DOI:10.1021/jf1020158

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Ling-Zhi Polysaccharides Potentiate Cytotoxic Effects of Anticancer Drugs against Drug-Resistant Urothelial Carcinoma Cells

Chao-Yuan Huang,[†,§,∥] Jeff Yi-Fu Chen,[#,∥,⊗] Jia-En Wu,[⊥] Yeong-Shiau Pu,[†] Guang-Yaw Liu,[△] Min-Hsiung Pan,[○] Ying-Tang Huang,[▽] A-Mei Huang,[⊥,⊗] Chi-Ching Hwang,[⊥] Shu-Ju Chung,[⊥] and Tzyh-Chyuan Hour*[,⊥,⊗]

[†]Department of Urology, National Taiwan University Hospital, and College of Medicine, National Taiwan University, Taipei, Taiwan, [§]Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, [#]Department of Biotechnology, [⊥]Institute of Biochemistry, [⊗]Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, [△]Institute of Microbiology & Immunology, Chung-Shan Medical University, and Division of Allergy, Immunology, and Rheumatology, Chung-Shan Medical University Hospital, Taichung, Taiwan, [○]Department of Seafood Science, and [▽]Department of Marine Biotechnology, National Kaohsiung Marine University, Kaohsiung, Taiwan. [∥]These authors contributed equally to this work.

The combined effects of ling-zhi polysaccharide fraction 3 (LZP-F3) and anticancer drugs (cisplatin and arsenic trioxide) were examined in three human urothelial carcinoma (UC) cells (parental, NTUB1; cisplatin-resistant, N/P(14); and arsenic-resistant, N/As(0.5)). MTT assay and median-effect analysis revealed that LZP-F3 could profoundly reverse the chemosensitivity of N/P(14) and N/As(0.5) to cisplatin and arsenic, respectively, in a dose-dependent manner, which involved activation of p38 and down-regulation of Akt and XPA. A dose of 10 μg/mL of LZP-F3 induced significant G1 arrest in N/P(14) and N/As(0.5) cells by flow cytometry, which may be mediated by the induction of $p21^{WAF1/CIP1}$. The combination of LZP-F3 and arsenic trioxide produced a significant synergistic growth inhibition of NTUB1 and N/As(0.5) cells. Similar results were also found in N/P(14) cells. These molecular events of combined effects involved significant and earlier induction of Fas, caspase 3 and 8 activation, Bax and Bad up-regulation, Bcl-2 and Bcl-x_L down-regulatuion, and cytochrome *c* release.

KEYWORDS: Ling-Zhi polysaccharides; chemosensitivity; urothelial carcinoma; drug resistance; cisplatin; arsenic trioxide; synergistic

INTRODUCTION

Bladder cancer is the second most common malignancy affecting the urinary system. Approximately 90% of urothelial tumors are urothelial carcinomas (UC). For the majority of patients presenting with superficial bladder tumors, death from metastatic disease results in approximately 20−40% of UC patients (*1*). Systemic chemotherapies including cisplatin, gemicitabine, or taxol are used for locally advanced disease or metastatic disease (*2*), but about 30−50% of advanced UC cells do not respond to these chemotherapeutic agent-based therapies (*3*). A common feature among chemoresistant and metastatic tumor cells is that they exhibit profound resistance to apoptosis (*4*). Thus, chemoresistance can be considered as the final stage during tumor progression and is mainly responsible for the majority of urothelial cancer-related death (*5*). The challenges of controlling UC are the prevention of recurrence and the inhibition of disease progression during the treatment course.

*Address correspondence to this author at the Institute of Biochemistry, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 807, Taiwan, ROC (phone +886 7 3211101, ext. 2242, ext. 16; fax +886 7 3218309; e-mail cliff@cc.kmu.edu.tw).

Resistance to multiple chemotherapeutic agents is a common clinical problem for cancer treatment; such resistance, termed multidrug resistance (MDR), may occur in primary therapy (intrinsic) or be acquired during or after treatment (*6*). Mechanisms involved in drug resistance include the presence of a range of drug transporters (such as P-glycoprotein or multidrug resistance-associated protein) (*7*−*9*); changes in the expression of topoisomerases (*8*, *10*); alterations in metabolic pathways that influence drug metabolism (*11*−*13*), DNA repair, or apoptosis (*4*, *14*−*16*); delivery and distribution of anticancer drugs to tumor cells (*17*, *18*); imbalance of the ratio of Bax to Bcl-2 (*19*−*21*); high level of constitutive nuclear factor-κB (NF-κB) activity (*22*, *23*), mitogen-activated protein kinase (MAPK) signaling pathway (such as high level of ERK1/2 phosphorylation) (*24*−*26*), cyclin D1 and B1 overexpression (*27*, *28*), and the microenvironment (*29*). Drug resistance in cancer cells can be overcome by administration of a nontoxic reversing agent together with the anticancer agent (*30*). This co-administration of an anticancer agent and a chemomodulator has been shown to increase the therapeutic effect of the drug. In particular, combined treatments involving inhibitors (such as glutathione, EGFR, mTOR, and NF-κB) plus conventional

 Published on Web 07/13/2010

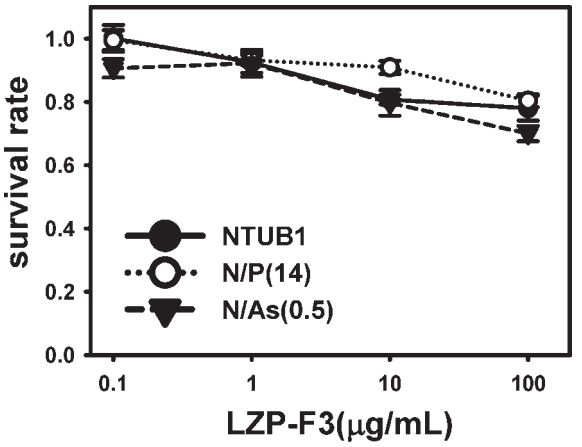

Figure 1. Cytotoxicity of LZP-F3 in UC cells. NTUB1, N/P(14), and N/As(0.5) cells treated with LZP-F3 alone at various doses of 0.1, 1, 10, and 100 μg/mL, respectively, showed nontoxicity to the three cells at concentrations of up to 100 μg/mL by MTT assay.

chemotherapy have been tested, showing significant improvements in antitumor effects (*31*−*35*).

Ganoderma lucidum (reishi or ling-zhi) has been used as traditional Chinese medicine for its beneficial activities in human health for several centuries (*36*). The major pharmacologically active constituents of *G. lucidum* are triterpenoids and polysaccharides (*37*). Previous studies have indicated that the crude or partially purified polysaccharides of *G. lucidum* (LZP) show antitumor activity as well as inhibit tumor metastasis in animal models (*36*, *38*, *39*). Triterpenoids have been reported to exhibit hepatoprotective, antihypertensive, hypocholesterolemic, antihistaminic, antitumor, platelet aggregation-inhibiting, and complement inhibition activities (*37*,*40*−*43*). Polysaccharides, such as β-D-glucans, have been well-known to possess antitumor effects by stimulating the expression of CD4 and T-cell counts during or after chemotherapy (*39*) and antiangiogenesis by suppression of VEGF induction (*44*). Additionally, polysaccharides have a protective activity against free radicals and suppress cell damage by mutagens (*37*,*41*). Although the active constituents responsible for the antitumor and immunomodulating activities have been qualitatively studied and the structure has been partially identified (*36*), the molecular roles of their actions have not been clearly explored.

It was apparent that more effective regimens, involving better combinations and novel agents, were needed. However, the cytotoxic effect of the combination of LZP and anticancer agent was unclear. In this study, we will examine the new combinations of cytotoxic agents with LZP to serve as a novel treatment for UC. Findings of this study can contribute to substantiating the clinical use of LZP in the treatment of human UC.

MATERIALS AND METHODS

Cell Lines and Chemicals. Three bladder urothelial carcinoma cell lines were used: NTUB1, N/P(14) (cisplatin-resistant subline), and N/As(0.5) (As_2O_3-resistant subline). N/P(14) and N/As(0.5) were generated by culturing NTUB1 in progressively increased concentrations of each drug and could thrive at 14 μM cisplatin and 0.5 μM As_2O_3, respectively (*34*, *45*). The cisplatin IC_{50} of N/P(14) and As_2O_3 and the IC_{50} of N/As(0.5) were 36.6 and 5.0 μM, respectively, being 15.3- and 4.2-fold higher than those of NTUB1. All cells were maintained in an RPMI-1640 medium (Gibco BRL, Gaithersburg, MD) containing 10% heat-inactivated fetal calf serum (Gibco BRL) at 37 °C in humidified air with 5% CO_2. Cisplatin was purchased from Sigma Chemical Co. (St. Louis, MO), and As_2O_3 was provided from TTY Biopharm Co. (Taipei, Taiwan). Anti-p21, anti-caspase-8, anti-Bad, anti-cytochrome *c*, and anti-Fas antibodies were purchased from BD Pharmingen (San Diego, CA). Anti-Akt, anti-Bcl-2, and anti-XPA antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase 3 antibody was obtained from IMGENEX (San Diego, CA). Anti-Bax antibody was purchased from GeneTex (Irvine, CA). Anti-Bcl-xL, anti-p38, and antiphosphorylated p38 antibodies were obtained from Cell Signaling (Boston, MA).

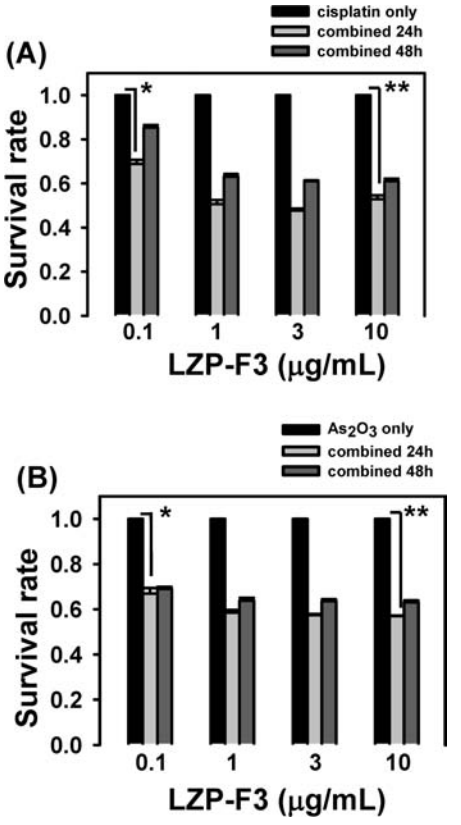

Figure 2. LZP-F3 reversed the chemosensitivity of cisplatin- and arsenic-resistant UC cells. Combinations of various doses of LZP-F3 (0.1, 1, 3, and 10 μg/mL) and (**A**) cisplatin (14 μM) in N/P(14) cells or (**B**) arsenic trioxide (0.5 μM) in N/As(0.5), respectively, were used. The survival rate was analyzed by MTT assay. Data are presented as mean ± standard error of the means of three separate experiments. *, $p < 0.05$; **, $p < 0.01$.

Purification of LZP-F3. Purification of the water-soluble extract of *G. lucidum* was as previously described (*36*). In short, 6 g of crude powder of reishi extract (6 g) was dissolved in 120 mL of double-distilled water and centrifuged (1000 rpm) for 1 h to remove the insoluble remains. The supernatant was purified by gel filtration chromatography using a Sephacryl S-500 column (100 × 1.6 cm) with 0.1 N Tris buffer (pH 7.0) as the eluent. Each 6.0 mL/tube was fractionated by the flow rate of 0.6 mL/min. Then, each fraction was subjected to carbohydrate detection with phenol−H_2SO_4 after chromatographic purification. Five fractions were collected (designated LZP-F1F2 and LZP-F3−LZP-F6), concentrated at 40−50 °C to give a small volume, dialyzed to remove excessive salts and sodium azide, and then lyophilized to give 520 mg (25%) of LZP-F3.

Cell Cycle and Sub-G1 Fraction Analysis by DNA Flow Cytometry. Cells (2×10^5 cells) were treated with LZP-F3 at 10 μg/mL for up to 48 and 72 h, respectively. These cells were harvested after varied culture intervals (24, 48, and 72 h) by trypsinization, washed with 1× PBS, resuspended in 200 μL of PBS, and fixed in 800 μL of ice-cold 100% ethanol at −20 °C. After overnight incubation, the cell pellets were collected by centrifugation, resuspended in 1 mL of a hypotonic buffer (0.1% Triton X-100 and 50 μg/mL RNase A), and incubated at 37 °C for 30 min. One milliliter of propidium iodide solution (50 μg/mL) was added to stain the nuclei. The mixture was allowed to stand on ice for 30 min. These samples were analyzed with a FACScan flow cytometer (Becton-Dickinson, San Jose, CA) for cell cycle and sub-G1 analysis.

Combined Treatment of Anticancer Drugs and LZP. Cellular chemosensitivity was determined using a modified 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium (MTT, Sigma Chemical Co., St. Louis,

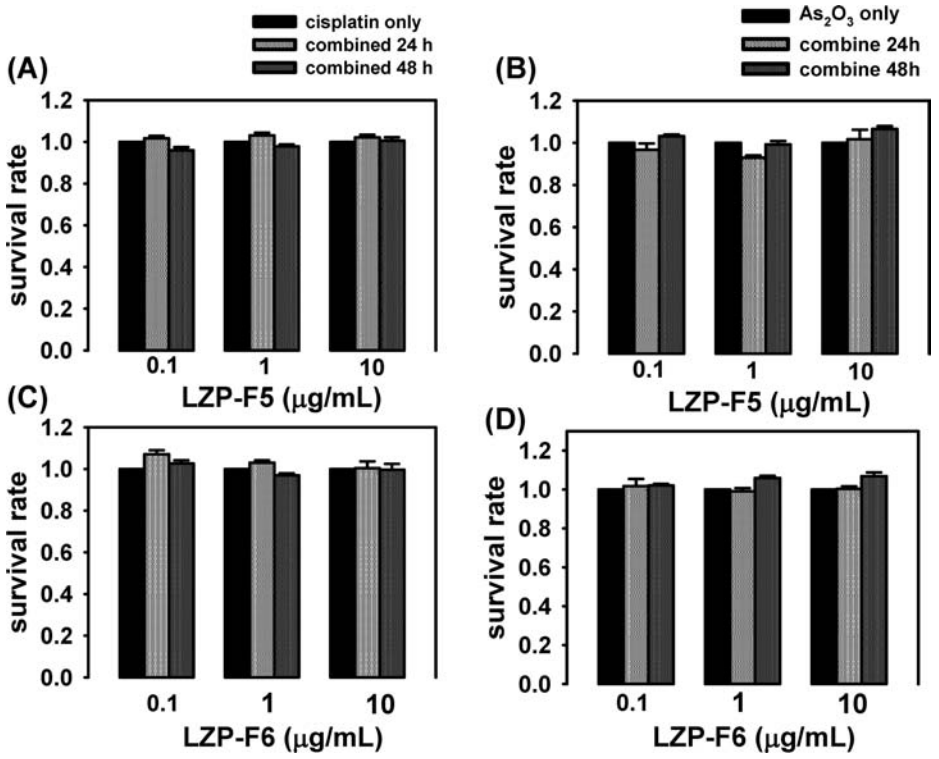

Figure 3. LZP-F5 and LZP-F6 fractions could not reverse the chemosensitivity of drug-resistant UC cells. Combinations of various doses of LZP-F5 (0.1, 1, and 10 μg/mL) and (**A**) cisplatin (14 μM) in N/P(14) cells or (**B**) arsenic trioxide (0.5 μM) in N/As(0.5), respectively, were used. Similarly, combinations of various doses of LZP-F6 (0.1, 1, and 10 μg/mL) and (**C**) cisplatin (14 μM) in N/P(14) cells or (**D**) arsenic trioxide (0.5 μM) in N/As(0.5), respectively, were used. Data are presented as mean $\pm$ standard error of the means of three separate experiments. $*$, $p < 0.05$, and $**$, $p < 0.01$, compared to the control value.

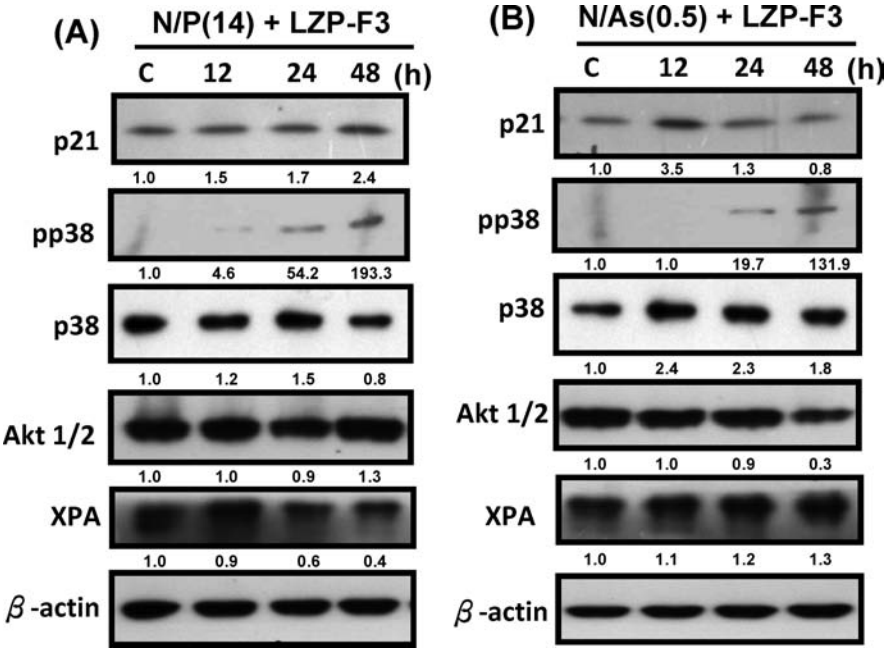

Figure 4. Induction of p21 and p38 and suppression of Akt and XPA by LZP-F3 in drug-resistant UC cells. N/P(14) and N/As(0.5) cells were treated with LZP-F3 at 10 μg/mL for 24 and 48 h, respectively. The levels of p21, p38, Akt, and XPA protein were examined by Western blotting analysis. β-Actin served as an internal control. The number under each band in the immunoblot indicates the relative intensity of the corresponding band. The staining intensity of protein was assessed by using a densitometer. The fold of protein level was normalized to β-actin and compared to control. C, control group.

MO) assay in vitro (*33*). In brief, cells in 100 μL of culture medium were seeded into 96-well microplates and incubated at 37 °C for 24 h prior to drug exposure. The number of cells was titrated to keep control cells growing in the exponential phase throughout the 72 h incubation period. For the combined treatment, cells were treated with LZP and anticancer drugs (each in 100 μL of culture medium) simultaneously and incubated for 72 h. At 72 h, 50 μL of MTT (2 mg/mL in RPMI medium) was added to each well and incubated for 2.5 h. Blue formazan crystals thus formed were pelleted to the bottom of the well by centrifugation, separated from the supernatant, and dissolved in 150 μL of dimethyl sulfoxamide. The optical density at 492 nm was determined by absorbance spectrometry using a microplate reader (MRX-2, Dynex Technologies, Inc., Chantilly, VA).

Three separate experiments with triplicate data were performed to obtain mean cell viability. Drug concentration that inhibited cell growth by 50% (IC_{50}) was determined by the dose−effect analysis model as previously described (*33*) and presented as mean ± standard error of the means (SEM).

Median-Effect Analysis of Combined Effects. By combining LZP-F3 and anticancer drugs at graded concentrations, numerous combined effects on growth inhibition were determined by median-effect analysis with the mutually nonexclusive model as previously described (*31*, *33*). The calculation was carried out using the computer software Calcusyn (version 1.1.1, 1996, Biosoft Inc., Cambridge, U.K.). Control experiments were performed in which either active agent was replaced with drug-free medium. For a given degree of growth inhibition (known as the "fraction affected" in the analysis), there is a corresponding combination index (CI) that reflects the combined effect. The combined effect is displayed in fraction-affected combination index plots, where a combination index of <1, =1, or >1 indicates synergism, additivism, or antagonism, respectively. Synergism or antagonism of various degrees may occur in different fraction-affected ranges with the same combination.

Western Blotting Analysis. Cells (2×10^6) were treated with LZP-F3 at 10 μg/mL for 24 and 48 h, respectively. Cells scraped from one 100-mm Petri dish were resuspended in 100 μL of RIPA lysis buffer (0.5% sodium deoxycholate, 1% NP-40, 150 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl (pH 7.5), 1 mM sodium ovanadate, 0.1% sodium dodecyl sulfate (SDS), 10 μg/mL aprotinin, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/mL leupeptin) and put on ice for 30 min. The lysate was then centrifuged at 18000*g* for 30 min at 4 °C to collect the supernatant for protein concentration determination. Cell extracts (50 μg) were separated on 10% SDS−polyacrylamide gels and transferred to immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking, the membranes were incubated with human specific antibodies, respectively, at 4 °C for 12 h, followed by the horseradish peroxidase-labeled second antibody, and developed with the ECL system (PiercE, Rockford, IL).

Statistical Methods. All symmetrical numeric data are presented as mean ± SEM and compared with the Student's *t* test.

RESULTS

Cytotoxicity of LZP-F3 in Urothelial Carcinoma Cells. NTUB1, N/P(14), and N/As(0.5) cells treated with LZP-F3 alone at various doses of 0.1, 1, 10, and 100 μg/mL, respectively, showed nontoxicity to the three cells at concentrations of up to 100 μg/mL by MTT assay (**Figure 1**). Similarly, sub-G1 fractions continued to show no significant difference after 72 h of exposure to LPZ-F3 (10 μg/mL) alone in all three cells (data not shown). These data suggested that the lower dose of LZP-F3 was almost noncytotoxic for the three cell lines.

LZP-F3 Reversed Chemosensitivity of N/P(14) and N/As(0.5). In this study, we established that both N/P(14) and N/As(0.5) sublines from NTUB1 could thrive at 14 μM cisplatin and 0.5 μM As_2O_3, respectively. With the combinations of various doses of LZP-F3 (0.1, 1, 3, and 10 μg/mL) and cisplatin (14 μM) in N/P(14) cells or arsenic trioxide (0.5 μM) in N/As(0.5), respectively, the survival rate in MTT assay analysis decreased dramatically in these two drug-resistant cells, indicating that LZP-F3 could reverse significant chemosensitivity in both resistant cells (**Figure 2**). The survival rates of N/P(14) and N/As(0.5) were significantly suppressed to approximately 50% by LZP-F3 at 10 μg/mL at 24 h. However, LZP-F5 or LZP-F6 could not reverse the chemosensitivity in both resistant cells (**Figure 3**).

Induction of p21 and p38 and Suppression of Akt and XPA by LZP-F3. For N/P(14) cells, LZP-F3 induced slight expression of p21 protein at 48 h (**Figure 4A**). LZP-F3 (10 μg/mL) up-regulated significantly p21 in the first 12 h and then decreased gradually at 24 and 48 h of treatment in N/As(0.5) (**Figure 4B**). In addition, we examined the effects of LZP-F3 treatment on two chemoresistant-related molecules, p38, phosphorylated p38 (pp38), and Akt. Treatment with LZP-F3 at 10 μg/mL down-regulated Akt expression

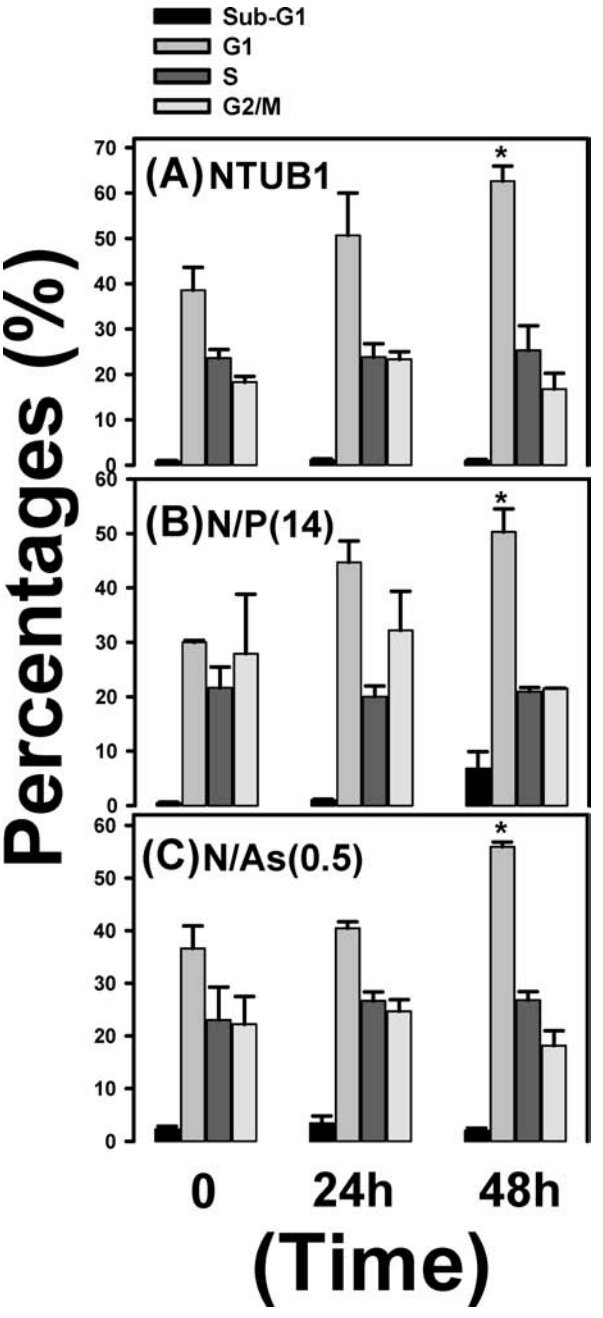

Figure 5. Induction of G1 arrest by LZP-F3 in UC cells. NTUB1, N/P(14), and N/As(0.5) cells were treated with LZP-F3 (10 μg/mL) alone for 48 h and then subjected to flow cytometric cell cycle analysis. Data are presented as mean ± standard error of the means of three separate experiments. ∗, $p < 0.05$ compared to the control value.

in N/As(0.5) (**Figure 4B**), but not in N/P(14) cells (**Figure 4A**). p38 activation was induced in N/As(0.5) after LZP-F3 treatment for up to 24 h (**Figure 4**). Similarly, p38 activation in N/P(14) cells was also significantly altered by LZP-F3. Using Western blot analysis, we demonstrated an evident down-regulation of xeroderma pigmentosum, complementation group A (XPA) protein expression, after LZP-F3 treatment in N/P(14) cells but not in N/As(0.5) cells. This decrease in expression was in a time-dependent manner.

Alteration of Cell Cycle Progression by LZP-F3. NTUB1, N/P(14), and N/As(0.5) cells were treated with 10 μg/mL LZP-F3 for 48 h and then subjected to flow cytometric cell cycle analysis (**Figure 5**). LZP-F3 caused a progressive accumulation of G0/G1

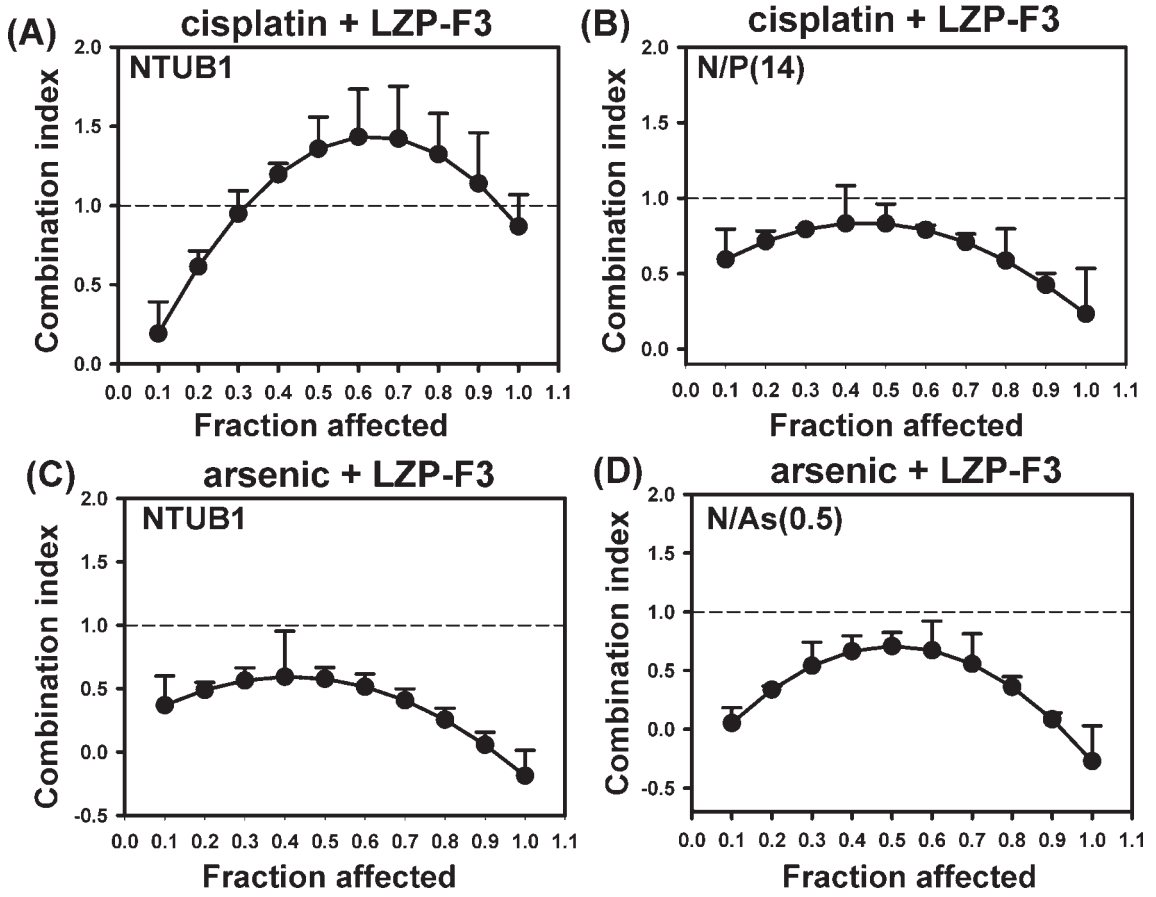

Figure 6. Median-effect analysis of the combined effects of LZP-F3 and anticancer drugs in NTUB1, N/P(14), or N/As(0.5) cells. By combining LZP-F3 and cisplatin or arsenic trioxide at graded concentrations in these three cells, respectively, numerous combined effects on growth inhibition were determined by median-effect analysis with the mutually nonexclusive model. Combination indices of <1, =1, and >1 indicate synergistic, additive, and antagonistic combinations, respectively. Among the three cells, the synergism of the combination appeared to be more profound in the two resistant cells than in the sensitive parental cell.

fractions (G1 arrest) in these cells for up to 48 h treatment. At the same time, there was a slight decrease in G2/M phase cells.

Median-Effect Analysis of Combined Effects of LZP-F3 and Anticancer Drugs. As shown in **Figure 6**, the combination of LZP-F3 and cisplatin showed a significant synergistic effect (combination indices < 1) in N/P(14) cells according to the median-effect analysis (**Figure 6B**). For N/P(14) cells, although synergism could be observed, additivism was present within a small range of the fraction affected (0.4−0.6 range) by this combination. In contrast, the combined effect of LZP-F3 and cisplatin generated an evident antagonistic effect (combination indices > 1) in NTUB1 cells (**Figure 6A**). It appeared to be the least effective in killing NTUB1 cells. Interestingly, median-effect analysis revealed that a combined treatment of LZP-F3 and arsenic trioxide generated profound synergistic cytotoxic effects in NTUB1 and N/As(0.5) cells (**Figure 6C**,**D**). Of particular note, the higher the fraction affected (or degree of growth inhibition), the more profound was the synergism with the two-drug combination. Among the three cells, the synergism of the combination appeared to be more profound in the two resistant cells than in the sensitive parental cell, indicating that LZP-F3 may potentiate cisplatin and arsenic cytotoxicity in cisplatin- and arsenic-resistant cells, respectively.

Combined Cytotoxic Mechanism by LZP-F3 and Anticancer Drugs. Next, we compared the extent of Bcl-2 family, apoptosis-related molecules, Fas, caspase 3 and 8, and cytochrome *c* induced by 10 μg/mL LZP-F3 alone or in combination with cisplatin (IC_{30} = 30 μM) and arsenic trioxide (IC_{30} = 2 μM) in N/P(14) and N/As(0.5) cells, respectively. More prominent as well as earlier caspase 3 and 8 activation at 48 h was seen with the combined treatment than with LZP-F3 alone in N/P(14) and N/As(0.5), respectively (**Figure 7**). Western blots, however, did not detect expression of caspase-9 in the two cells under identical conditions (data not shown). Commitment to apoptosis is largely a mitochondrial event controlled by proteins in the Bcl-2 family. Compared to LZP-F3 treatment alone, the pro-apoptotic proteins Bad and Bax were significantly up-regulated and showed earlier expressions in cells incubated with the combined treatment (**Figure 7**). In contrast, the combined treatment mediated significant Bcl-2 and Bcl-x_L reduction after 56 h of continuous treatment compared to LZP-F3 alone in N/P(14) and N/As(0.5) cells, respectively (**Figure 7**).

To examine whether or not the apoptotic effect induced by LZP-F3 alone or in combination with anticancer drugs was through the mitochondrial signaling pathway, release of cytochrome *c* was determined. The combined treatment showed a substantial amount of cytochrome *c* release after 48 h into the cytosol as well as caspase 3 activation compared to LZP-F3 alone in N/P(14) and N/As(0.5) cells (**Figure 7**), as shown by Western blot analysis. Interestingly, we also found Fas was significantly up-regulated and showed earlier expression in combined treatment than in LZP-F3 alone in these two cells (**Figure 7**). Similarly, FasL also was more significantly induced in combined treatment than in LZP-F3 alone (data not shown).

DISCUSSION

In this study, we have shown that the combination of LZP-F3 and chemotherapeutic agents (cisplatin and arsenic trioxide) produced synergistic cytotoxicity in UC (**Figure 6**). The apoptotic

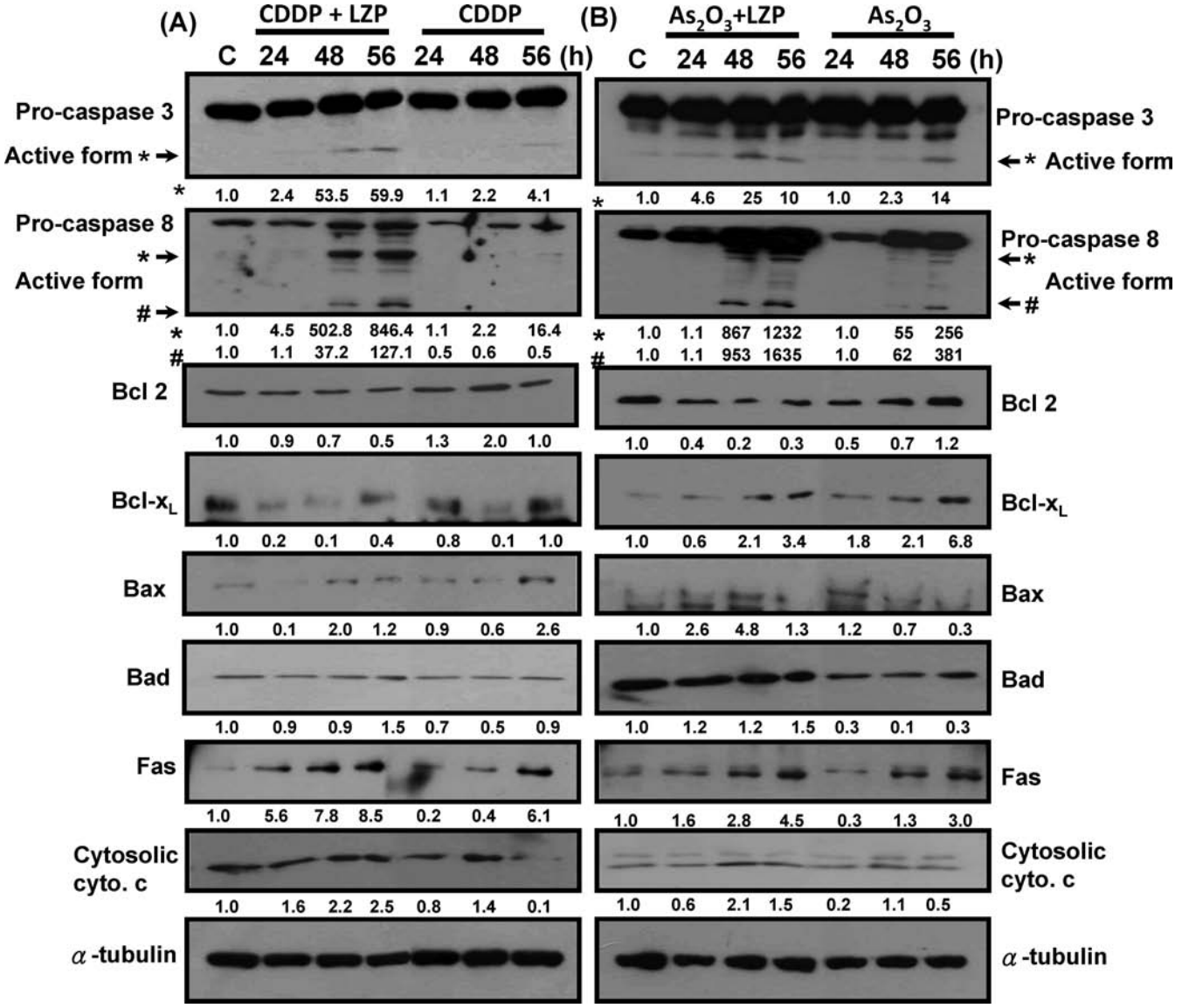

Figure 7. Combined effects of LZP-F3 and anticancer drugs on cytochrome *c* release, Bcl-2 family proteins, Fas expression, and caspase 3 and 8 activation in N/P(14) and N/As(0.5) cells. N/P(14) and N/As(0.5) cells were treated with 10 μg/mL LZP-F3 alone or in combination with cisplatin (IC_{30} = 30 μM) and arsenic trioxide (IC_{30} = 2 μM) for 56 h, respectively. The levels of Bcl-2 family, Fas, cytochrome *c*, and caspase 3 and 8 proteins were examined by Western blotting analysis. α-Tubulin served as an internal control. The number under each band in the immunoblot indicates the relative intensity of the corresponding band. The staining intensity of protein was assessed by using a densitometer. The fold of protein level was normalized to β-actin and compared to control. C, control group.

molecules of combined effects involved significant and earlier induction of Fas, caspase 3 and 8 activation, up-regulation of Bax and Bad, Bcl-2 and Bcl-x_L down-regulatuion, and cytochrome *c* release (**Figure 7**). From these findings, we hypothesized LZP-F3 enhanced cytotoxicity of chemotherapeutic agents in N/P(14) and N/As(0.5) cells and perhaps involved Fas/FasL-mediated apoptosis through signal pathway of cytochrome *c* release and caspase 8 and 3 activation.

LZP-F3 itself at concentrations of up to 100 μg/mL had little impact on viability of UC cells (**Figure 1**). Upon exposure to LZP-F3, induction of G1 arrest (**Figure 5**) and a number of critical molecular events occurred, which included induction of p21 and p38 expressions and suppression of the Akt and xeroderma pigmentosum and complementation group A (XPA) expressions in N/P(14) or N/As(0.5) cells (**Figure 4**). Interestingly, we also found a synergistic effect generated by combining LZP-F3 and docetaxel in hormone refractory prostate cancer (data not shown). By doing so, we can sensitize cancer cells to drugs that normally would not be able to kill cells effectively. Because advanced UC cells are known to be relatively chemoresistant, any efforts to potentiate chemotherapeutic effects by modulating phenotypes of cancer cells could be a novel strategy of treatment. These results may at least in part explain the potentiating effects of LZP-F3 on the cytotoxicity to UC cells.

According to our data, the potential ability of LZP-F3 was associated with the p21 expression and subsequent induction of cell cycle G1 arrest in UC cells (**Figure 5**). Thus, induction of p21 appeared to play a key role in the ability to inhibit cell cycle progression and suppress tumor cell growth (*33*). We have examined the expression of Akt, which was associated with drug resistance in cancer cells (*46*). Our results indicated that the suppression of Akt by LZP-F3 rendered N/As(0.5) cells more susceptible to the cytotoxic effect of arsenic trioxide. However, LZP-F3 had no effect on the Akt protein in N/P(14) cells. A previous study showed that XPA, a DNA repair protein, was involved in the cisplatin-mediated resistance in some cancer cells (*47*). Our data demonstrated that LZP-F3 inhibited the XPA expression in N/P(14) but had no effect on N/As(0.5) cells.

These findings may partly explain why LZP-F3 can specifically inhibit the expression of drug resistance-related molecules, such as Akt and XPA, to reverse the chemosensitivity of N/P(14) and N/As(0.5), respectively.

Cisplatin is among the most active cytotoxic agents against human UC. Arsenic trioxide has recently demonstrated significant activity for the treatment of cisplatin-resistant UC (*48*). However, approximately 30−50% of advanced UC cells do not respond to chemotherapeutic agent-based therapies. Therefore, chemoresistance is still a major problem for successful treatment of cancer. Many studies have reported that chemoresistance in cancer cells can be overcome by administration of a nontoxic reversing agent together with the anticancer agent (*30*). This coadministration of an anticancer agent and a chemomodulator has been shown to enhance the therapeutic effect of the drug (*31*−*35*). In this study, we found a novel modulator, LZP-F3, that showed a synergistic combination with anticancer drugs (cisplatin and arsenic trioxide) in UC cells. It was noteworthy that synergism of the combination appeared to be more profound in the two resistant cells than in the sensitive parental cell. However, the combined effect of LZP-F3 and cisplatin showed an evident antagonistic effect in NTUB1 cells, indicating that this combination might be not suitable for chemosensitive UC.

Previous findings showed that LZP-F3 could stimulate spleen cell proliferation and expression of cytokines, including IL-1, IL-2, and IFN-γ through Toll-like receptor 2 and 4 (TLR2 and 4) pathways (*36*). The composition of the LZP-F3 fraction has been analyzed, and the presence of fucose has been determined to be essential for induction of immunomodulating activities and antitumor activity (*49*, *50*). Additionally, Lin et al. have found and demonstrated that LZP-F3 could induce the innate immune response through the TLR2/TLR4-mediated p38 MAPK signal pathway (*51*). Of note, we also found that LZP-F3 could induce p38 expression in N/As(0.5) and N/P(14) cells. Therefore, LZP-F3 may be an effective chemomodulator agent for treatment of human drug-resistant UC.

Cheng et al. showed that LZP-F3 may induce death receptor ligands, such as tumor necrosis factor-α (TNF-α), tumor necrosis factor-related apoptosis inducing ligand (TRAIL), and Fas (CD95/APO-1) to mediate cellular apoptosis through death receptor oligomerization, recruitment of specialized adaptor proteins, activation of the caspase 3 and 8 cascade, and Bcl-2 down-regulation (*52*). Interestingly, we also found the similar feature that LZP-F3 potentiates cytotoxic effects of anticancer drugs against N/P(14) and N/As(0.5) cells via a Fas/FasL signaling pathway (a cell surface "death receptor" of the TNF receptor family), caspase 3 and 8 activation, and Bcl-2 down-regulation in this study. Wang et al. have also indicated the main active component of LZP-F3 is a glycoprotein fraction containing essential terminal fucose residues with 1,2-linkages for cellular cytotoxicity (*36*) and antitumor activity (*49*, *50*). Furthermore, the presence of fucose in the saccharide fraction is required for the activities. We hypothesized LZP-F3 may mimic a death ligand by the terminal fucose residues to bind the death receptor for induction of cell apoptosis. However, the detailed structures of LZP-F3 and other LZP fractions are still unknown due to their huge size and complex structures. Therefore, the real carbohydrate epitope responsible for the antitumor activity and its receptor remain to be identified.

Very few studies on evaluating the combined effects of LZP and chemotherapeutic agents in cancer cells have been performed. To our knowledge, this is the first study showing that LZP-F3 potentiates the cytotoxic effects of cisplatin and arsenic trioxide in human UC cells. Our results have clinical implications and represent one of the few efforts to substantiate the potential use of LZP-F3 as a chemomodulator in the treatment of human urothelial carcinomas.

ACKNOWLEDGMENT

We are grateful to Dr. Wen-Bin Yang and Dr. Chi-Huey Wong (Genomics Research Center, Academia Sinica, Taiwan) for kindly supplying the LZP-F3, F5, and F6 fractions from water-soluble extract of Ling-Zhi in this study.

LITERATURE CITED

(1) Kaufman, D. S.; Shipley, W. U.; Feldman, A. S. Bladder cancer. *Lancet* **2009**, *374* (9685), 239–249.

(2) Cheng, T. Systemic therapy for unresectable and metastatic transitional cell carcinoma of the urothelium: first-line and beyond. *Curr. Opin. Support. Palliat. Care* **2008**, *2* (3), 153–160.

(3) Bosl, G. J.; Fair, W. R.; Herr, H. W.; Bajorin, D. F.; Dalbagni, G.; Sarkis, A. S.; Reuter, V. E.; Cordon-Cardo, C.; Sheinfeld, J.; Scher, H. I. Bladder cancer: advances in biology and treatment. *Crit. Rev. Oncol. Hematol.* **1994**, *16* (1), 33–70.

(4) Chresta, C. M.; Arriola, E. L.; Hickman, J. A. Apoptosis and cancer chemotherapy. *Behring Inst. Mitt.* **1996**, *97*, 232–240.

(5) Leppert, J. T.; Shvarts, O.; Kawaoka, K.; Lieberman, R.; Belldegrun, A. S.; Pantuck, A. J. Prevention of bladder cancer: a review. *Eur. Urol.* **2006**, *49* (2), 226–234.

(6) Fojo, A. T.; Ueda, K.; Slamon, D. J.; Poplack, D. G.; Gottesman, M. M.; Pastan, I. Expression of a multidrug-resistance gene in human tumors and tissues. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84* (1), 265–269.

(7) Siu, L. L.; Banerjee, D.; Khurana, R. J.; Pan, X.; Pflueger, R.; Tannock, I. F.; Moore, M. J. The prognostic role of p53, metallothionein, P-glycoprotein, and MIB-1 in muscle-invasive urothelial transitional cell carcinoma. *Clin. Cancer Res.* **1998**, *4* (3), 559–565.

(8) Kim, W. J.; Kakehi, Y.; Wu, W. J.; Fukumoto, M.; Yoshida, O. Expression of multidrug resistance-related genes (mdrl, MRP, GST-pi and DNA topoisomerase II) in urothelial cancers. *Br. J. Urol.* **1996**, *78* (3), 361–368.

(9) Kubo, H.; Sumizawa, T.; Koga, K.; Nishiyama, K.; Takebayashi, Y.; Chuman, Y.; Furukawa, T.; Akiyama, S.; Ohi, Y. Expression of the multidrug resistance-associated protein (MRP) gene in urothelial carcinomas. *Int. J. Cancer* **1996**, *69* (6), 488–494.

(10) Floyd, J. W.; Lin, C. W.; Prout, G. R., Jr. Multi-drug resistance of a doxorubicin-resistant bladder cancer cell line. *J. Urol.* **1990**, *144* (1), 169–171.

(11) Masters, J. R.; Thomas, R.; Hall, A. G.; Hogarth, L.; Matheson, E. C.; Cattan, A. R.; Lohrer, H. Sensitivity of testis tumour cells to chemotherapeutic drugs: role of detoxifying pathways. *Eur. J. Cancer* **1996**, *32A* (7), 1248–1253.

(12) Yang, C. R.; Ou, Y. C.; Kuo, J. H.; Kao, Y. L.; Chen, C. L.; Yean, S. Y.; Horng, Y. Y.; Yang, C. S. Intracellular glutathione content of urothelial cancer in correlation to chemotherapy response. *Cancer Lett.* **1997**, *119* (2), 157–162.

(13) Saika, T.; Tsushima, T.; Ochi, J.; Akebi, N.; Nasu, Y.; Matsumura, Y.; Ohmori, H. Over-expression of metallothionein and drug-resistance in bladder cancer. *Int. J. Urol.* **1994**, *1* (2), 135–139.

(14) Shirakawa, T.; Sasaki, R.; Gardner, T. A.; Kao, C.; Zhang, Z. J.; Sugimura, K.; Matsuo, M.; Kamidono, S.; Gotoh, A. Drug-resistant human bladder-cancer cells are more sensitive to adenovirus-mediated wild-type p53 gene therapy compared to drug-sensitive cells. *Int. J. Cancer* **2001**, *94* (2), 282–289.

(15) Strano, S.; Dell'Orso, S.; Di, A. S.; Fontemaggi, G.; Sacchi, A.; Blandino, G. Mutant p53: an oncogenic transcription factor. *Oncogene* **2007**, *26* (15), 2212–2219.

(16) Levenson, V. V.; Davidovich, I. A.; Roninson, I. B. Pleiotropic resistance to DNA-interactive drugs is associated with increased expression of genes involved in DNA replication, repair, and stress response. *Cancer Res.* **2000**, *60* (18), 5027–5030.

(17) Kuo, M. T.; Chen, H. H.; Song, I. S.; Savaraj, N.; Ishikawa, T. The roles of copper transporters in cisplatin resistance. *Cancer Metastasis Rev.* **2007**, *26* (1), 71–83.

(18) Niell, H. B.; Webster, K. C.; Smith, E. E. Anticancer drug activity in human bladder tumor cell lines. *Cancer* **1985**, *56* (5), 1039–1044.

(19) Cho, H. J.; Kim, J. K.; Kim, K. D.; Yoon, H. K.; Cho, M. Y.; Park, Y. P.; Jeon, J. H.; Lee, E. S.; Byun, S. S.; Lim, H. M.; Song, E. Y.; Lim, J. S.; Yoon, D. Y.; Lee, H. G.; Choe, Y. K. Upregulation of Bcl-2 is associated with cisplatin-resistance via inhibition of Bax translocation in human bladder cancer cells. *Cancer Lett*. **2006**, *237* (1), 56–66.

(20) Hong, J. H.; Lee, E.; Hong, J.; Shin, Y. J.; Ahn, H. Antisense Bcl2 oligonucleotide in cisplatin-resistant bladder cancer cell lines. *BJU Int*. **2002**, *90* (1), 113–117.

(21) Kirsh, E. J.; Baunoch, D. A.; Stadler, W. M. Expression of bcl-2 and bcl-X in bladder cancer. *J. Urol*. **1998**, *159* (4), 1348–1353.

(22) Kamat, A. M.; Sethi, G.; Aggarwal, B. B. Curcumin potentiates the apoptotic effects of chemotherapeutic agents and cytokines through down-regulation of nuclear factor-κB and nuclear factor-κB-regulated gene products in IFN-α-sensitive and IFN-α-resistant human bladder cancer cells. *Mol. Cancer Ther*. **2007**, *6* (3), 1022–1030.

(23) Tapia, M. A.; Gonzalez-Navarrete, I.; Dalmases, A.; Bosch, M.; Rodriguez-Fanjul, V.; Rolfe, M.; Ross, J. S.; Mezquita, J.; Mezquita, C.; Bachs, O.; Gascon, P.; Rojo, F.; Perona, R.; Rovira, A.; Albanell, J. Inhibition of the canonical IKK/NF κB pathway sensitizes human cancer cells to doxorubicin. *Cell Cycle* **2007**, *6* (18), 2284–2292.

(24) Shrader, M.; Pino, M. S.; Lashinger, L.; Bar-Eli, M.; Adam, L.; Dinney, C. P.; McConkey, D. J. Gefitinib reverses TRAIL resistance in human bladder cancer cell lines via inhibition of AKT-mediated X-linked inhibitor of apoptosis protein expression. *Cancer Res*. **2007**, *67* (4), 1430–1435.

(25) Liu, S. Q.; Yu, J. P.; Yu, H. G.; Lv, P.; Chen, H. L. Activation of Akt and ERK signalling pathways induced by etoposide confer chemoresistance in gastric cancer cells. *Dig. Liver Dis*. **2006**, *38* (5), 310–318.

(26) Liu, S. Q.; Yu, J. P.; Yu, H. G.; Lv, P.; Chen, H. L. Activation of Akt and ERK signalling pathways induced by etoposide confer chemoresistance in gastric cancer cells. *Dig. Liver Dis*. **2006**, *38* (5), 310–318.

(27) Gomez, L. A.; de Las, P. A.; Reiner, T.; Burnstein, K.; Perez-Stable, C. Increased expression of cyclin B1 sensitizes prostate cancer cells to apoptosis induced by chemotherapy. *Mol. Cancer Ther*. **2007**, *6* (5), 1534–1543.

(28) Kilker, R. L.; Planas-Silva, M. D. Cyclin D1 is necessary for tamoxifen-induced cell cycle progression in human breast cancer cells. *Cancer Res*. **2006**, *66* (23), 11478–11484.

(29) Koiso, K. Bladder cancer: chemotherapy of advanced bladder cancer. *Gan To Kagaku Ryoho* **1982**, *9* (3), 377–386.

(30) Chambers, S. K.; Chambers, J. T.; Davis, C. A.; Kohorn, E. I.; Schwartz, P. E.; Lorber, M. I.; Handschumacher, R. E.; Pizzorno, G. Pharmacokinetic and phase I trial of intraperitoneal carboplatin and cyclosporine in refractory ovarian cancer patients. *J. Clin. Oncol*. **1997**, *15* (5), 1945–1952.

(31) Pu, Y. S.; Hsieh, M. W.; Wang, C. W.; Liu, G. Y.; Huang, C. Y.; Lin, C. C.; Guan, J. Y.; Lin, S. R.; Hour, T. C. Epidermal growth factor receptor inhibitor (PD168393) potentiates cytotoxic effects of paclitaxel against androgen-independent prostate cancer cells. *Biochem. Pharmacol*. **2006**, *71* (6), 751–760.

(32) Pu, Y. S.; Hour, T. C.; Chen, J.; Huang, C. Y.; Guan, J. Y.; Lu, S. H. Arsenic trioxide as a novel anticancer agent against human transitional carcinoma − characterizing its apoptotic pathway. *Anticancer Drugs* **2002**, *13* (3), 293–300.

(33) Hour, T. C.; Chen, J.; Huang, C. Y.; Guan, J. Y.; Lu, S. H.; Pu, Y. S. Curcumin enhances cytotoxicity of chemotherapeutic agents in prostate cancer cells by inducing p21(WAF1/CIP1) and C/EBPβ expressions and suppressing NF-κB activation. *Prostate* **2002**, *51* (3), 211–218.

(34) Hour, T. C.; Chen, J.; Huang, C. Y.; Guan, J. Y.; Lu, S. H.; Hsieh, C. Y.; Pu, Y. S. Characterization of chemoresistance mechanisms in a series of cisplatin-resistant transitional carcinoma cell lines. *Anticancer Res*. **2000**, *20* (5A), 3221–3225.

(35) Wysocki, P. J. mTOR in renal cell cancer: modulator of tumor biology and therapeutic target. *Expert. Rev. Mol. Diagn*. **2009**, *9* (3), 231–241.

(36) Wang, Y. Y.; Khoo, K. H.; Chen, S. T.; Lin, C. C.; Wong, C. H.; Lin, C. H. Studies on the immuno-modulating and antitumor activities of *Ganoderma lucidum* (reishi) polysaccharides: functional and proteomic analyses of a fucose-containing glycoprotein fraction responsible for the activities. *Bioorg. Med. Chem*. **2002**, *10* (4), 1057–1062.

(37) Boh, B.; Berovic, M.; Zhang, J.; Zhi-Bin, L. *Ganoderma lucidum* and its pharmaceutically active compounds. *Biotechnol. Annu. Rev*. **2007**, *13*, 265–301.

(38) Wang, S. Y.; Hsu, M. L.; Hsu, H. C.; Tzeng, C. H.; Lee, S. S.; Shiao, M. S.; Ho, C. K. The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. *Int. J. Cancer* **1997**, *70* (6), 699–705.

(39) Wang, G.; Zhang, J.; Mizuno, T.; Zhuang, C.; Ito, H.; Mayuzumi, H.; Okamoto, H.; Li, J. Antitumor active polysaccharides from the Chinese mushroom *Songshan lingzhi*, the fruiting body of *Ganoderma tsugae*. *Biosci., Biotechnol., Biochem*. **1993**, *57* (6), 894–900.

(40) Yuen, J. W.; Gohel, M. D. Anticancer effects of *Ganoderma lucidum*: a review of scientific evidence. *Nutr. Cancer* **2005**, *53* (1), 11–17.

(41) Lin, Z. B. Cellular and molecular mechanisms of immuno-modulation by *Ganoderma lucidum*. *J. Pharmacol. Sci*. **2005**, *99* (2), 144–153.

(42) Sliva, D. *Ganoderma lucidum* (reishi) in cancer treatment. *Integr. Cancer Ther*. **2003**, *2* (4), 358–364.

(43) Opara, E. I. The efficacy and safety of Chinese herbal medicines. *Br. J. Nutr*. **2004**, *91* (2), 171–173.

(44) Cao, Q. Z.; Lin, Z. B. Antitumor and anti-angiogenic activity of *Ganoderma lucidum* polysaccharides peptide. *Acta Pharmacol. Sin*. **2004**, *25* (6), 833–838.

(45) Hour, T. C.; Huang, C. Y.; Lin, C. C.; Chen, J.; Guan, J. Y.; Lee, J. M.; Pu, Y. S. Characterization of molecular events in a series of bladder urothelial carcinoma cell lines with progressive resistance to arsenic trioxide. *Anticancer Drugs* **2004**, *15* (8), 779–785.

(46) Szanto, A.; Bognar, Z.; Szigeti, A.; Szabo, A.; Farkas, L.; Gallyas, F., Jr. Critical role of bad phosphorylation by Akt in cytostatic resistance of human bladder cancer cells. *Anticancer Res*. **2009**, *29* (1), 159–164.

(47) Leichman, L.; Lawrence, D.; Leichman, C. G.; Nava, H.; Nava, E.; Proulx, G.; Clark, K.; Khushalani, N. I.; Berdzik, J.; Greco, W.; Smith, P.; Creaven, P. J.; Kepner, J. L.; Javle, M. M.; Pendyala, L. Expression of genes related to activity of oxaliplatin and 5-fluorouracil in endoscopic biopsies of primary esophageal cancer in patients receiving oxaliplatin, 5-flourouracil and radiation: characterization and exploratory analysis with survival. *J. Chemother*. **2006**, *18* (5), 514–524.

(48) Lin, C. C.; Pu, Y. S.; Hsu, C. H.; Keng, H. Y.; Cheng, A. L.; Yang, C. H. Acute encephalopathy following arsenic trioxide for metastatic urothelial carcinoma. *Urol. Oncol*. **2008**, *26* (6), 659–661.

(49) Hsu, H. Y.; Hua, K. F.; Lin, C. C.; Lin, C. H.; Hsu, J.; Wong, C. H. Extract of Reishi polysaccharides induces cytokine expression via TLR4-modulated protein kinase signaling pathways. *J. Immunol*. **2004**, *173* (10), 5989–5999.

(50) Haltiwanger, R. S. Fucose is on the TRAIL of colon cancer. *Gastroenterology* **2009**, *137* (1), 36–39.

(51) Lin, K. I.; Kao, Y. Y.; Kuo, H. K.; Yang, W. B.; Chou, A.; Lin, H. H.; Yu, A. L.; Wong, C. H. Reishi polysaccharides induce immunoglobulin production through the TLR4/TLR2-mediated induction of transcription factor Blimp-1. *J. Biol. Chem*. **2006**, *281* (34), 24111–24123.

(52) Cheng, K. C.; Huang, H. C.; Chen, J. H.; Hsu, J. W.; Cheng, H. C.; Ou, C. H.; Yang, W. B.; Chen, S. T.; Wong, C. H.; Juan, H. F. *Ganoderma lucidum* polysaccharides in human monocytic leukemia cells: from gene expression to network construction. *BMC Genomics* **2007**, *8*, 411–428.

Received for review February 1, 2010. Revised manuscript received June 17, 2010. Accepted June 27, 2010.