약제학회지(2007), 제 37 권 제 5 호
J. Kor. Pharm. Sci., Vol. 37, No. 5, 305-309 (2007)

Anti-proliferative Effect of Tetra-arsenic Oxide (TetraAs®) in Human Gastric Cancer Cells in Vitro

Won-Heui Chung, Hye-Jin Koo and Hyo-Jeong Kuh†

Department of Biomedical Sciences, College of Medicine, The Catholic Univ. of Korea, Seoul, Korea

(Received October 9, 2007 · Accepted October 20, 2007)

ABSTRACT – Arsenic compounds have been used to treat various diseases including cancer in oriental medicine. Arsenic trioxide (As_2O_3, Trisenox®) has been used for the treatment of leukemia and its anti-solid tumor activity has also been reported recently. Tetra-arsenic oxide (As_4O_6, TetraAs®) is a newly developed arsenic compound which has shown an anti-cancer activity in some human cancer cell lines. The purpose of this study was to evaluate the anti-gastric cancer potential of TetraAs and to search for an agent with synergistic interaction with TetraAs against human gastric cancers. We analysed anti-proliferative effect of TetraAs when given alone and in combination with other chemotherapeutic agents such as 5-FU, paclitaxel, and cisplatin in SNU-216, a human gastric cancer cell line. The IC_{50} of these 4 anti-cancer drugs ranged from 5.8 nM to 7.5 μM with a potency rank of order paclitaxel > TetraAs > cisplatin > 5-FU. TetraAs showed 10-fold greater potency than 5-FU and cisplatin at the same effect level of IC_{50}. TetraAs + 5-FU and TetraAs + paclitaxel showed synergistic and additive interaction, respectively. On the other hand, TetraAs with cisplatin group appeared to be strongly antagonistic. Apoptotic population was measured and compared between single and combination treatment. The apoptotic cells for the combination of TetraAs + 5-FU showed significant increase compared to single TetraAs treatment. On the contrary, TetraAs+cisplatin showed less apoptotic cells compared to TetraAs or cisplatin alone treatment. Overall, our results indicate that TetraAs can be effectively combined with 5-FU or paclitaxel, but not with cisplatin for synergistic anti-cancer effect, which warrants further evaluation using in vivo models.

Key words – TetraAs, 5-FU, Paclitaxel, Cisplatin, Synergistic interaction

Gastric cancer accounts for 9.9% of all new cancer diagnoses and 12.1% of all cancer deaths.[1,2] Chemotherapy is important in gastric cancer treatment for palliation of symptoms and survival improvement. The most commonly used single agent is 5-fluorouracil (5-FU) with response rates less than 30%. Cisplatin and paclitaxel also have been used as a single chemotherapeutic agent with similar response rates. Several combination regimens including ECF (epirubicin, cisplatin and 5-FU), FAM (5-FU, adriamycin and mitomycin), FAMTX (5-FU, adriamycin and methotrexate) are widely accepted.[3,4] However, significant improvement in response rate has not been obtained.[1] Recently, new concept of molecular targeting or anti-angiogenesis inhibition has gained much attention and new agents on these new targets are being studied for gastric cancer therapy.

Arsenic compound has been used clinically for the treatment of acute promyelocytic leukemia (APL) since early 1990.[5,6] Prominent effectiveness of arsenic compound in APL has led to investigation of its effectiveness against gastric and cervical cancers. It has been reported that arsenic trioxide (As_2O_3, ATO, Trisenox®) induced apoptosis and cell cycle arrest in MKN-45, human gastric cancer cell line and showed anti-cancer effect in human gastric tumor xenograft model via disturbing blood vessel formation.[5,6]

Tetra-arsenic oxide (As_4O_6, TetraAs®) is a newly developed arsenic compound. Anti-proliferative and apoptosis inducing effect of TetraAs has been demonstrated against human leukemic cells.[7] Reactive oxygen species (ROS) and cytochrome c release were thought to be a major mechanism of its anti-proliferative effect.[7,8] In a phase I clinical trial, 10 of 15 patients with solid tumors showed stable disease phase when given 15 mg~45 mg of TetraAs everyday for 28 days. And further clinical evaluation is underway.

Clinical efficacy along with low toxicity of TetraAs has been demonstrated in a phase I study. Due to the issue of less cytotoxic (size reductive) effect, combination with other conventional cytotoxic agents is considered to improve the anti-cancer efficacy of TetraAs. In the present study, we evaluated anti-proliferative effect of TetraAs when given alone and in combination with other conventional chemotherapeutic agents such as 5-FU, paclitaxel, and cisplatin in gastric cancer cells, in order to investigate its chemotherapeutic potential for gastric

†본 논문에 관한 문의는 이 저자에게로
Tel : 02)590-2422, E-mail : hkuh@catholic.ac.kr

cancer treatment.

Materials and methods

Cell culture

The human stomach adenocarcinoma cell line SNU-216 was obtained from the Korean Cell Line Bank (Seoul, South Korea). This cell line was maintained using RPMI 1640 (Gibco BRL, Grand Island, NE) supplemented with 10% heat-inactivated FBS (Welgene Inc., Daegu, South Korea), penicillin 100 U/ml (Sigma Chemical Co., St. Louis, MO) and streptomycin 100 μg/ml (Sigma Chemical Co., St. Louis, MO) in a humidified 5% CO_2 atmosphere at 37°C.

Reagents

Tetra-arsenic oxide was obtained from Chonjisan Co., LTD. (Seoul, South Korea). Cisplatin was kindly provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). Paclitaxel was donated by Hanmi Research Center Co. LTD. (Giheung, South Korea). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless mentioned otherwise. TetraAs was dissolved in 1 N NaOH, whereas cisplatin, paclitaxel and 5-FU in DMSO (dimethyl sulfoxide), then further diluted with PBS as needed.

Cytotoxicity assay

The sulforhodamine B (SRB) assay was conducted as previously described.[9] Cells in the log phase were harvested and plated in 96-well plates at a predetermined density. After 24 hr incubation, cells were exposed to drug-containing media for 72 hr. Cells were then fixed with 10% trichloroaceticacid (TCA) and stained with 0.4% SRB for 10 min. After washes, protein-bound dye was extracted with 10 mM buffered Tris (Amresco, Solon, OH) and absorbance was measured.

Apoptosis detection assay

Apoptosis was measured using annexin V fluorescein isothiocyanate (FITC) apoptosis detection kit I (BD Pharmingen, San Diego, CA). Cells were washed twice with cold PBS and then resuspended in binding buffer and stained with annexin V-FITC and propidium iodide (PI) for 15 min at 25°C in the dark. Samples were then analyzed using FACS Caliber and data were acquired using Cell Quest software (Becton Dickinson, San Jose, CA).

Data analysis

Inhibition of cell growth (% cell survival) was calculated using (Eq. 1). Median effect model was used to analyse the cell survival data (Eq. 2). ICx was defined as the drug concentration required to reduce the absorbance to (100-x)% of the control in each test and was determined from model parameters and model equation (Eq. 2).

$$\%\ \text{Cell Survival} = \left(\frac{mean\ absorbance\ of\ treated\ cells}{mean\ absorbance\ of\ control\ cells}\right) \times 100 \qquad \text{(Eq. 1)}$$

$$\%\ \text{Cell Survival} = (100-R) \times \left(1 - \frac{[D]^m}{K_d^m + [D]^m}\right) + R \qquad \text{(Eq. 2)}$$

[D] is the drug concentration, K_d is the concentration of drug that produces a 50% reduction of maximum inhibition rate (Emax), m is a Hill-type coefficient and R is the residual unaffected (resistance) fraction (R=100-Emax). IC_{50} was defined as the drug concentration required to reduce viability to 50% of that of the control (i.e., $K_d = IC_{50}$ when R=0). Combination index (CI) was calculated using the following equation (Eq. 3).

$$CI_X = \frac{(D)_A}{(D_X)_A} + \frac{(D)_B}{(D_X)_B} + \alpha\frac{(D)_A(D)_B}{(D_X)_A(D_X)_B} \qquad \text{(Eq. 3)}$$

CIx is CI at a fixed effect x for a combination of drug A and B. $[Dx]_A$ and $[Dx]_B$ are the concentration of drug A or B alone giving an effect x, $[D]_A$ and $[D]_B$ are the concentration of drug A or B in combination A+B giving an effect x. α is 0 when A and B are mutually exclusive and 1 when mutually non-exclusive.[10] CI less than 0.8 was considered as synergistic, 0.8 ~1.2 as additive, and greater than 1.2 as antagonistic interaction.

Statistical analysis: Analysis of Variance (ANOVA) followed by Bonferroni post hoc test was performed using SPSS® for windows, version 8.0. A $p<0.05$ was considered statistically significant.

Results

Anti-proliferative activity of TetraAs, 5-FU, paclitaxel or cisplatin in SNU-216 cells

The anti-proliferative activity of TetraAs, 5-FU, paclitaxel and cisplatin were determined in SNU-216 cells after 72 hr continuous drug exposure (Fig. 1). IC_{50} of paclitaxel was in nM range (i.e., 5.83 nM) whereas those of other drugs in μM range, i.e., 0.76 to 7.54 μM (Table I). Among latter three agents, TetraAs showed 10-fold greater potency at same effect level (50% inhibition) and lowest resistant fraction among the four agents in SNU-216 cells (Table I). It was noted that paclitaxel showed significant fraction of resistant cells (25.5%)

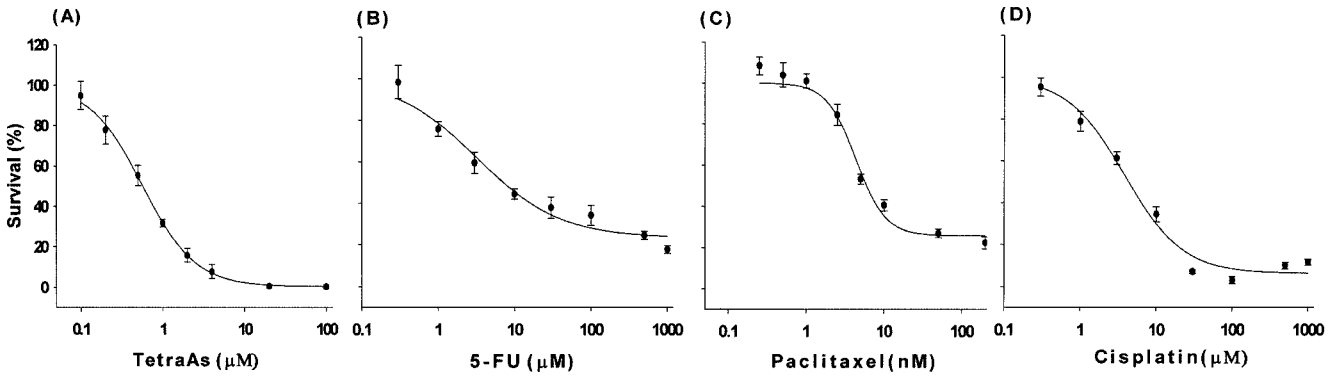

Figure 1—Representative dose-response curves for TetraAs (A), 5-FU (B), paclitaxel (C) and cisplatin (D) in SNU-216 human gastric cancer cells. Cell viability was assessed by using SRB assay after 72 hr drug exposure and % survival was calculated using Eq.1.

against SNU-216 (Table I). The ratio of IC_{70}/IC_{50} reflects the Hill type coefficient, m. The m value was smallest for 5-FU (0.64), hence, more than 7 fold higher dose was required for 20% further reduction in proliferative inhibition (Table I). To the contrary, paclitaxel showed largest m value and only 3 fold higher concentration was required to induce 20% further reduction. TetraAs as well as cisplatin showed steep dose-response curve with IC_{70}/IC_{50} ratio of 1.9, suggesting these agents can induce much greater anti-cancer activity than other agents by increasing dose intensity to a similar extent.

Table I—*Anti-proliferative Activity of TetraAs, 5-FU, Paclitaxel, Cisplatin in SNU-216, a Human Gastric Cancer Cells. Median Effect Model (Eq. 2) was Fitted to Dose-response Data. Inhibitory Concentrations (IC_{50}, IC_{70} and IC_{90}) were Calculated to Represent Absolute Value to Produce the Designated % Reduction in Survival. Each Value Indicates the Mean of More than Three Independent Experiments*

Parameter	TetraAs	5-FU	Paclitaxel[1]	Cisplatin
IC_{50} (μM)	0.76±0.40	7.54±1.89	5.83±0.50	6.70±2.01
IC_{70} (μM)	1.49±0.71	59.1±26.2	16.4±1.36	12.9±2.35
IC_{90} (μM)	4.53±1.92	>1000	>200	96.9±30.2
m^2	1.25±0.15	0.64±0.13	2.00±0.31	1.51±0.33
R (%)[3]	0.53±0.72	14.9±10.4	25.5±0.27	8.56±1.69

[1]Paclitaxel in nM.
[2]m is the Hill-type coefficient. (see Eq.2)
[3]R is the residual unaffected fraction (the resistance fraction). (see Eq.2)

Table II—*Combinatory Interaction of TetraAs with 5-FU, Paclitaxel or Cisplatin in SNU-216 Cells. CIs[1] were Calculated at the 50%, 70% and 90% Growth Inhibition Levels, i.e., CI_{50}, CI_{70} and CI_{90}. Drug Treatments were Given Simultaneously for 72 hr at Equitoxic Ratios*

	TetraAs+5-FU	TetraAs +Paclitaxel	TetraAs +Cisplatin
CI index[50]	0.44±0.04	0.87±0.25	4.63±0.62
CI index[70]	0.34±0.01	0.76±0.17	4.62±0.40
CI index[90]	ND[2]	ND[2]	2.83±0.13
R (%)	3.81±0.88%	9.52±3.02%	2.56±1.10%

[1]CIs were calculated with Eq.3 with $\alpha=1$ assuming two agents are mutually non-exclusive.
[2]Not determined due to R>10.

Combinatory effect of TetraAs with 5-FU, paclitaxel or cisplatin

Anti-proliferative effect of TetraAs when combined with 5-FU, paclitaxel or cisplatin was examined at equitoxic ratios which were determined as 1:10, 1:0.01, or 1:10, respectively, based on the IC_{50} of each drug. TetraAs with 5-FU showed the most synergistic effect among three combination groups with CI index 0.44 (Table II). TetraAs+paclitaxel group showed additive interaction with CI index of 0.87 (Table II). These results indicate that when TetraAs was combined with 5-FU or paclitaxel, each agent was required at only 24% or 49% of single treatment dose, respectively, to induce 50% inhibition of cell proliferation in SNU-216 cells. On the contrary, TetraAs+cisplatin group showed strong antagonistic effect with CI index of 4.63 (Table II), indicating that more than 240% of single treatment dose of each agent was used to reach the same 50% inhibition level. The synergistic interaction between TetraAs and 5-FU was enhanced at higher effect level i.e., CI decreased to 0.34 at IC_{70} level (Table II). These data demonstrate that synergistic interaction with 5-FU may increase with higher dose, but, not either with paclitaxel or cisplatin.

Apoptosis induction of TetraAs combined with 5-FU, paclitaxel or cisplatin in SNU-216 cells

In order to confirm the synergistic and antagonistic interaction, apoptosis was measured and compared after TetraAs single or combination treatments of TetraAs with 5-FU, paclitaxel or cisplatin. Apoptosis induction was determined as % apoptotic cells (annexin V-positive cells) compared with

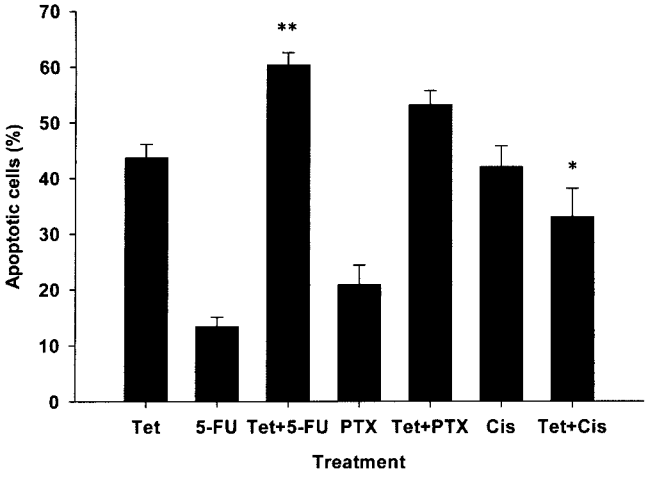

Figure 2—The percent apoptotic cells were determined as annexin positive cells. Cells were exposed for 48 hr to TetraAs (10 μM), paclitaxel (0.1 μM), cisplatin (100 μM), and 5-FU (100 μM) either alone or in combination as indicated. Tet:TetraAs ; PTX : Paclitaxel ; Cis:Cisplatin. $^{**}P<0.01$, $^{*}P<0.05$ when compared to TetraAs only.

untreated cell population. 10 μM of TetraAs induced 43.7% of apoptotic cells. When given in combination with 100 μM 5-FU or 0.1 μM paclitaxel, 60.3% and 53.2% of apoptotic cells were induced, respectively (Fig. 2), i.e., apoptotic cell % increased by 16.6% (P value < 0.01) and 9.5% when combined with 5-FU or paclitaxel, respectively, when compared with TetraAs only treated group. On the other hand, when combined with 100 μM of cisplatin, TetraAs induced less apoptosis (33.0%) compared to that of TetraAs single treatment (43.7%).

Discussion and Conclusions

In this study, we investigated the chemotherapeutic potential of TetraAs given as a single or doublet combination in vitro for the treatment of human gastric cancers. Although mechanism of action is still unclear, it has been reported that TetraAs induced loss of mitochondrial membrane potential and cytochrome c release through ROS production in human cancer cells.[7,8] Another arsenic compound, ATO, used for the treatment of APL induces apoptosis via bcl-2 down regulation and degradation of PML-RARα (promyelocytic leukemia retinoic acid receptor α).[5,11,12] Recent studies have also reported that ATO can decrease microvessel density and disturb angiogenesis in gastric tumors.[6] Interestingly, TetraAs also showed anti-angiogenesis activity in a pre-clinical and phase I clinical trial, suggesting that two agents may have similar mechanisms of action.

We evaluated the anti-proliferative activity of 5-FU, paclitaxel and cisplatin along with TetraAs in SNU-216 human gastric cancer cells. These agents are commonly used as single or combined regimen in gastric cancer chemotherapy.[3,4] The IC_{50} of TetraAs in SNU-216 cells was similar to that of U937 human leukemia cells reported in the literature (0.76 μM v.s 0.20 μM).[7] TetraAs showed significantly low resistant fraction (0.53%) compared to other agents (Table I). These data suggest that TetraAs may be an active agent against human gastric cancer and a good chemotherapeutic agent to combine with the agent with significant resistant fraction, such as 5-FU or paclitaxel (Fig. 1 and Table I). Along with low toxicity demonstrated in phase I studies, high m value and low R value of dose-response curve of TetraAs constitutes a good profile for a potent yet safe anti-tumor agent.

We demonstrated combinatory effect of TetraAs and other anti-cancer drugs (Table II). TetraAs+5-FU showed synergism, however, TetraAs+paclitaxel and TetraAs+cisplatin was additive and antagonistic, respectively. Combinatory effect of TetraAs has not been reported yet, but some literature data are available for the combination of ATO and many chemotherapeutic agents. Phytosphingosine showed synergy with ATO in human myeloid leukemia cells through the mitochondrial translocation of Bax and the PARP-1 activation.[13] Sulindac exerted a synergy on apoptosis induction in human non-small lung cancer cells, which was attributed to the ROS dependent down regulation of survivin.[14] It has been shown that glutathione (GSH) reduction system can modulate ROS mediated anti-cancer effect of ATO.[11,15] Moreover, it was confirmed that combinatory mechanism between ATO and other agents was mainly determined by intracellular GSH level.[15] Hence, the possible involvement of GSH level in combinatory interaction of TetraAs with 5-FU, paclitaxel and cisplatin warrants further investigation.

For apoptosis induction experiments, cells were exposed to the drug concentration that was 10 fold higher than IC_{50} of each agent. The level of apoptotic induction was consistent with CI shown in Table II, indicating that combinatory interaction between three agents is reserved not only at 50% growth inhibitory level, but also at 10 fold higher concentration levels in SNU-216 cancer cells. However, the difference in % apoptotic cells between single treatment and combination treatment appeared to be smaller than expected from the CI values. It suggests that apoptosis induction may not be sole mechanism to explain synergistic interaction, and other mechanisms for synergy should be studied in order to provide insight into combination therapy development including TetraAs.

Our study demonstrated anti-proliferative activity and apoptosis induction of TetraAs when given alone and in combination with other conventional chemotherapeutic agents.

TetraAs+5-FU and TetraAs+paclitaxel showed synergistic and additive interaction, respectively, in SNU-216 human gastric cancer cells in vitro. On the contrary, antagonistic interaction was observed in TetraAs+cisplatin combination. We also showed that combinatory interaction is related to apoptosis induction, however, it seems that other factors also contribute to these synergistic interaction. Overall, these data support chemotherapeutic potential of TetraAs against human gastric cancer in single or combination treatment, which warrants further evaluation using in vivo models and detailed studies on combinatory mechanisms.

Acknowledgments

The present work was supported by a grant from Chonjisan. Co., LTD. and a grant from Ministry of Science & Technology (F104AA010007-06A0101-00710), Republic of Korea.

References

1) D.H. Roukos, Current status and future perspectives in gastric cancer management, *Cancer Treat Rev.*, **26**, 243-255 (2000).
2) L. Lim, M. Michael, G.B. Mann and T. Leong, Adjuvant therapy in gastric cancer, *J. Clin. Oncol.*, **23**, 6220-6232 (2005).
3) J.A. Meyerhardt and C.S. Fuchs, Chemotherapy options for gastric cancer, *Semin Radiat Oncol.*, **12**, 176-186 (2002).
4) A.D. Wagner, W. Grothe, S. Behl, G. Kleber, A. Grothey, J. Haerting and W.E. Fleig, Chemotherapy for advanced gastric cancer, *Cochrane Database of Systemic reviews*, (2005).
5) Q.S. Shao, Z.Y. Ye, Z.Q. Ling and J.J. Ke, Cell cycle arrest and apoptotic cell death in cultured human gastric carcinoma cells mediated by arsenic trioxide, *World J. Gastroenterol.*, **14**, 3451-3456 (2005).
6) Y.F. Xiao, S.X. Liu, D.D. Wu, X. Chen and L.F. Ren, Inhibitory effect of arsenic trioxide on angiogenesis and expression of vascular endothelial growth factor in gastric cancer, *World J. Gastroenterol.*, **28**, 5780-5786 (2006).
7) I.C. Park, M.J. Park, S.H. Woo, H.C. Lee, S. An, H.S. Gwak, S.H. Lee, S.I. Hong, I.J. Bae, K.M. Seo and C.H. Rhee, Tetraarsenic oxide induces apoptosis in U937 leukemic cells through a reactive oxygen species-dependent pathway, *Int. J. Oncol.*, **23**, 943-948 (2003).
8) M.J. Park, I.C. Park, I.J. Bae, K.M. Seo, S.H. Lee, S.I. Hong, C.K. Eun, W. Zhang and C.H. Rhee, Tetraarsenic oxide, a novel orally administrable angiogenesis inhibitor, *Int. J. Oncol.*, **22**, 1271-1276 (2003).
9) J.L. Au, D. Li, Y. Gan, X. Gao, A.L. Johnson, J. Johnston, N.J. Millenbaugh, S.H. Jang, H.J. Kuh, C.T. Chen and M.G. Wientjes, Pharmacodynamics of immediate and delayed effects of paclitaxel: role of slow apoptosis and intracellular drug retention, *Cancer Res.*, **15**, 2141-2148 (1998).
10) T.C. Chou and P. Talalay, Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors, *Adv. Enzyme Regul.*, **22**, 27-55 (1984).
11) B. Kong, S. Huang, W. Wang, D. Ma, X. Qu, J. Jiang, X. Yang, Y. Zhang, B. Wang, B. Cui and Q. Yang, Arsenic trioxide induces apoptosis in cisplatin-sensitive and -resistant ovarian cancer cell lines, *Int. J. Gynecol. Cancer.*, **15**, 872-877 (2005).
12) W.M. Cheung, P.W. Chu and Y.L. Kwong, Effects of arsenic trioxide on the cellular proliferation, apoptosis and differentiation of human neuroblastoma cells, *Cancer Lett.*, **8**, 122-128 (2007).
13) M.T. Park, Y.H. Kang, I.C. Park, C.H. Kim, Y.S. Lee, H.Y. Chung and S.J. Lee, Combination treatment with arsenic trioxide and phytosphingosine enhances apoptotic cell death in arsenic trioxide-resistant cancer cells, *Mol. Cancer Ther.*, **6**, 82-92 (2007).
14) H.O. Jin, S.I. Yoon, S.K. Seo, H.C. Lee, S.H. Woo, D.H. Yoo, S.J. Lee, T.B. Choe, S. An, T.J. Kwon, J.I. Kim, M.J. Park, S.I. Hong, I.C. Park and C.H. Rhee, Synergistic induction of apoptosis by sulindac and arsenic trioxide in human lung cancer A549 cells via reactive oxygen species-dependent down-regulation of survivin, *Biochem. Pharmacol.*, **15**, 1228-1236 (2006).
15) L.M. Lin, B.X. Li, J.B. Xiao, D.H. Lin and B.F. Yang, Synergistic effect of all-trans-retinoic acid and arsenic trioxide on growth inhibition and apoptosis in human hepatoma, breast cancer, and lung cancer cells in vitro, *World J. Gastroenterol.*, **28**, 5633-5637 (2005).