Synergistic interaction between tetra-arsenic oxide and paclitaxel in human cancer cells *in vitro*

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Abstract. Similar to arsenic trioxide (As_2O_3), tetra-arsenic oxide (As_4O_6, TAO) has shown anti-proliferative and apoptosis-inducing effects against human leukemic and solid tumor cells. In order to assess the increase in efficacy, we evaluated the combinatory interaction of TAO combined with paclitaxel, 5-FU or cisplatin and studied its mechanism of action in the cell lines of human gastric, cervix and head and neck tumors. Two agents were combined at equitoxic ratios based on the IC_{50} of each drug. Efficacy improvement was evaluated using a combination index and isobologram at 50% inhibition level. Apoptosis induction and expression of apoptosis-related proteins was determined and the effect on microtubule polymerization was monitored. TAO combined with paclitaxel showed synergistic interaction in all three of gastric, cervix and head and neck cancer cell lines. On the other hand, TAO when combined with 5-FU or cisplatin showed an antagonistic interaction in head and neck or cervix cancer cell lines, respectively. Simultaneous treatment with TAO with paclitaxel resulted in an increased percentage of apoptotic cells and a significant increase in PARP cleavage and caspase-3 activation in the gastric and cervix cancer cells compared to TAO alone as well as the antagonistic groups (TAO with 5-FU or cisplatin). TAO suppressed the tubulin polymerization in the presence and absence of paclitaxel in a concentration-dependent manner, suggesting mitotic catastrophe as a potential mechanism of the synergism with paclitaxel. Overall, the present study suggests that TAO may have a greater potential as an anti-cancer agent against human gastric, cervix and head and neck tumors, in combination with paclitaxel. The synergistic interaction with paclitaxel may be associated with increased apoptosis via inhibition of paclitaxel-induced tubulin polymerization. Further detailed studies of combinatory mechanisms and evaluation using *in vivo* models are warranted.

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Introduction

Arsenic compounds have been used as therapeutic agents for rheumatism, syphilis and epilepsy since ancient times (1). Arsenic trioxide (ATO, Trisenox) has been used for the treatment of acute promyelocytic leukemia (APL) since 2000 and clinical trials for hepatocellular carcinoma and melanoma are now underway (2,3). Arsenic sulfide also recently showed an anti-cancer effect against APL (4). Currently, other arsenic anti-cancer agents such as tetra-arsenic oxide [As_4O_6, 2,4,6,8,9,10-Hexaoxa-1,3,5,7-tetraarsatricyclo(3.3.1.1^{3,7}) decane, TAO] and sodium meta arsenite are currently being developed (5-7).

Previous studies have shown that ATO induces apoptosis not only in human leukemic cells but also in human solid tumor cells (8-12). Activation of capase-3 following down-regulation of bcl-2 and degradation of PML-RARα protein was shown in the leukemic cancer cell lines (13,14). In the solid tumor cells, the production of reactive oxygen species (ROS), loss of the mitochondrial membrane potential and caspase-3 activation were shown (15). ATO also showed a possibility of a synergistic interaction with sulindac, which is attributable to ROS-dependent regulation of p53 and survivin (16).

TAO has shown anti-proliferative and apoptosis-inducing effects and ROS production, leading to apoptosis is thought to be the major mechanism of the anti-cancer effect in leukemic cells (6). TAO and other arsenic compounds also showed potent activity against human solid tumors, such as prostate and cervix cancers (7,17). Similar to ATO, efficacy of TAO may be improved via combination with other chemotherapeutic agents (16,18,19). TAO also showed an inhibitory effect on tube formation of endothelial cells *in vitro* and an anti-angiogenic effect in a rat model (20). These data testify to the potential of TAO against human solid tumors.

In the present study, we evaluated the effect of TAO in human gastric, cervix and head and neck tumor cells when in combination with paclitaxel, 5-FU or cisplatin, which are agents commonly used to treat these tumor types in the clinical setting. TAO showed the most synergistic interaction with

paclitaxel, hence we measured the levels of apoptosis-related proteins and apoptosis induction to better understand the synergistic mechanisms. We further evaluated the effect of TAO on paclitaxel-induced microtubule polymerization and confirmed its reversal effect on polymerization.

Materials and methods

Cell culture. Human gastric (SNU-484, AGS and SNU-216), cervix (SiHa, CaSki and ME-180) and head and neck (FaDu) cancer cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea). Two head and neck cancer cell lines (PCI-1 and AMC-HN-4) were kindly provided by Seoul National University (Seoul, Korea) and Asan Medical Center (Seoul, Korea), respectively. These cell lines were maintained using appropriate media such as RPMI-1640, EMEM and DMEM (Gibco BRL, Grand Island, NE) supplemented with 10% heat-inactivated FBS (Welgene Inc., Daegu, Korea), penicillin 100 U/ml (Sigma Chemical Co., St. Louis, MO) and streptomycin 100 μg/ml (Sigma Chemical Co.) in a humidified 5% CO_2 atmosphere at 37˚C.

Reagents. Tetra-arsenic oxide (As_4O_6) was obtained from Chonjisan (Seoul, Korea). Cisplatin was kindly provided by the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program at the National Cancer Institute (Bethesda, MD). Paclitaxel was donated by the Hanmi Research Center Co., Ltd. (Giheung, Korea). The other reagents were purchased from Sigma Chemical Co. unless otherwise noted. Tetra-arsenic oxide was dissolved in 1 N NaOH, whereas the other agents were dissolved in dimethyl sulfoxide (DMSO) and both further diluted with PBS as needed.

Cytotoxicity assay. The sulforhodamine B (SRB) assay was performed, as previously described (21). Cells in the log phase of growth were harvested and plated on 96-well plates at a predetermined density (1000-3000 cells/well). After 24 h of incubation, the cells were exposed to drug-containing media for 72 h. The cells were then fixed with 10% tri-chloroacetic acid (TCA) and stained with 0.4% SRB solution for 10 min. After washing, the protein-bound dye was extracted with 10 mM buffered Tris (Amresco, Solon, OH) and the optical absorbance was measured.

Western blot analysis. After drug exposure, cells were harvested, washed with ice-cold PBS and lysed in lysis buffer supplemented with protease inhibitors. Protein concentrations were measured by Bradford assay. Lysate was separated on SDS-PAGE gels (12% of acrylamide) and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Richmond, CA). After blocking using 5% skimmed milk, the membranes were incubated with primary antibodies. The following primary antibodies were used at the dilution indicated in the manufacturer's manual. a) Anti-PARP antibody (BD Transduction Laboratories, Lexington, KY, b) anti-cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, MA), c) anti-p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), d) anti-Bax antibody (Santa Cruz Biotechnology), e) anti-ß-actin antibody (Santa Cruz Biotechnology). After washing with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and immunoblots were visualized by enhanced chemi-luminescence (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's protocol. The cytosolic fractions isolated from the cells were used for Western blot analysis of cytochrome-c as previously described (22,23). Cytochrome-c protein was detected using anti-cytochrome-c antibody (Santa Cruz Biotechnology).

Apoptosis detection assay. Apoptosis was measured using an annexin V fluorescein isothiocyanate (FITC) apoptosis detection kit I (BD Pharmingen, San Diego, CA). After drug exposure, 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 a FACSCalibur and data were acquired using Cell Quest software (Becton-Dickinson, San Jose, CA).

Tubulin polymerization assay and staining. Mouse embryonic fibroblasts were generously provided by S.K. Hanks (Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN). For tubulin staining, mouse embryonic fibroblast cells were incubated for 24 h on a coverslip. After drug exposure for 24, 48 and 72 h, cells were washed with PBS and stained with anti-ß-tubulin antibody (Sigma Chemical Co.) at 4˚C for 1 h. After the incubation, slides were washed with PBS and stained with Alexa-488-conjugated anti-mouse IgG antibody (Invitrogen, Carlsbad, CA). Slides were washed with PBS and then examined using the Leica Confocal TCS 4D Laser Scanning Microscope (Leica, Nussuloch, Germany).

For the tubulin polymerization assay, the tubulin polymerization assay kit was used according to the manufacturer's protocol (Cytoskeleton, Inc., Denver, Co.). Drug solution diluted with general tubulin buffer (80 mM PIPES, 2 mM $MgCl_2$, 0.5 mM EGTA) was loaded into the 96-well plate and then, incubated at 37˚C for 2 min. Tubulin was defrosted and diluted with ice-cold tubulin polymerization buffer (general tubulin buffer containing 15% glycerol and 1 mM of GTP). The diluted protein was then immediately loaded into the pre-warmed 96-well plate using a multi-channel pipette and the polymerization reaction was initiated. Tubulin polymerization was monitored spectrophotometrically by the change in absorbance at 340 nm. The observance was measured at 1 min intervals for 30 min at 37˚C.

Data analysis. Changes in cell growth (% of cell survival) were calculated using (Eq. 1). The median effect model was used to analyse the cell survival data (Eq. 2).

$$\% \ Cell \ survival = \frac{mean \ absorbance \ of \ treated \ cells}{mean \ absorbance \ of \ control \ cells} \quad (Eq. 1)$$

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

where (D) is the drug concentration, K_d is the concentration of drug that produces a 50% reduction of the maximum

Table I. Anti-proliferative activity of tetra-arsenic oxide in comparison with paclitaxel, 5-FU and cisplatin in human gastric, cervix and head and neck cancer cell lines.

	Cell line	Paclitaxel (nM)	5-FU (μM)	Cisplatin (μM)	TAO (μM)[a]
Gastric cancer	SNU-484	4.86±1.01	15.5±2.05	64.9±7.02	0.94±0.03
	AGS	8.07±2.52	3.44±0.31	14.7±0.95	0.68±0.12
	SNU-216	5.83±0.50	7.54±1.89	6.70±2.01	0.76±0.40
Average ± SD		6.25±1.64	8.82±6.13	28.7±31.5	0.79±0.13
Cervix cancer	SiHa	364±310	153±54.7	22.6±1.35	2.20±1.11
	CaSki	12.1±3.30	3.60±0.60	15.9±1.18	0.47±0.004
	ME-180	4.25±1.12	2.10±0.49	2.55±1.17	1.52±0.20
Average ± SD		126±205	52.9±86.6	13.6±10.2	1.39±0.87
Head and neck cancer	PCI-1	11.7±2.47	3.29±1.31	4.49±0.77	1.13±0.18
	AMC-HN-4	1.38±0.19	7.58±1.10	4.38±1.63	0.74±0.12
	FaDu	3.58±0.77	8.75±0.61	14.3±1.54	1.03±0.21
Average ± SD		5.55±5.43	6.54±2.87	7.72±5.69	0.96±0.20
Total average ± SD		46.1±119	22.7±49.0	16.7±19.2	1.02±0.52

The median effect model (Eq. 2) was fitted to the dose-response data. The inhibitory concentration (IC$_{50}$) was calculated as a value to the absolute 50% reduction in survival. [a]TAO, tetra-arsenic oxide.

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 the control (i.e., K_d = IC$_{50}$ when R = 0).

The combination index (CI) was calculated using the following equation (Eq. 3),

$$CI = \frac{(D)_A}{(D_{50})_A} + \frac{(D)_B}{(D_{50})_B} + \alpha \frac{(D)_A (D)_B}{(D_{50}) (D_{50})_B} \qquad \text{(Eq. 3)}$$

where $(D_{50})_A$ and $(D_{50})_B$ are the concentrations of drug A or B alone giving an 50% reduction in cell viability compared to a control. $(D)_A$ and $(D)_B$ are the concentration of drug A and B in combination producing an 50% reduction in cell viability compared to a control. α is 0 when A and B are mutually exclusive or 1 when they are mutually non-exclusive (24). CI <0.8 was considered synergistic, 0.8-1.2 was considered additive and >1.2 was considered an antagonistic interaction.

Isobologram analysis was performed using Steel and Peckham's model, as previously described (25,26). An isobologram consists of three iso-effect lines, which were constructed based on the dose-response curves of each agent. Mode I line was created under the assumption that the action mechanism between two agents was mutually exclusive. On the contrary, Mode II line was created based on non-exclusive interaction. When the coordinate of the combination dose expressed as a fraction of IC$_{50}$ located below or above to the additivity envelope (area surrounded by mode I and mode II lines), a synergistic or antagonistic interaction was assigned for the combination, respectively.

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 TAO. The anti-proliferative activity of TAO was determined after 72 h of continuous exposure and compared to that of 5-FU, paclitaxel and cisplatin. Paclitaxel showed IC$_{50}$'s in the nM range, whereas the other drugs including TAO in the μM range (Table I). In gastric cancer cells, TAO showed a 10- to 30-fold lower IC$_{50}$ compared to 5-FU or cisplatin based on the average IC$_{50}$ in three cell lines tested i.e., 0.79 vs. 8.82 or 28.7 μM, respectively. The lower IC$_{50}$ of TAO also was shown in cervix cancer cell lines, with a 10- to 40-fold difference, but to a lesser extent than the 10-fold difference in head and neck cancer cell lines. These results indicate the higher potency of TAO in gastric and cervical cancers.

The average IC$_{50}$ of TAO was 0.79, 1.39 and 0.96 μM against gastric, cervix and head and neck cancer cell lines, respectively (Table I), indicating a relative resistance in cervix cancer cell lines compared to the gastric and head and neck. Nonetheless, TAO showed a similar IC$_{50}$ among the tumor types tested, i.e., 0.79 to 1.39 μM, whereas the other agents showed a wide range of IC$_{50}$'s (Table I). These data suggest similar activity of TAO against these gastric, cervix and head and neck tumors in comparison with other agents.

Synergy evaluation of TAO-containing combination. The synergistic interactions of TAO with 5-FU, paclitaxel or cisplatin were examined in cell lines of human gastric, cervix and head and neck tumors. Each agent was combined at an equitoxic ratio based on IC$_{50}$. TAO with paclitaxel showed a synergistic interaction with CIs of 0.55, 0.55 and 0.73 in gastric, cervix and head and neck cancer cell lines, respectively (Table II). The synergistic interaction was obtained in all

Table II. Combination index (CI) of tetra-arsenic oxide when combined with paclitaxel, 5-FU and cisplatin in human gastric, cervix and head and neck cancer cell lines.

	Cell line	TAO[a] + PTX[b]	TAO[a] + 5-FU	TAO[a] + Cis[c]
Gastric cancer	SNU-484	0.21±0.02	1.32±0.05	0.31±0.002
	AGS	0.73±0.03	1.12±0.05	0.56±0.04
	SNU-216	0.73±0.18	0.40±0.03	2.47±0.26
Average ± SD		0.55±0.30	0.94±0.48	1.11±1.18
Cervix cancer	SiHa	0.54±0.13	0.38±0.15	1.66±0.20
	CaSki	0.50±0.06	0.68±0.05	0.92±0.04
	ME-180	0.62±0.03	1.15±0.09	0.88±0.06
Average ± SD		0.55±0.06	0.73±0.38	1.15±0.43
Head and neck cancer	PCI-1	0.18±0.01	1.57±0.14	0.22±0.04
	AMC-HN-4	0.88±0.03	1.15±0.02	0.95±0.05
	FaDu	1.14±0.10	1.11±0.33	1.29±0.02
Average ± SD		0.73±0.49	1.27±0.25	0.82±0.54
Total average ± SD		0.61±0.30	0.98±0.40	1.02±0.70

CI was calculated at the 50% growth inhibition levels with $\alpha = 0$ assuming two agents are mutually exclusive (Eq. 3). Drug treatments were given simultaneously for 72 h at equitoxic ratios. [a]TAO, tetra-arsenic oxide; [b]PTX, paclitaxel and [c]Cis, cisplatin.
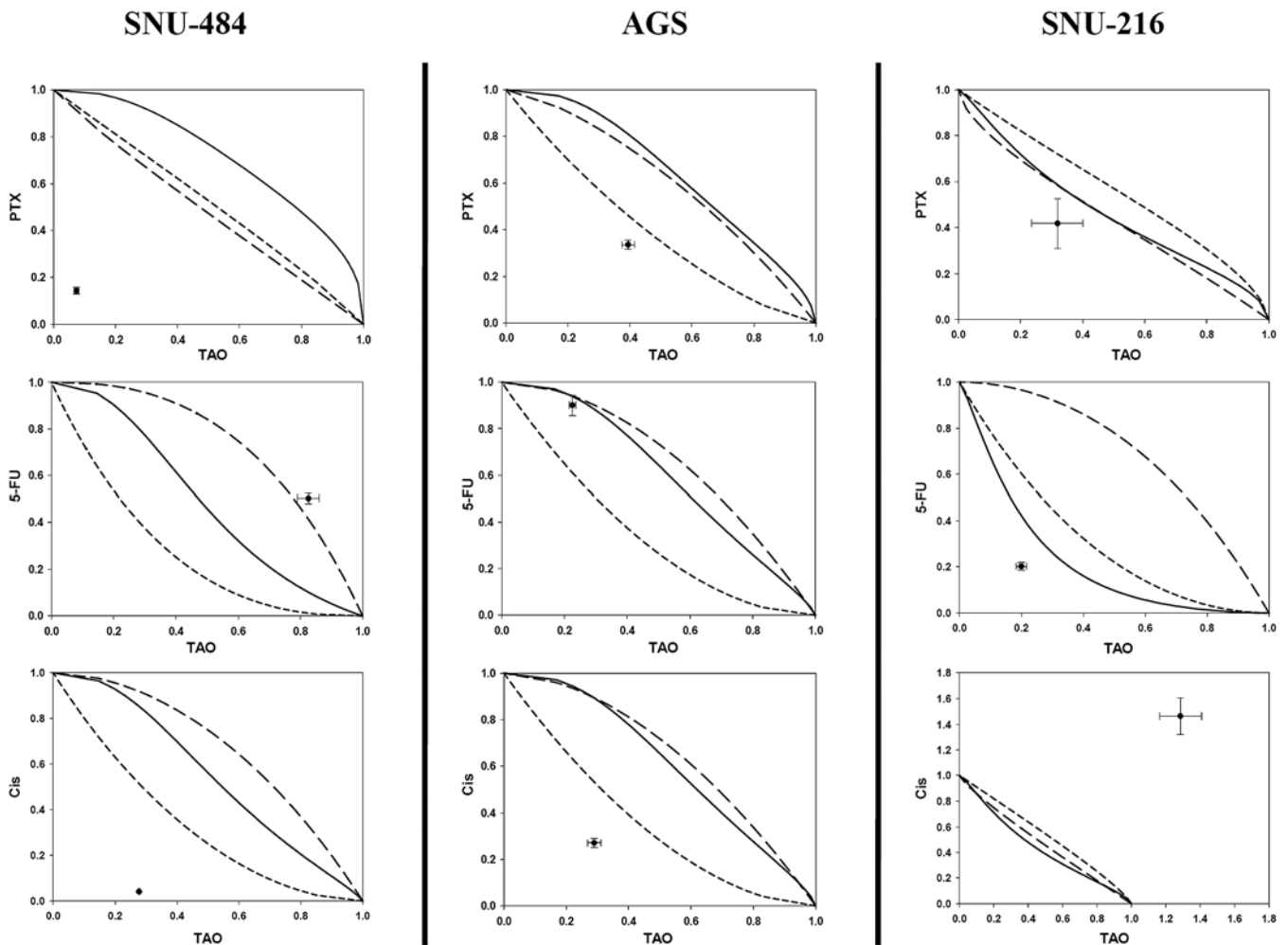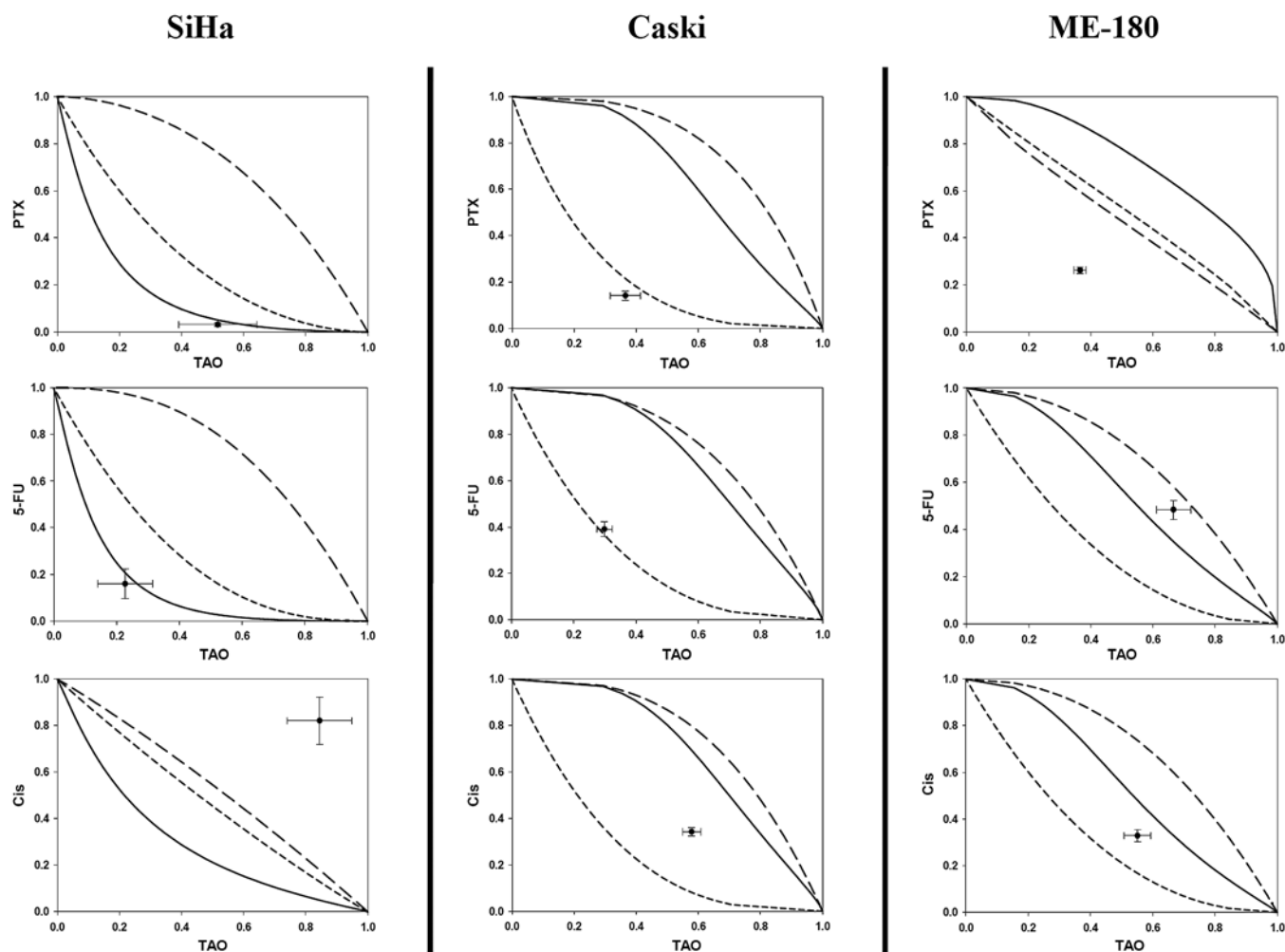

Figure 1. Combined interaction between tetra-arsenic oxide (TAO) and other anti-cancer agents (paclitaxel, 5-FU and cisplatin) in 3 human gastric cancer cell lines. PTX, paclitaxel; Cis, cisplatin; ——, MODE I; – –, MODE IIa; – – –, MODE IIb.

Figure 2. Combined interaction between tetra-arsenic oxide (TAO) and other anti-cancer agents (paclitaxel, 5-FU and cisplatin) in 3 human cervix cancer cell lines. PTX, paclitaxel; Cis, cisplatin; —, MODE I; - -, MODE IIa; - - -, MODE IIb.

three cell lines of gastric and cervix and in one out of three cell lines of head and neck cancer as shown with CI index (Table II) and isobologram analysis (Figs. 1-3). TAO combined with 5-FU showed an additive or synergistic interaction with CIs of 0.94 and 0.73 in gastric and cervix cancer cell lines, respectively. However, an additive to antagonistic interaction was seen in head and neck cell lines (Table II). The same results were obtained using isobologram analysis (Figs. 1-3). TAO with cisplatin showed an additive interaction in cervix and head and neck cancer cell lines (Table II). In gastric cancer cell lines, TAO with cisplatin showed a synergistic to antagonistic interaction depending on the cell line (Table II). Overall, in gastric cancer cell lines and in head and neck cancer cell lines, TAO only when combined with paclitaxel showed synergistic interaction. In cervix cancer cell lines, TAO combined with paclitaxel as well as 5-FU showed synergistic interaction.

Apoptosis induction of TAO alone or in combination. In order to evaluate the association of apoptotic pathway in synergistic interaction, the changes in expression level of apoptosis-related proteins in SNU-484 (gastric), SiHa (cervix) and PCI-1 (head and neck) cells were determined. In SNU-484 cells, single treatment with TAO showed PARP cleavage and caspase-3 activation (17 kDa). The TAO combined with paclitaxel (synergistic group) also showed PARP cleavage, whereas the TAO with 5-FU (antagonistic group) showed decreased PARP cleavage resulting from no active caspase-3 level. In the case of p53, single treatment of TAO showed no significant change and combination with 5-FU (antagonistic group) resulted in increased expression compared to combination with paclitaxel (synergistic group) (P-value <0.05), (Fig. 4B). No significant change in Bax level was noted in any of the treatment groups (Fig. 4B).

In SiHa cells, the TAO alone group showed weak PARP cleavage along with a subtle increase in active caspase-3 (19 kDa) (Fig. 5). The TAO with paclitaxel (synergistic group) showed prominent PARP cleavage and caspase-3 activation (19 kDa) compared to combination with cisplatin (antagonistic group) (P-value <0.05) (Fig. 5). In the case of p53, Bax and cytochrome-c, no significant change was noted either between the negative control and TAO single treatment or TAO with paclitaxel (synergistic group) and TAO with cisplatin (antagonistic group) (Fig. 5B).

In PCI-1 cells, the TAO alone group showed clear PARP cleavage and an increase in active caspase-3 (19 kDa) expression compared to the untreated group (Fig. 6A). Expression of cleaved PARP, activated caspase-3 (19 kDa),
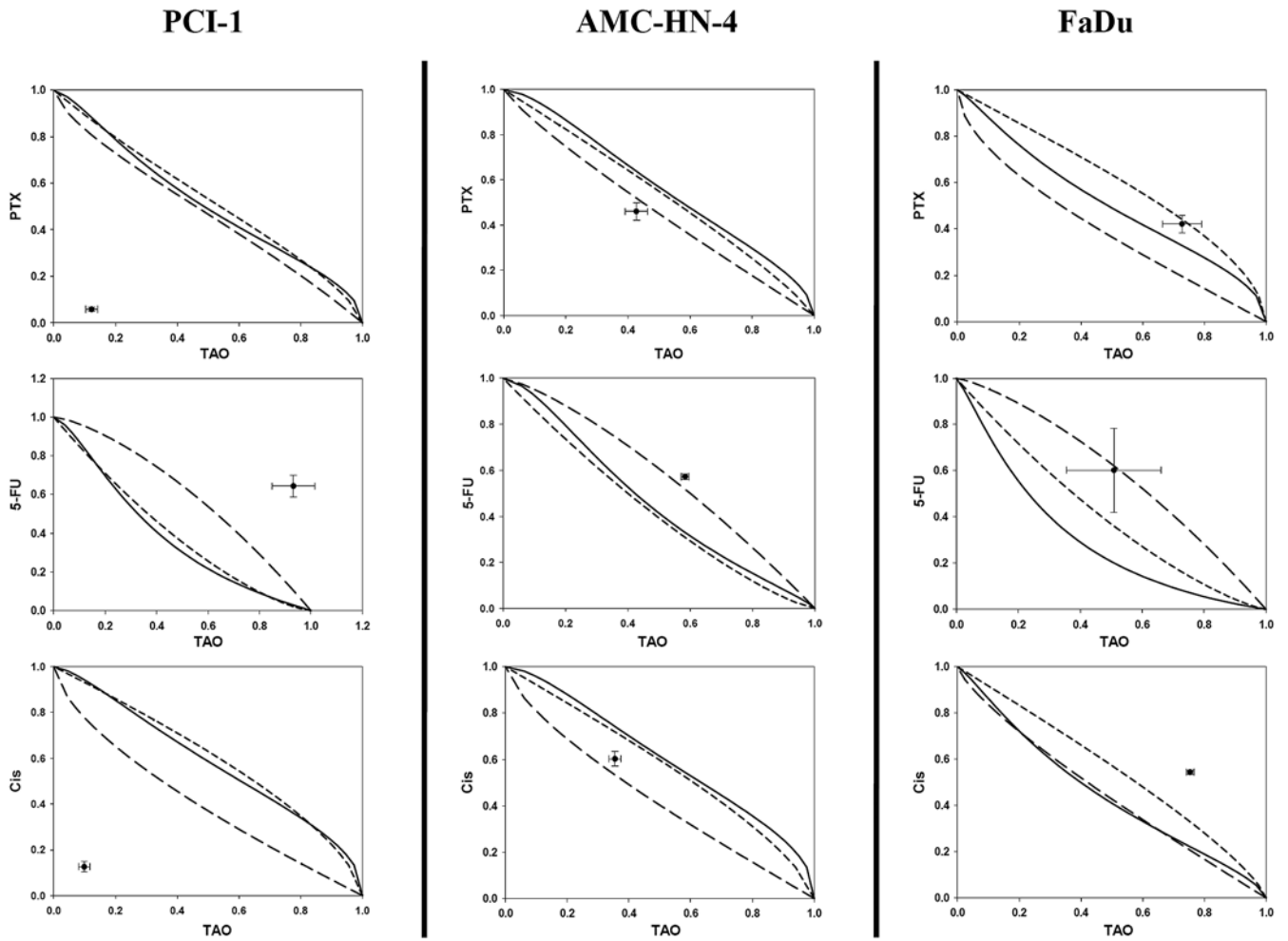
Figure 3. Combined interaction between tetra-arsenic oxide (TAO) and other anti-cancer agents (paclitaxel, 5-FU and cisplatin) in 3 human head and neck cancer cell lines. PTX, paclitaxel; Cis, cisplatin; —, MODE I; --, MODE IIa; ---, MODE IIb.
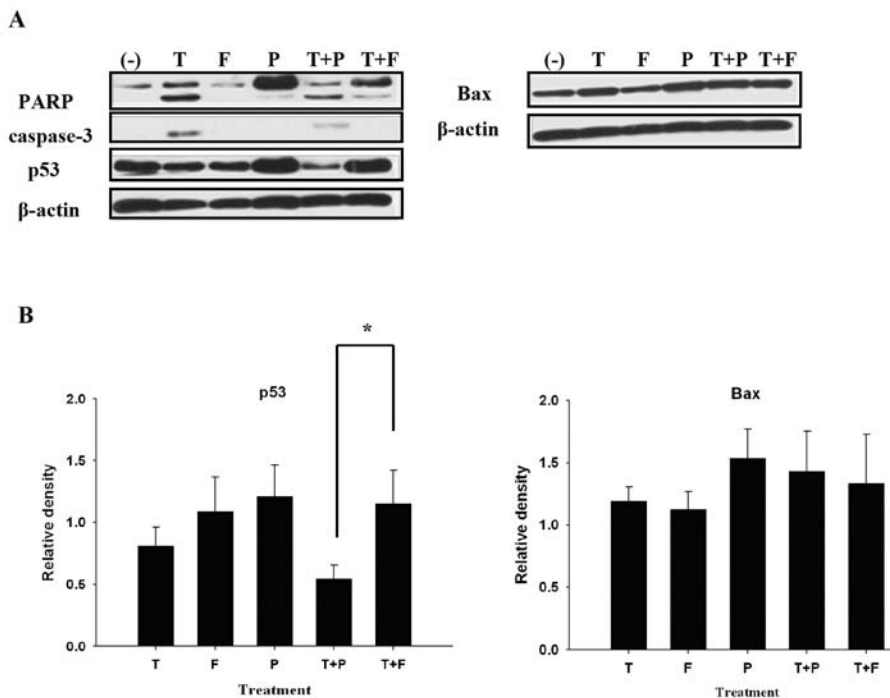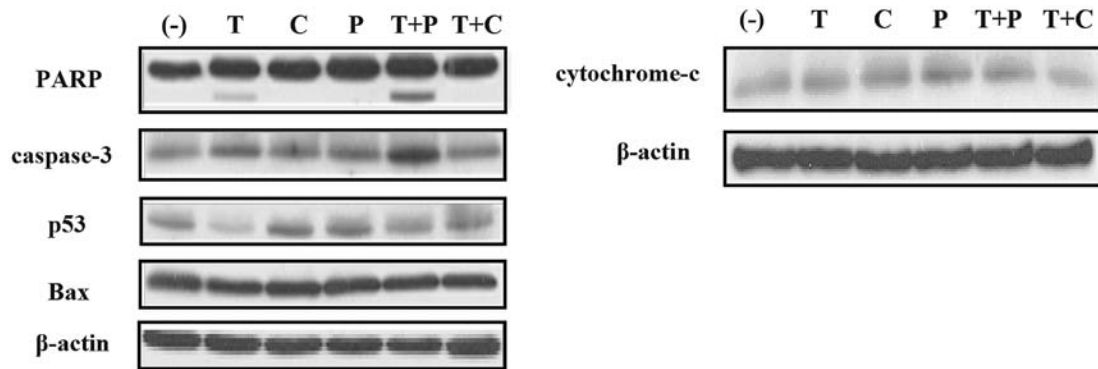

Figure 4. SNU-484 cells were exposed to tetra-arsenic oxide (1 μM), 5-FU (10 μM) and paclitaxel (0.01 μM) either alone or in combination for 12 h. PARP, active caspase-3, p53 and Bax were detected by Western blot analysis. (A) Representative gel image. (B) Densitometry analysis of p53 and Bax levels. (-), Untreated control; T, tetra-arsenic oxide; F, 5-FU and P, paclitaxel. Densitometry data were expressed as a relative density to a negative control. *P<0.05.
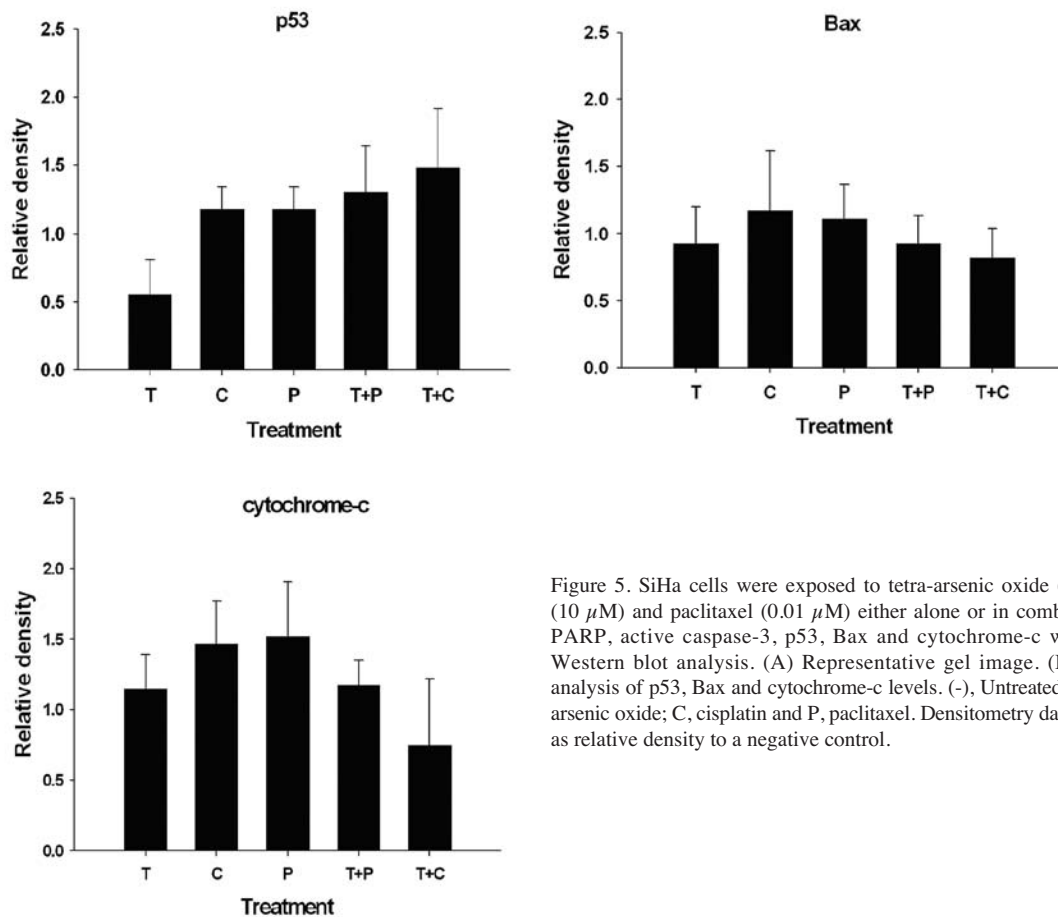
Figure 5. SiHa cells were exposed to tetra-arsenic oxide (1 μM), cisplatin (10 μM) and paclitaxel (0.01 μM) either alone or in combination for 24 h. PARP, active caspase-3, p53, Bax and cytochrome-c were detected by Western blot analysis. (A) Representative gel image. (B) Densitometry analysis of p53, Bax and cytochrome-c levels. (-), Untreated control; T, tetra-arsenic oxide; C, cisplatin and P, paclitaxel. Densitometry data were expressed as relative density to a negative control.

p53 and Bax were similar among the drug-treated groups including the TAO with paclitaxel (synergistic group) and 5-FU (antagonistic group) (Fig. 6).

In order to investigate the contribution of apoptosis to the synergistic effect, the percentage of apoptotic cells was measured and compared between the synergistic and antagonistic groups in SNU-484 human gastric cancer cells. TAO at 10 μM induced apoptosis in 61.1% of cells and combination with 0.1 μM of paclitaxel (synergistic group), increased apoptotic cells to 74.7% (Fig. 7), indicating an increase in 13.6% (P<0.01). On the contrary, when TAO was combined with 100 μM of 5-FU (antagonistic group), the percentage of apoptotic cells decreased by 7.0% from TAO single treatment (P<0.05). Although paclitaxel (0.1 μM) or 5-FU (100 μM) showed similar levels of apoptotic induction when given alone (37.3, 39.6%, respectively), significant differences in the percentage of apoptotic cells were observed when combined with TAO. i.e., 74.7 vs. 54.1% (Fig. 7).

Effect of TAO on tubulin polymerization. In order to investigate the mechanism of synergistic interaction between
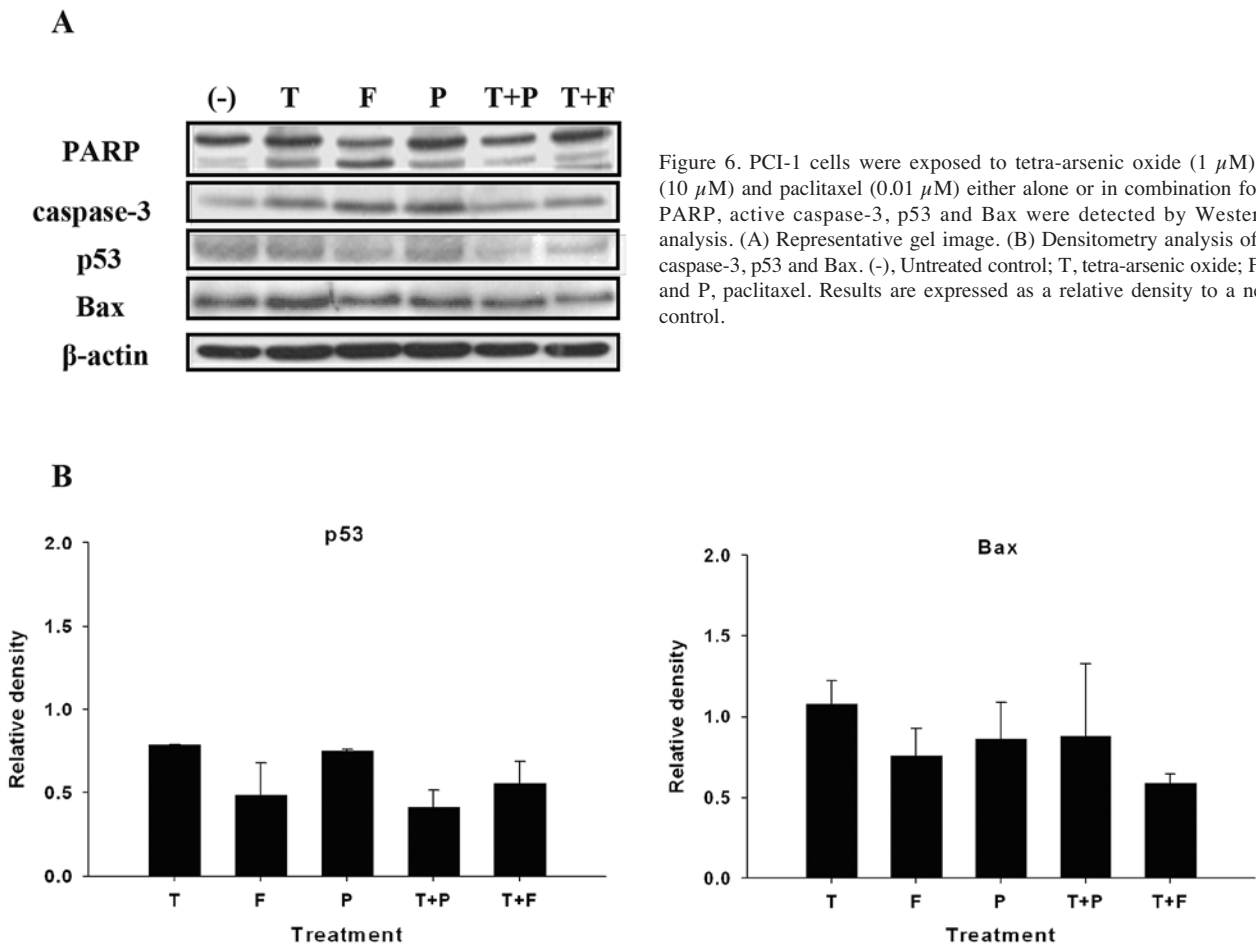
A

Figure 6. PCI-1 cells were exposed to tetra-arsenic oxide (1 μM), 5-FU (10 μM) and paclitaxel (0.01 μM) either alone or in combination for 12 h. PARP, active caspase-3, p53 and Bax were detected by Western blot analysis. (A) Representative gel image. (B) Densitometry analysis of active caspase-3, p53 and Bax. (-), Untreated control; T, tetra-arsenic oxide; F, 5-FU and P, paclitaxel. Results are expressed as a relative density to a negative control.

B

TAO and paclitaxel, we evaluated the effect of TAO either alone or in combination with paclitaxel using a tubulin staining assay as well as an *in vitro* microtubule polymerization assay. TAO treatment inhibited microtubule polymerization resulting in a decrease in the microtubule density compared to the untreated control (Fig. 8A). The microtubule polymerization assay also showed polymerization inhibition in a concentration-dependent manner, resulting in complete blockage of polymerization at 1000 μM of TAO (Fig. 8B). When combined with paclitaxel, TAO also inhibited paclitaxel-induced polymerization and showed a 50% decrease of maximum polymerization at 1000 μM of TAO (Fig. 8C). These data indicated that TAO antagonized the effect of paclitaxel on microtubule polymerization and this may be associated with the synergistic interaction between these two drugs.

Discussion

The present study evaluated the interaction between TAO and other cytotoxic agents in human gastric (3 cell lines), cervix (3 cell lines) and head and neck (3 cell lines) cancer cells. The IC$_{50}$ of TAO in these cell lines ranged from 0.47 to 2.20 μM and the average value (1.04 μM) was higher than that seen in U937 human leukemic cells (0.20 μM) as reported elsewhere (Table I) (6). This indicates that leukemic cells may be more sensitive to TAO compared to solid cancer cells. Among the solid tumor types tested in the present study, gastric cancer cell lines showed the highest sensitivity with

sub μM IC$_{50}$ values in all three cell lines (Table I). When compared to 5-FU and cisplatin, TAO showed greater cytotoxicity with less difference among tumor types, suggesting the potent anti-tumor activity of TAO against gastric, cervix and head and neck cancers. When dose-response curves were analyzed by using the median effect model (Eq. 2), the m (Hill-type coefficient) of TAO (1.99) was significantly higher compared to 5-FU (1.03) in gastric, cervix and head and neck cancer cells (P<0.05), indicating that TAO may require a smaller increase in dose intensity to induce greater anti-proliferative effect than 5-FU. On the other hand, cisplatin showed a similar m value (1.60) to TAO. Considering the fact that TAO showed minimal toxicity in phase I trial, these pharmacodynamic characteristics may add an additional benefit to TAO-based regimen in terms of potency and adverse reactions.

ROS production, cytochrome-c release and caspase-3 activation have been reported to be the mechanism of action for TAO-induced apoptosis (6). ATO also has been reported to induce loss of mitochondrial trans-membrane potential through ROS generation. This mitochondrial damage results in caspase-3 activation via up-regulation of p53 and sequential activation of Bax (8,10,15). We selected one cell line that showed a significant synergy between TAO and paclitaxel from each tumor type, then, determined changes in the level of caspase-3 activation and PARP cleavage along with p53, Bax and cytochrome-c release. No significant difference was observed in cells treated for different duration of time (data
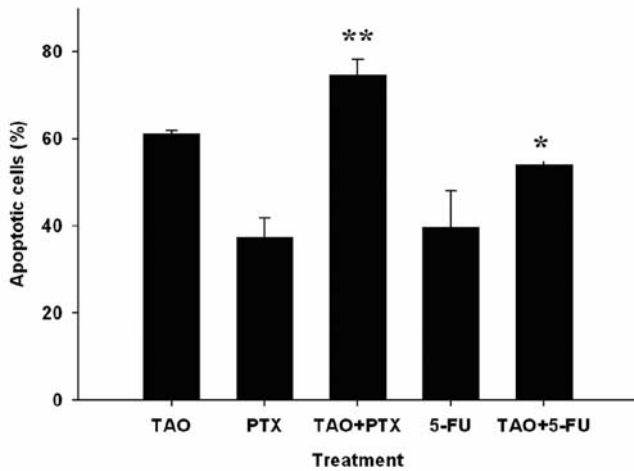
Figure 7. The percentage of apoptotic cells were determined in SNU-484 cells exposed to tetra-arsenic oxide (10 μM), paclitaxel (0.1 μM) and 5-FU (100 μM) either alone or in combination for 24 h. TAO, tetra-arsenicoxide and PTX, paclitaxel. **P<0.01, *P<0.05 when compared to TAO only.

not shown), hence, the data obtained after 12 or 24 h exposure are shown in Figs. 4, 5 and 6 (27). Caspase-3 activation was confirmed along with PARP cleavage in TAO-treated gastric, cervix and head and neck cells in our study (Figs. 4, 5 and 6). However, a significant increase was not observed either in p53 or Bax or cytochrome-c in any of the cell lines tested (Figs. 4B, 5B and 6B). These results suggest a different mechanism of TAO-induced apoptosis from ATO, e.g., a p53-independent pathway to apoptosis as reported for ATO in the neuroblastoma cell line (28).

Paclitaxel showed a stronger synergistic interaction with TAO than 5-FU or cisplatin in all three types of tumor cell lines tested in our study (Table II). In particular, the most synergistic interaction between paclitaxel and TAO was shown in SNU-484 gastric cancer cell line with a CI value of 0.21. This value indicates that when combined, only 11% of single treatment dose of TAO and paclitaxel can induce the same 50% inhibition in cell proliferation. Synergistic interaction may depend on the dose ratio of drugs to be combined, nonetheless,
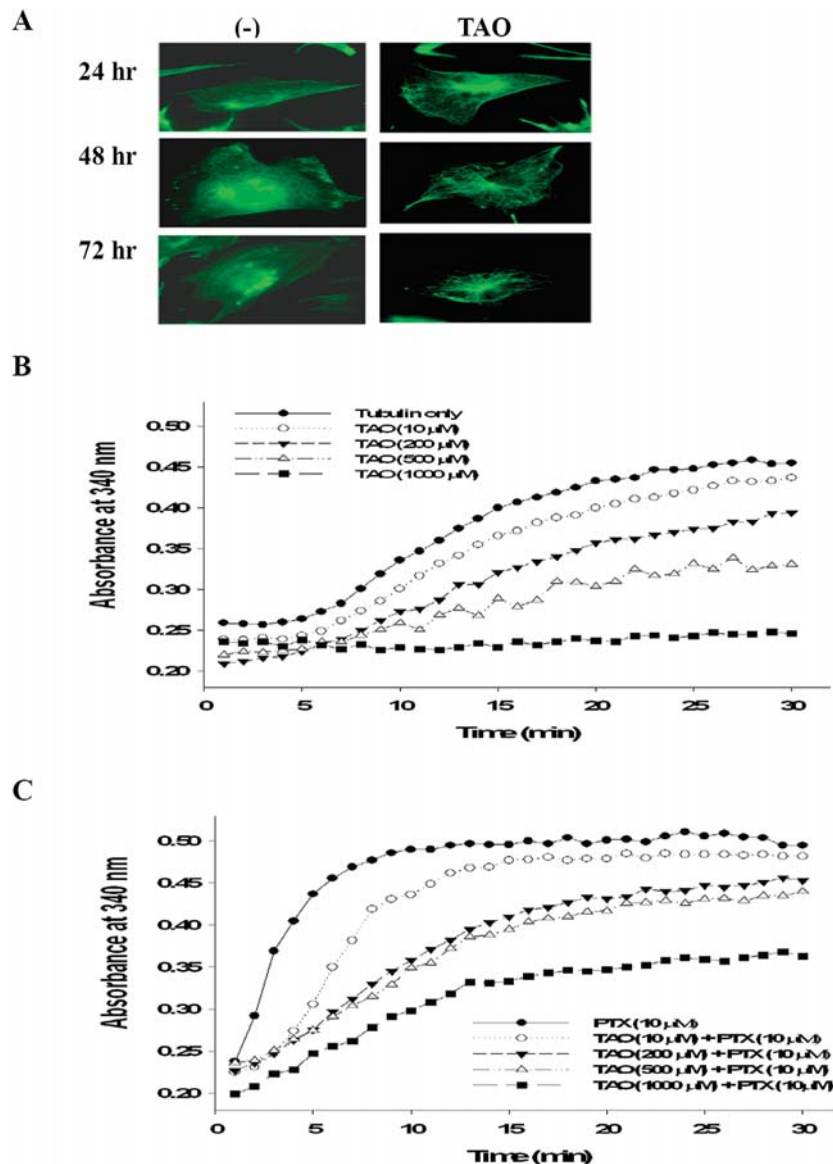

Figure 8. Effect of TAO on microtubule polymerization in the presence or absence of paclitaxel. (A) Effect of TAO (2 μM) on the organization of microtubule in mouse embryonic fibroblast cells. (-), Untreated control. (B) TAO inhibited tubulin polymerization in a concentration-dependent manner (10, 200, 500 and 1000 μM). (C) Paclitaxel (10 μM)-induced tubulin polymerization was inhibited by TAO in a concentration-dependent manner (10, 200, 500 and 1000 μM).

the use of equitoxic ratios are most frequently reported in the literature (16). We also used the equitoxic ratios in order to measure the interaction when both drugs can affect the cell viability to a similar extent.

The synergistic interaction between TAO and paclitaxel was confirmed by an increase in the percentage of apoptotic cells as determined through analysis of annexin (+) cells and it seemed to be related to caspase-dependent apoptotic induction as supported by an increased level of active caspase-3 when compared to antagonistic combination of TAO with 5-FU in SNU-484 cells (CI = 1.32) (Figs. 4 and 7). Combination interaction with 5-FU was dependent on cell lines, i.e., TAO with 5-FU showed synergy in SNU-216 cells with a CI of 0.4 (Table II), which was confirmed through increased apoptotic cell death (60.3%) compared against the TAO alone group (43.7%) or TAO with cisplatin (antagonistic group, CI = 2.47, 33.0%), (data not shown).

The increase in the percentage of apoptotic cells for synergistic combinations, nonetheless, appeared to be less than expected from CI index or from respective single treatment. We speculate that induction of apoptosis may not be the sole mechanism for the synergism. In recent studies, autophage (type II programmed cell death), instead of apoptosis was observed in malignant glioma cells as well as in breast cancer cells treated with ATO (2μM) and paclitaxel analogs (AGS115 and EFDAC), respectively (29,30). These data suggest that autophagic cell death may be another potential mechanism of synergistic interaction between TAO and paclitaxel. We can not rule out the possibility that the degree of apoptosis is not represented by the percentage of apoptotic cells seen at a single time point.

ATO has shown synergistic interaction with sulindac and phytosphingosine via ROS-dependent regulation of p53 and Bax in both leukemia and lung cancer cells. These signals induced activation of caspases and PARP, in sequence (16,19). In our study, increases in PARP cleavage and caspase-3 activation were also seen in the synergistic combination in gastric and cervix cancer cells (Figs. 4 and 5). However, no significant difference was noticed in the level of Bax or p53. The level of p53 expression was even higher in the antagonistic combination (TAO + 5-FU) than in the synergistic group (TAO + paclitaxel) in SNU-484 gastric cancer cells (Fig. 4B). This suggests a p53-independent mechanism for the synergistic interaction of TAO-based combination, which warrants further investigation.

In order to further investigate the mechanism of synergism between TAO and paclitaxel, we measured the effect of TAO on microtubule polymerization in the presence and absence of paclitaxel (Fig. 8). TAO suppressed tubulin polymerization in both conditions. The combination of paclitaxel with oxaliplatin also has shown synergistic interaction in colon and gastric cancer cells (31,32). The mechanism underlying this synergism was attributed to mitotic catastrophe associated with suppression of survivin expression (31). Since platinum compounds have shown an inhibitory activity on tubulin polymerization (33), synergism between paclitaxel and TAO may also act via a mitotic catastrophe mechanism. However, in contrast to our results, ATO has previously shown an antagonistic interaction with paclitaxel and it has been attributed to inhibition of tubulin polymerization by ATO (34).

Taken together, it can be speculated that the inhibition of paclitaxel-induced tubulin polymerization may result in either synergism or antagonism depending on the cellular context, which warrants further investigation.

Overall, our study demonstrated the pharmacodynamic interaction and its mechanism when TAO is given in combination with other conventional chemotherapeutic agents. Paclitaxel showed a synergistic interaction with TAO in gastric, cervix and head and neck cancer cell lines. We showed that this synergistic interaction may be related to caspase-3 and PARP-dependent, but p53-independent induction of apoptosis. Furthermore, TAO suppressed paclitaxel-induced microtubule polymerization to a similar extent as ATO. These data support the chemotherapeutic potential of TAO, especially in combination with paclitaxel against human gastric, cervix and head and neck cancer and warrants further evaluation using *in vivo* models as well as detailed studies on combinatory mechanisms including cell cycle effects.

Acknowledgements

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