination. The determination of AcCoA-dependent N-acetylation of AF was performed as previously described (18).

c. Protein determination. Protein concentration in the human T24 cell cytosols was determined using bovine serum albumin as the standard as described by Bradford (1976). All of the samples were assayed in triplicate.

Detection and measurement of DNA adducts in T24 cells. Detection and measurement of DNA adducts were performed as described previously (18, 19). About $5x10^6$ cells/well were incubated with AF (30, 60, 90 μM) and shikonin (0, 16 μM) and recovered by centrifugation. A shikonin concentration of zero was used as the control. The DNA was prepared by the GENOME DNA isolation kit protocol (BIO 101, La Jolla, CA,

USA), then the AF-DNA adduct was determined and examined as described previously (18, 19).

Preparations of polyclonal antibodies. We immunized 6-week-old female BALB/c mice with prepared recombinant proteins (NAT). Each mouse was initially injected with 0.5 ml of pristane. About 100 mg 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 and boosted again for another 10-20 days. The serum-free myeloma cells (0.5-1x10^6/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 (20).

Detection of NAT enzyme by flow cytometry. The level of intracellular NAT of the T24 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using the prepared polyclonal antibody mentioned above. Cells were co-treated with various concentrations (0, 1.6, 3.2, 4, 8, 16, 24 μM) of shikonin for 24 h to detect the intracellular NAT enzyme. A shikonin concentration of zero was used as the control. 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. 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 (20).

Electrophoresis and reverse transcriptase polymerase chain reaction (RT-PCR) for NAT mRNA. The cells were incubated with various concentrations (0, 3.2, 4, 8, 16, 24 μM) of shikonin for 24 h, then collected to extract total RNA by using the Qiagen RNeasy Mini Kit (50) at the indicated time. Total RNA (1.5 μg), 0.5 μg of oligo-dT primer and DEPC (diethyl pyrocarbonate)-treated water were combined in a micro-centrifuge 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 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 the target cDNA was amplified, 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 and VPKHGD-X-NAT1 for NAT1, FP1-NAT2 and RP1-NAT2 for NAT2, Act b1 and Act b2 for beta-actin), cDNA template corresponding to the amount synthesized from 50 ng of total RNA and 2 units of DNAzyme DNA polymerase. The sequence of primers were 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'-TAACGTG AGGGTAGAGA GGA-3', nt 1073-1054, GenBank accession number NM-000015; Act b1, 5'-GCTCGTCGTCGACAACG GCTC-3', nt 94-114, GenBank accession number NM-001101; Act b2, 5'-CAAACATGATCT GGGTCATCTTCTC-3', nt 446-422, GenBank accession number NM-001101 (21, 22). The extracted RNA was subjected to RT-PCR analysis using specific primers for NAT1, NAT2 and β-actin, and then PCR-amplified cDNA derived from mRNA were applied to agarose gel electrophoresis. The mRNA levels of NAT1, NAT2 and β-actin on the gel electrophoresis were quantified by densitometric analysis of the gel photograph and expressed as NAT1/β-actin ratio.

Statistical treatment of data. Statistical analysis of the data was performed by an unpaired Student's *t*-test. The kinetic constants were calculated with the Cleland HYPER Program (1976), which performs linear regression using a least-squares method (23).

Results

AF acetylation by NAT in the T24 intact cells under various concentrations of shikonin and for various time periods. HPLC was used in this assay to examine the levels of acetylated and non-acetylated AF in T24 intact cells cotreated with various concentrations of shikonin and various time periods (Figure 1). The data indicated that there was a decrease in N-acetylation of AF associated with an increase in the concentration of shikonin in T24 cells. In the control group, N-acetylation of AF varied from 8% to 68%, depending on the timing for AF acetylation. In the presence of 1.6, 3.2, 4.0, 8.0, 16 and 24 μM shikonin, the N-acetylation of AF was inhibited. Higher concentrations of shikonin induced less AF acetylation. The longer the incubation time, the greater the difference in AF acetylation with the same shikonin concentrations.

Effect of incubation time with shikonin on human T24 cells. T24 cells incubated with AF produced AAF in the culture media, whereas cells without AF and AF without cells did not lead to any detectable AAF in the media at all of the examined times. Increased time of incubation led to increasing AAF production. In the presence of 16 μM of shikonin, the N-acetylation of AF was decreased by about 72-84% (Figure 2).

Effects of AF concentration and shikonin on human T24 cells. The data indicated that increased AF concentrations led to an increase in the AAF production in examined cells. Overall, the data indicated that shikonin decreased the amount of AAF formation in all examined AF doses (15, 30, 60 and 90 μM) (Figure 3).

Effects of 16 μM shikonin on kinetic constants of NAT in human T24 cell cytosols. In the presence or absence of shikonin, specific concentrations of AF (0.087, 0.174, 0.348,

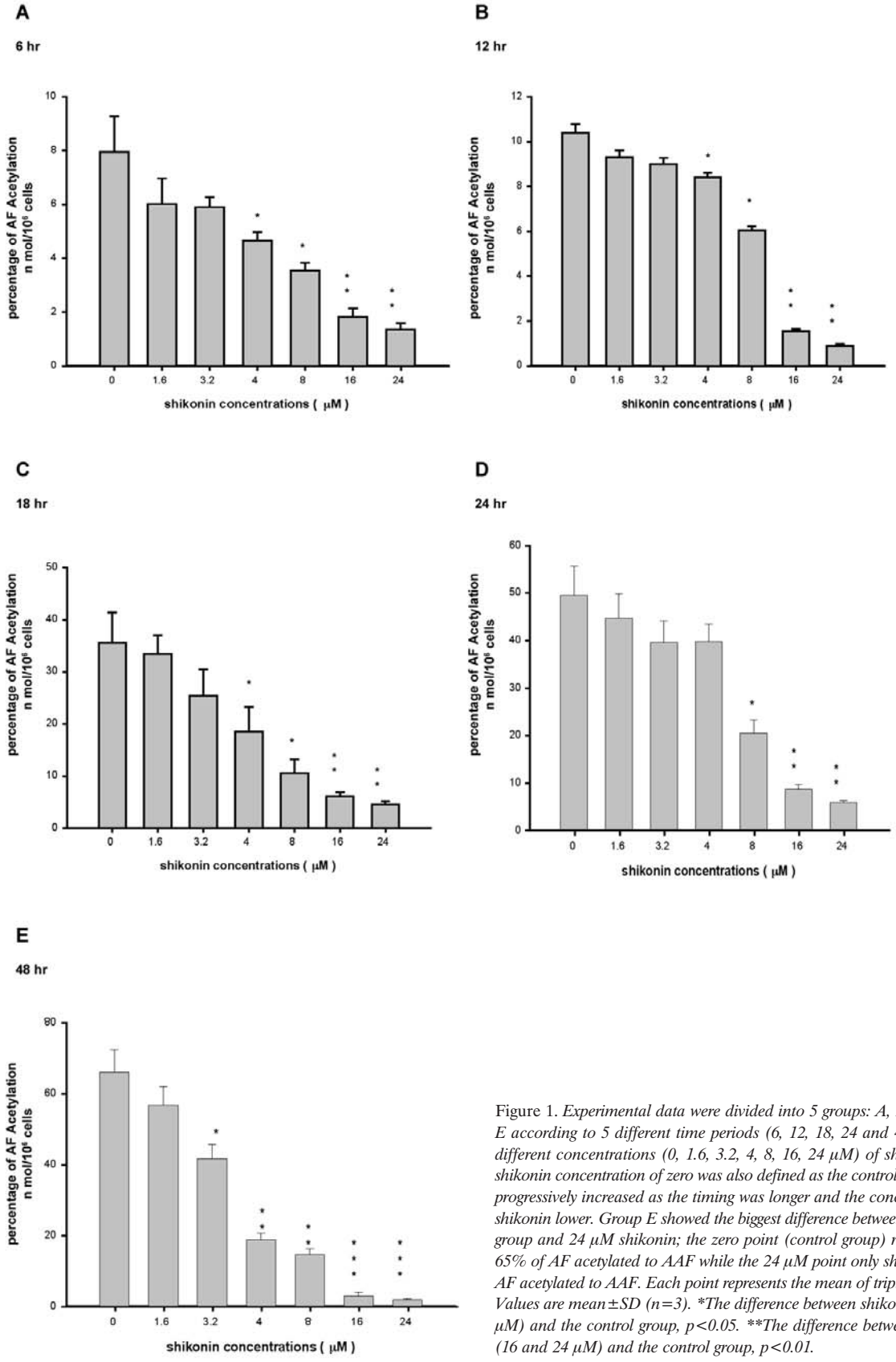

Figure 1. *Experimental data were divided into 5 groups: A, B, C, D and E according to 5 different time periods (6, 12, 18, 24 and 48 h) with 7 different concentrations (0, 1.6, 3.2, 4, 8, 16, 24 μM) of shikonin. The shikonin concentration of zero was also defined as the control group. AAF progressively increased as the timing was longer and the concentration of shikonin lower. Group E showed the biggest difference between the control group and 24 μM shikonin; the zero point (control group) revealed over 65% of AF acetylated to AAF while the 24 μM point only showed 2% of AF acetylated to AAF. Each point represents the mean of triplicate assays. Values are mean±SD (n=3). *The difference between shikonin (4 and 8 μM) and the control group, $p<0.05$. **The difference between shikonin (16 and 24 μM) and the control group, $p<0.01$.*

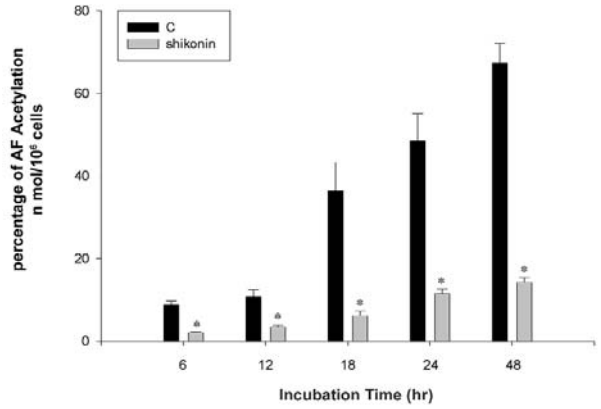

Figure 2. *AF acetylation in fixed concentrations of AF (22.5 μM) and shikonin (0, 16 μM) when the T24 cells were incubated for different time periods (6, 12, 18, 24 and 48 h). The shikonin concentration of zero was also defined as the control group. The longer incubation time increased the level of AF acetylation. The percentage of acetylated AF increased in the control group from 10% (6 h) to 70% (48 h) and in the experimental group from 2% (6 h) to 14% (48 h). Each point represents the mean of triplicate assays. Values are mean±SD (n=3). *The difference between experimental and control group, p<0.05.*

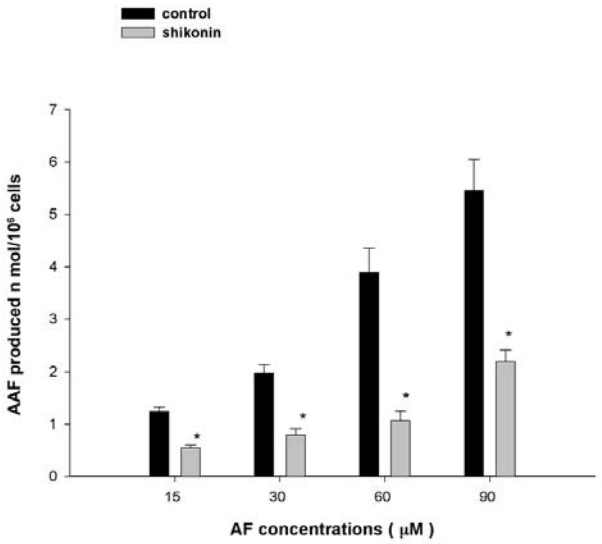

Figure 3. *AAF production in fixed time (24 h), fixed concentrations (0, 16 μM) of shikonin and different concentrations of AF incubated in T24 cells. The shikonin concentration of zero was also defined as the control group. There were 4 different concentrations (15, 30, 60, and 90 μM) of AF. AAF increased in proportion to an increase in AF concentration. The difference between the control and experimental groups was significant (p<0.05). Each point represents the mean of triplicate assays. Values are mean±SD (n=3). *The difference between experimental and control group.*

0.696 and 1.392 mM for cytosol examination) were added to the recycling mixtures in order to determine cytosolic NAT kinetic constants. When 16 μM shikonin was added to the reaction mixtures, the apparent Km and Vmax values were decreased by 45% and 52%, respectively (Table I).

Table I. *Kinetic data for the AF acetylated in T24 bladder tumor cells.*

	Km (mM)	Vmax (nmol/min/ mg protein)
Control	2.24±0.41	7.38±0.91
Shikonin	[a]1.22±0.24	[b]3.49±0.62

Values are mean±S.D. n=3. The concentrations of acetyl coenzyme A and shikonin are 0.1 mM and 16 μM in separate, and the kinetic constants were calculated from the modified HYPER Program of Cleland (23).
[a]differ between 16 μM shikonin and control, $p<0.05$
[b]differ between 16 μM shikonin and control, $p<0.05$

Effects of shikonin on DNA-2-AF adducts in T24 cells. In order to determine AF-DNA adducts, cells were recovered and DNA was prepared, hydrolyzed to nucleotides, the adducted nucleotides were extracted into butanol and analyzed by HPLC. The data indicated that increased AF concentration led to an increase of AF-DNA adduct formation in examined cells. Overall, the data indicated that shikonin decreased the amount of AAF production and AF-DNA adducts formation in human T24 cells in all examined AF doses (Figure 4).

Effects of shikonin on the NAT Ag-Ab reaction in T24 cells. The variations of NAT protein were measured by the ability of the NAT antibody to form an antigen-antibody complex. The amount of NAT-Ag-Ab complex was measured by flow cytometry (Figure 5 and Table II). The expression of NAT-Ag-Ab complex in the cells treated with different concentrations (0, 1.6, 3.2, 4, 8, 16, 24 μM) of shikonin led to a dose-dependent decrease in the NAT-Ag-Ab complex in the T24 cells. The percentages of cells stained by antibody after treatment with shikonin were 62.56±8.89, 50.67±7.86, 47.74±6.42, 40.66±5.98, 24.89±3.08, 16.42±2.77 and 7.95±1.06, respectively. The data were significant for the higher concentrations (8, 16, 24 μM) of shikonin.

Dose-dependent effects of shikonin on NAT mRNA expression in intact T24 cells examined by polymerase chain reaction (PCR). The extracted RNA was subjected to RT-

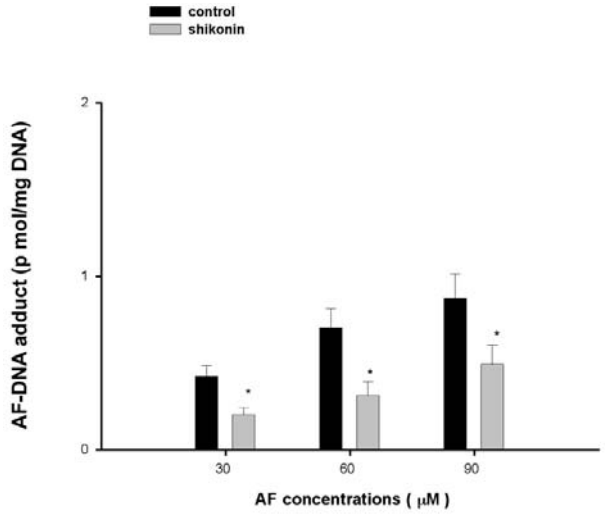

Figure 4. *AF-DNA adduct formation in fixed time (24 h), fixed concentrations (0, 16 μM) of shikonin and different concentrations of AF in T24 cells. The shikonin concentration of zero was defined as the control group. There were 3 different concentrations (30, 60 and 90 μM) of AF for AF-DNA adducts. The AF-DNA adducts increased with increased concentrations of AF. The difference between the control and experimental group was significant. Each point represents the mean of triplicate assays. Values are mean±SD (n=3). *The difference between experimental and control group, p<0.05.*

Table II. *The percentages of (T24 cells) which were treated by shikonin and stained by the NAT antibody.*

Shikonin (μM)	Percentage of cells stained by anti-NAT
0 (Control)	62.56±8.89
1.6	50.67±7.86
3.2	47.74±6.4 2
4	40.66± 5.98
8	*24.89±3.08
16	*16.42±2.77
24	*7.95±1.06

Values are mean±S.D. n=3. The T24 cells (1x10^6 cells/ml) were co-treated with various concentrations of shikonin. The zero concentration of shikonin was defined as control. The percentages of cells stained by NAT antibody were determined by flow cytometry as described in the Materials and Methods section.
*differs between 8-24 μM shikonin and control, $p<0.05$

PCR analysis using specific primers for NAT1, NAT2 and β-actin, and then PCR-amplified cDNA derived from mRNA was applied to agarose gel electrophoresis. The data presented in Figure 6 A show that NAT1 mRNA levels decreased at higher levels (16-24 μM) of shikonin significantly differently from the low levels (3.2-8 μM) of shikonin. NAT1 and NAT2 mRNA were present in T24 cells, but NAT2 was not affected by shikonin. The data and gel photographs demonstrated that shikonin decreased NAT1 mRNA expression in a dose-dependent manner but that NAT2 did not change in these cells. The mRNA levels of NAT1, NAT2 and β-actin on gel electrophoresis were quantified by densitometric analysis of gel photographs and expressed as NAT1/β-actin (Figure 6B).

Discussion

Bladder cancer is the eleventh most common cancer in the world, accounting for 3-4% of all malignancies (24), and it is the thirteenth most common cause of mortality for males in Taiwan (People's Health Bureau, Taiwan, 2001). The first known cause of human bladder cancer was occupational exposure to arylamines, including 2-naphthylamine, 4-aminobiphenyl (ABP) and benzidine, principally among workers in the textile dye and rubber tyre industries (25).

Most carcinogenic chemicals are not reactive *per se* and require metabolic activation before reacting with the genetic material, which may lead to mutations and development of carcinoma. Metabolic activation of chemical compounds is through their oxidation, hydrolysis or reduction and involves phase I enzymes for biotransformation. Phase II enzymes catalyse detoxification of the activated compounds (26, 27). N-acetyltransferases, which belong to phase II of xenobiotic transformation, are involved in reactions of acetylation of arylamines, components of both carcinogenic compounds and various drugs. In humans, two isoforms, NAT1 and NAT2 with different acetylation phenotypes (rapid and slow) characterized by different response to certain drugs, are observed. Thus, the N-acetylation polymorphism determines individual variability in biotransformation of aromatic amines (4-aminobiphenyl), hydrazines and heterocyclic amines (carcinogen responsible for urinary bladder cancer) present in tobacco smoke and supplied with the diet (28, 29). In the human genome, three genetic loci are connected with NAT expression. One of them, *NATP*, seems to be a pseudogene, which codes for a non-functional protein. The nucleotide sequences of the other two expressed genes, *NAT1* and *NAT2,* are 87% similar. Different nucleotide changes occur in the *NAT* sequence: nucleotide substitutions (all *NAT2*, some *NAT1* alleles), and insertions or deletions (*NAT1*). So far, 24 different *NAT1* alleles and 26 different *NAT2* alleles have been found (28-33).

The high frequency of the NAT1 and NAT2 acetylation polymorphism in human populations together with ubiquitous exposure to aromatic and heterocyclic amines suggest that NAT1 and NAT2 acetylator

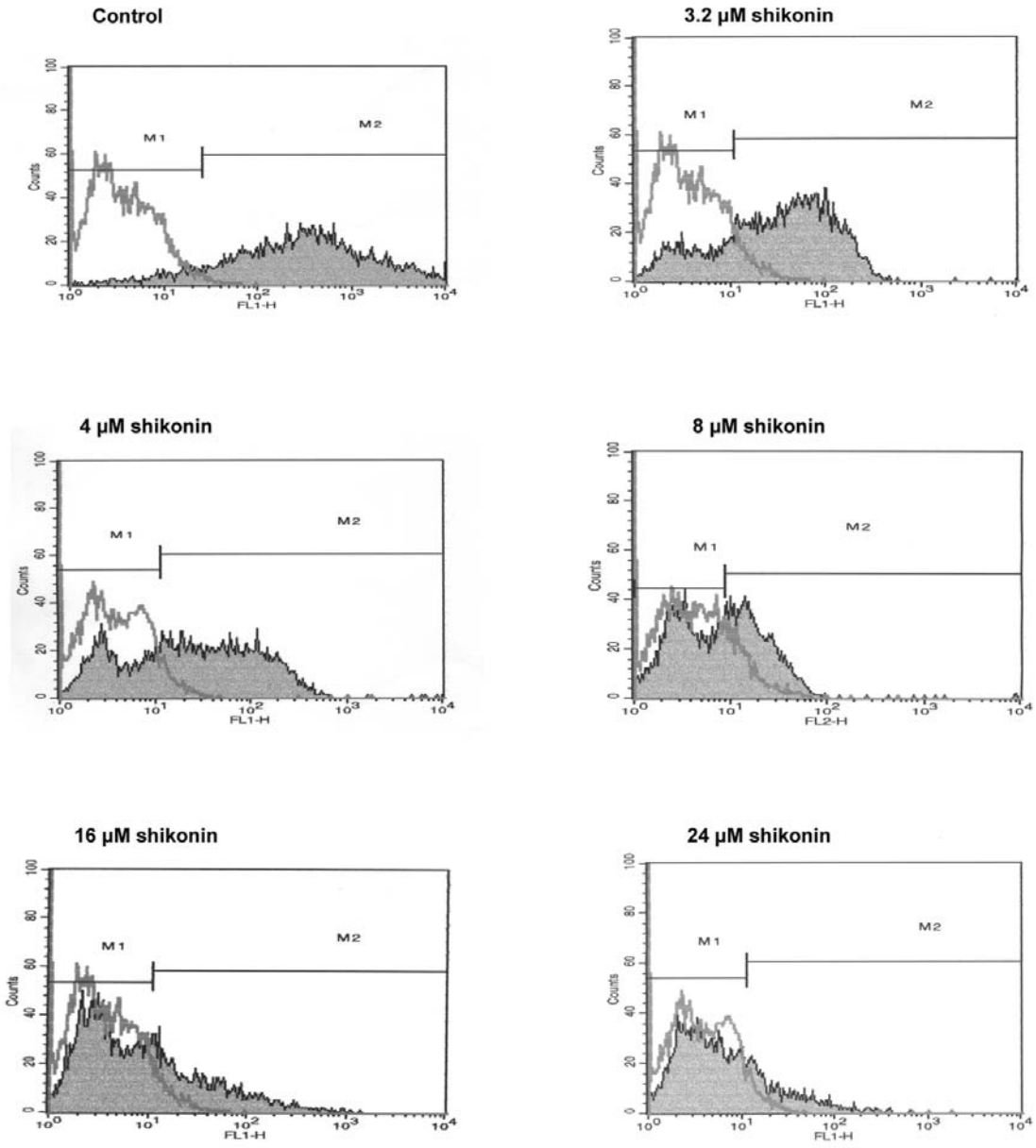

Figure 5. *Effects of shikonin on NAT expression in T24 human bladder cancers cells. T24 cells (1x10^6/ml) were treated with 0, 3.2, 4, 8, 16, 24 μM shikonin for 24 hours followed by evaluation of NAT expression. NAT expression was estimated by flow cytometry. Details are described in Materials and Methods.*

genotypes are important modifiers of human cancer susceptibility, in which N-acetylation is a detoxification step, such as aromatic amine related to urinary bladder cancer (14, 34, 35).

In addition, the cytochrome P450 enzyme is reportedly involved in the metabolism of N-acetylated AF (AAF). For example, cytochrome P450-dependent formation of N-hydroxy-AAF plays an important role in the initial rate-limiting step of AAF metabolism, which leads to the mutation of the potentially carcinogenic products (36). Other investigators demonstrated that P4501A1 (cytochrome P4501A1) is particularly efficient in the catalytic conversion of AAF to 7-OH-AAF (37, 38).

An important point is 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, and c) the rates of glucuronide hydrolysis and NAT-mediated activation in the target tissue (39).

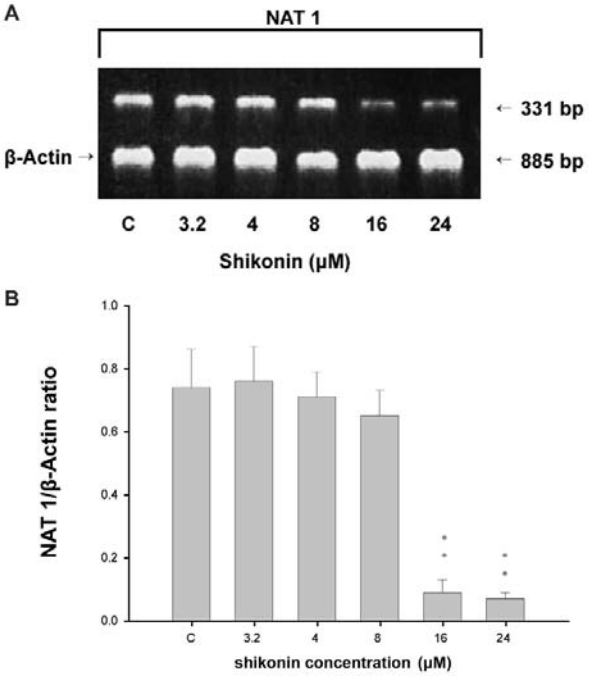

Figure 6. *A. Treatment of NAT1 m-RNA and beta-actin with different concentrations (0, 3.2, 4, 8, 16, 24 μM) of shikonin. The shikonin concentration of zero was defined as control. The gel electrophoresis was quantified by densitometric analysis of gel photograph that showed NAT1 mRNA was obviously depressed by shikonin at the higher concentrations (16, 24 μM). The mRNA levels of NAT1 and β-actin expressed as NAT1/β-actin ratio. The ratio revealed a marked decrease in NAT1 activity at higher shikonin concentrations (16, 24 μM).*

The efficacy of shikonin in the treatment of cancer has been rigorously tested *in vivo* in animal models. In mice, following intraperitoneal injection of sarcoma 180 (S-180) ascites cells, shikonin inhibited tumor growth and resulted in a 92.5% increase in life-span when administered at a dose of 5-10 mg/kg/day (40, 41). A number of recent studies have shown that shikonin derivatives exert antineoplastic effects by (a) inhibiting cancer cell growth and inducing apoptosis, (b) enhancing antitumor activity, (c) inhibiting DNA topoisomerase, (d) possessing antimitogenic action, (e) reducing carcinogenesis and (f) reducing angiogenesis. More detailed information is given below:

1). Cancer cells can be induced to differentiate and undergo apoptosis by various chemotherapeutic agents, *e.g.* taxol (42), a well known plant-derived anticancer compound. Therefore, chemical agents with potent differentiation-inducing or apoptosis-inducing activity, but acceptable toxic side-effects, have potential as anticancer drugs. A number of studies suggest that shikonin derivatives meet this criterion (43). β-Hydroxyisovalerylshikonin (β-HIVS) inhibited the growth of various cancer cell lines, including VMRCMELG, a colon malignant melanoma, COLO320DM, a colon adenocarcinoma, AZ-521 gastric cancer, MIA Paca 2 pancreatic cancer, HL-60, an acute promyelocytic leukemia and U937, a histiocytic lymphoma at low concentrations (between 0.001 and 1 mM). Among the cells tested, HL-60 cells were most sensitive to β-HIVS, the IC_{50} for 18-h treatment being 0.41 mM and 0.0035 mM after 4 days treatment. HL-60 cells treated with 1 mM β-HIVS for 3 h showed characteristic features of apoptosis (44). These studies indicate that the β-HIVS variant of shikonin efficiently activates the classic caspase-3-mediated apoptosis pathway, possibly resulting in a reduction of tumor survival *in vivo* by an apoptotic pathway.

2). One study showed that a recently synthesized shikonin derivative, 2-hyim-DMNQ-S33, significantly inhibited proliferation of radiation-induced fibrosarcoma (RIF) in a dose-dependent manner during a 4-h treatment with 1.5-15 mM of the compound. A 3.0 mM treatment of 2-hyim-DMNQ-S33 significantly down-regulated active phosphorylation of extracellular signal-regulated kinase (ERK) within 1 h without any change in inactive ERK. On the other hand, it markedly up-regulated active c-jun-N-terminal kinase (pJNK) and protein kinase C-α without any change in inactive JNK after a 4 h treatment. In contrast, 2-hyim-DMNQ-S33 had no effect on the expression or phosphorylation of p38. Treatment with 6 mg/kg 2-hyim-DMNQ-S33 prolonged the survival time of sarcoma 180 tumor-bearing mice by 239%. This result is similar to adriamycin treatment (45).

3). DNA topoisomerases are a class of enzymes that alter DNA conformation through a concerted breakage and rejoining of DNA molecules, thereby controlling the topological state of DNA. Topoisomerases are involved in many important processes of DNA processing including replication, transcription, recombination and chromosome segregation (46). In addition, DNA topoisomerases are the target of many anticancer drugs, which are already widely used in the clinic (47, 48). Shikonin, at a concentration above 10 μM, has been reported to induce topoisomerase II-mediated DNA cleavage, forming a cleavable complex (49). The ability of shikonin to inhibit topoisomerase activity is not as impressive as its ability to inhibit the production of reactive oxygen species, which suggests that topoisomerase is not a primary mechanism for the anticancer effects of shikonin, but rather an accessory effect (2).

4). An analogue of β,β-dimethylacryloylshikonin (93/637, SA) was found to inhibit prostate cancer cell (DU 145, LNCaP and PC-3) growth in a dose-dependent manner, where the lowest efficacious dose was 250nM. Results from lactate dehydrogenase (LDH) activity assays showed

that cellular damage occurred when SA concentrations exceeded 1 μM, suggesting that the inhibition of tumor cell growth mediated by SA is not based on cell damage (50).

5). Shikonin added to the diet (0.02%) significantly inhibited the incidence and average number of intestinal tumors in rats treated with azoxymethane, suggesting that shikonin might be a promising chemopreventive agent for intestinal neoplasia (51).

6). The growth of new blood vessels, or angiogenesis, plays an important role in the growth of solid tumors (52). In a murine model, angiogenesis induced by TNF-α (100 ng) was inhibited when shikonin (1-5 mg) was injected along with TNF-α. Shikonin (0.2 mg) co-injected with B16 melanoma cells strongly inhibited tumor growth and tumor-induced angiogenesis. The histological appearance of the B16 was not affected, suggesting that the effects of shikonin were not due to cytotoxicity, but to prevention of neovascularization and subsequent nutrient delivery. Normal developmental angiogenesis in the yolk-sac membrane of chicken embryos was inhibited by 0.25-0.5 mg of shikonin. However, shikonin had no effect on embryo development at these doses, again suggesting that the mechanism of action for shikonin is not based on toxicity alone (53).

In the present study, shikonin induced changes of the N-acetylation of AF, NAT activity (by HPLC), AF-DNA adducts formation (by HPLC), NATs' antibody (by flow cytometry) and NATs mRNA genes expression (RT-PCR) in a human bladder transitional cell carcinoma (T24 cell line). Our results demonstrated that shikonin decreased the levels of AF N-acetylation (AAF production) in both cytosols and intact T24 cells based on the following observations: (i) the levels of AAF in T24 intact cells and cytosol decreased in a dose-dependent manner; (ii) apparent values of Km and Vmax from NAT in T24 cell's cytosols decreased; and (iii) time-course-dependent experiments also showed decreased amounts of AAF in T24 cells co-treated with shikonin. The treatment with shikonin down-regulated the quantity of NATs' antigen. The NAT2 gene was not affected by shikonin treatment, but the NAT1 gene was inhibited by higher concentrations (16, 24 μM).

The attenuation of liver NAT activity is associated with the progress of several diseases, such as breast and bladder cancer (13, 54). The results also indicated that shikonin decreased NAT kinetic constants (apparent values of Km and Vmax) in the cytosols of T24 cells. The inhibitors of enzyme activity can be classified into 3 types: type 1 is a competitive inhibitor which competes with the substrate for the binding site on the enzyme (Km values are not changed, but Vmax values are); type 2 is a noncompetitive inhibitor which binds to a different site of the enzyme but blocks the conversion of the substrate to products (Km values are changed, but Vmax values are not); and type 3 is an uncompetitive inhibitor that binds only to the enzyme-substrate complex (both values of Km and Vmax are changed) (55). Therefore, shikonin is an uncompetitive inhibitor of NAT in T24 cells. Shikonin may be a useful tool to distinguish different NAT enzymes based on different extent of inhibition.

Apparently shikonin affected NAT1 mRNA expression, which led to a decrease in NAT activity and finally to lower levels of AAF production in the examined T24 cells. Of course, *in vivo* studies should be considered in the future. We also found that shikonin induced cytotoxicity in T24 cells (data not shown) in our primary studies. Although shikonin had been shown to induce apoptosis in human HL60 leukemia cells (44), the exact mechanism is still not clear. Therefore, the biological activity of shikonin on T24 cells still needs further investigation.

The exact role of arylamine-NAT reactive situation in the development of bladder cancer and other tissues remains unclear. It has been reported that liver NAT enzyme is involved in the detoxification of exogenous amines. Other investigators found that this enzyme could be involved in the acetylation of other drugs (13). Although the exact mechanism is not well understood, it is clear that conversion of AF to mutagenic metabolites involves hydroxylation and acetylation in liver tissue, with additional acetylation of the circulating hydroxyl metabolites occurring in other tissues. Currently, the results of our study offer some information for the human T24 cells. This report is the first to demonstrate that shikonin inhibits NAT activity, depresses gene expression (NAT1 mRNA), lowers the NAT-Ag-Ab complex formation and decreases the amount of AF-DNA adducts formation in cultured cells. So we hypothesize that shikonin could be a potential candidate to prevent bladder cancer formation by reducing the acetylation of arylamines by NAT, to minimize mutation to a higher grade tumor by decreasing AF-DNA adduct formation and to treat the bladder transitional cell carcinoma by inducing apoptosis and anti-angiogenesis.

Acknowledgements

This work was supported by grant DMR-091-067 from the Research Section of the China Medical University Hospital, Taichung City, Taiwan, Republic of China.

Referrences

1 Wang WJ, Bai JY,Liu DP,Xue LM and Zhu XY: The anti-inflammatory activity of shikonin and its inhibitory effect on leukotriene B4 biosynthesis . Yao Xue Xue Bao *29*: 161-165, 1994.

2 Chen X, Yang L, Oppenheim JJ *et al*: Cellular pharmacology studies of shikonin derivatives. *16*: 199-209, 2002.
3 Fu SL, Shang TM and Xiao PG: Analysis of naphthaquinone pigments in Chinese medicinal 'Zicao', *Arnebia euchroma*. Yao Xue Xue Bao *19*: 921-952, 1984.
4 Jian Y, Zhou Y and Zhang H: Quality of Chinese drug Zicao. Zhongg Zhong Yao Za Zhi *16*: 524-526, 573-574, 1991.
5 Luo SR and Li T: Determination of naphthaquinones in tissue cultured Zicao. Yao Xue Xue Bao *26*: 953-955, 1991.
6 Luo SR and Li T: Determination of naphthaquinone in Zicao. Zhongg Zhong Yao Za Zhi *17*: 552-554, 1992.
7 Lee H and Lin JY: Antimutagenic activity of extracts from anticancer drugs in Chineses medicine. Mutat Res *204*: 229-234.
8 Guo XP, Zhang XY and Zhang SD: Clinical trial on the effects of shikonin mixture on later lung cancer. Zhong Xi Yi Jie He Za Zhi *11*: 598-599, 580, 1991.
9 Vineis P, Malats N and Lang M: Metabolic polymorphisms and susceptibility to cancer. IARC Scientific Publications No. 148. Lyon: IARC. 1999.
10 Dybing E and Huitfeldt MS: Species differences in carcinogen metabolism and interspecies extrapolation. IARC Scientific Publications *116*: 501-522, 1992.
11 Vineis P and Pirastu R: Aromatic amines and cancer. Cancer Causes and Control *8*: 346-355, 1997.
12 Miller EC and Miller JA: Search for the ultimate chemical carcinogens and their reaction with cellular macromolecules. Cancer *47*: 2327-2345, 1981.
13 Weber WW and Hein DW: N-acetylation pharmacogenetics. Pharmacol Rev *37*: 25-99, 1985.
14 Hein DW: Molecular genetics and function of NAT1 and NAT2: role in aromatic amine metabolism and carcinogenesis. Mutat Res *506-507*: 65-77, 2002.
15 Cartwright RA, Glashami RW, Rogers HJ *et al*: Bladder cancer. Lancet *2*: 842-846, 1982.
16 Lang NP, Chu DZJ, Hunter CF *et al*: Role of aromatic amine acetyltransferase in human colorectal cancer. Arch Surg *121*: 1259-1261,1986.
17 Iletti KF, David BM, Detchon P *et al*: Acetylator phenotype in colorectal carcinoma. Cancer Res *47*: 1466-1469, 1987.
18 Chung JG, Levy GN and Webber WW: Distribution of 2-aminoflurene and p-aminobenzoic acid N-acetyltransferase activity in tissues of C57BL/6J rapid and B6.A-NATs slow acetylator congenic mice. Am Soc Pharmacol Exper Therap *21* (*6*): 1057-1063, 2000.
19 Chung JG: Paeonol promotion of DNA adduct formation and arylamines N-acetyltransferase activity in human colon tumour cells. Food Chem Toxicol *37*: 327-334, 1999.
20 Chung JG, Yeh KT, Wu SL *et al*: Novel transmembrane GPTase of non-small cell lung cancer identified by mRNA differential display. Cancer Res *61*: 8873-8879, 2001.
21 Ponte P, Ng SY, Engel J *et al*: Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. Nucleic Acids Res *12*: 1687-1696, 1984.
22 Blum M, Grantum DM, McBride W *et al*: Human arylamine N-acetyltransferase genes: isolation, chromosomal localization and functional expression. DNA Cell Biol *9*: 193-203, 1990.
23 Cleland WW: The statistical analysis of enzyme kinetics data. Adv Enzymol *29*: 1-32,1976
24 Parkin DM, Whelan SL, Ferlay J *et al*: Cancer incidence in five continents, Vol. VII, IARC Scientific Publications No. 143, Lyon: IRAC 1997.
25 Yu MC, Skipper PL, Tannenbaum SR *et al*: Arylamine exposures and bladder cancer risk. Mutat Res *506-507*: 21-28, 2002.
26 Autrup H: Genetic polymorphisms in human xenobiotica metabolizing enzymes as susceptibility factors in toxic response. Mutat Res *464*: 65-76, 2000.
27 Hirvonen A: Polymorphisms of xenobiotic-metabolising enzymes and susceptibility to cancer. Environ Hlth Perspect, *107 Suppl 1*: 34-47, 1999.
28 Gross M, KruisselbrinK T, Anderson K *et al*: Distribution and concordance of N-acetyltransferase genotype and phenotype in an American population. Cancer Epidemiol Biomark Prev *8*: 683-692, 1999.
29 Hirvoven A: Polymorphic NATs and cancer predisposition. *In*: Vineis P, Malats N, Lang M, D'Errico A, Caporsa N, Cuzick J, Boffeta P, editors. Metabolic Polymorphism and Susceptibility to Cancer. Lyon: IARC Scientific Publications No. *148*: 251-270, 1999.
30 Bell DA, Stephens EA, Castranio T *et al*: Polyadenylation polymorphismin the acetyltransferase 1 gene (NAT1) increases risk of colorectal cancer. Cancer Res *55*: 3537-3542, 1995.
31 Bell DA, Taylor JA, Butler MA *et al*: Genotype/phenotype discordance for human arylamine N-acetyltransferase (NAT2) reveals a new-acetylator allele common in African-Americans. Carcinogenesis *14*: 1689-1692, 1995.
32 Hein DW, Doll MA, Fretland AJ *et al*: Molecular genetics and epidemiology of the NAT1 and NAT2 acetylation polymorphisms. Cancer Epidemiol Biomark Prev *9*: 29-42, 2000.
33 Hein DW: N-acetyltransferase genetics and their role in predisposition to aromatic and heterocyclic amine-induced carcinogenesis. Toxicol Lett *112-113*: 349-356, 2000.
34 Grant DM, Blum M, Beer M *et al*: Monomorphic and polymorphic human arylamine N-acetyltransferase: A comparison of liver isozymes and expressed products of two cloned genes. Mol Pharmacol *39*: 184-191, 1991.
35 Minchin RF, Reeves PT, Teitel CH *et al*: N-and O-acetylation of aromatic and heterocyclic amine carcinogens by human monomorphic and polymorphic acetyltransferase expressed in COS-1 cells. Biochem Biophys Res Commun *185*: 839-844, 1992.
36 Thorgeirsson SS, Glowinski IB and McManus ME: Metabolism, mutagenicity, carcinogenicity of aromatic amines. *In*: Hodgson J, Bend J, Philpot RM (Editors). Reviews in Biochemical Toxicity, Vol. 6, Elsevier/North Holland Biomedical Press, New York, pp. 349-386, 1983.
37 Ferriola PC, Cody V and Middleton JE: Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. Biochem Pharmacol *15*: 1617-1624, 1989.
38 Hayes MA, Roberts E, Safe SH *et al*: Influences of different polychlorinated biphenyls on cytocidal, mitoinhibitory, and nodule-selecting activities of N-2-fluorenylacetamide in rat liver. JNCI *76*: 683-690,1986.
39 Smith G, Stanley LA, Sim E *et al*: Metabolic polymorphisms and cancer susceptibility. Cancer Surv *25*: 27-65, 1995.
40 Sankawa U, Ebizuka Y, Miyazaki T *et al*: Antitumor activity of shikonin and its derivatives. Chem Pharm Bull (Tokyo) *25*: 2392-2395, 1977.

41 Sankawa U, Otsuka H, Katoaka Y *et al*: Antitumor activity of shikonin, alkannin and their derivatives. II. X-ray analysis of cyclo-alkannin leucoacetate, tautomerism of alkannin and cyclo-alkannin and antitumor activity of alkannin derivatives. Chem Pharm Bull (Tokyo) *29*: 116-122, 1981.
42 Bhalla K, Ibrado AM, Tourkina E *et al*: Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. Leukemia *7*: 563-568, 1993.
43 Lu G and Liao J: Detection of the anti-cancer biological effect of naphthoquinone pigment-LIII. Zhong Xi Yi Jie He Za Zhi *10*: 422-425, 390, 1990.
44 Yoon Y, Kim YO, Lim NY *et al*: Shikonin,an ingredient of *Lithospermum erythrorhizon* induced apoptosis in HL60 human premyelocytic leukemia cell line. Planta Med *65*: 532-535, 1999.
45 Kim SH, Kang IC, Yoon TJ *et al*: Antitumour activities of a newly synthesized shikonin derivative, 2-hyim-DMNQ-S33. Cancer Lett *172*: 171-175, 2001.
46 Wang JC: DNA topoisomerases. Annu Rev Biochem *54*: 665-697, 1985.
47 Withoff S, De Jong S, De Vries EG *et al*: Human DNA topoisomerase II: biochemistry and role in chemotherapy resistance. Anticancer Res *16*: 1867-1880, 1996.
48 Wang JC: DNA topoisomerases: from a laboratory curiosity to subject in cancer chemotherapy. NCI Monogr *4*: 3-6, 1987.
49 Fujii N, Yamashita Y, Arima Y *et al*: Induction of topoisomerase II-mediated DNA cleavage by the plant naphthoquinones plumbagin and shikonin. Antimicrob Agents Chemother *36*: 2589-2594, 1992.
50 Gaddipati JP, Mani H, Shefali R *et al*: Inhibition of growth and regulation of IGFs and VEGF in human prostate cancer cell lines by shikonin analogue 93/637 (SA). Anticancer Res *20*: 2547-2552, 2000.
51 Yoshimi N, Wang A, Morishita Y *et al*: Modifying effects of fungal and herb metabolites on azoxymethane-induced intestinal carcinogenesis in rats. Jpn J Cancer Res *83*: 1273-1278, 1992.
52 Folkman J, Watson K, Ingber D *et al*: Induction of angiogenesis during the transition from hyperplasia to neoplaisa. Nature *339*: 58-61, 1989.
53 Hisa T, Kimura Y, Takata K *et al*: Shikonin, an ingredient of *Lithospermum erythrorhizon*, inhibit angiogenesis *in vivo* and *in vitro.* Anticancer Res *18*, *2A*: 783-790, 1998.
54 Levy GN, Chung JG and Weber WW: 2-Aminofluorene metabolism and DNA adduct formation by mononuclear leukocytes from rapid and slow acetylator mouse strains. Carcinogenesis *15*: 353-357,1994.
55 Zubay GL, Parson WW and Vance DE: Principles of Biochemistry. pp.135-153. Wm. C. Brown Publishers, 1995.

Received June 24, 2003
Revised October 10, 2003
Accepted November 4, 2003