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Synthetic isothiocyanate indole-3-ethyl isothiocyanate (homoITC) enhances sensitivity of human ovarian carcinoma cell lines A2780 and A2780/CP to cisplatin

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Isothiocyanates (ITCs), popular chemopreventive agents present in cruciferous vegetables, prove growth-inhibiting and apoptosis-inducing activities in cancer cell lines in vitro. Our study presents a new synthetic ITC derivate indol-3-ethyl isothiocyanate (homoITC) as an effective modulator of cellular proliferation and inducer of apoptosis with potential utility as an anticancer drug or a sensitizer to routinely used chemotherapeutic agent cisplatin (cis-Pt).

We analyzed the growth inhibitory effects of homoITC in the human ovarian carcinoma cell line A2780 and its cisplatin-resistant variant A2780/CP using MTT-test and its apoptosis-inducing properties by flow cytometry and caspase 3 activation. Combination index (CI) values from Calcusyn software were used to characterize the interactions of homoITC and cis-Pt as synergistic (CI<1), additive (CI=1), or antagonistic (CI>1). Significant synergistic effect in growth inhibition of homoITC (5 – 15 μM) and cis-Pt (2.5 – 10 μM) on A2780 parental cell line (CI from 0.42 to 0.85) was also observed on A2780/CP resistant subline (CI from 0.18 to 0.73) for 10-50 μM cis-Pt concentrations and the same concentrations of homoITC. Synergy in growth inhibition correlated with the potential of homoITC to stimulate apoptosis induced by cis-Pt.We conclude that homoITC may be worth of further studies assessing its value in the ovarian cancer treatment and elucidating mechanisms of its action.

Key words: ovarian carcinoma, isothiocyanate, cisplatin, growth inhibition, apoptosis

The anticancer effects of various chemopreventive agents [1-6]or their combinations with chemotherapeutic drugs [7-11] have been studied extensively.

Anticarcinogenic properties of hydrolytic products from specific glucosinolates - isothiocyanates (ITCs) were discovered more than 40 years ago [12] and consumption of ITCs and indoles found in cruciferous vegetables has been associated with reduced risk of cancer [13].

HomoITC is a structural homolog of 3-indolylmethyl isothiocyanate, a key intermediate of metabolic pathway to brassinin (3-(S-methyldithiocarbamoyl) aminomethyl indole) – an indole phytoalexin. Cruciferous vegetables synthesize brassinin and others phytoalexins, in contrast to glucosinolates, in response to diverse forms of stress/patogenes. Anticancer properties of brassinin were documented by Mehta et al. in 1995[14] Recently, Banerjee at al. [15] showed that anticancer activity of brassinin is mediated through the inhibition of indoleamine 2,3-dioxygenase, a pro-toleragenic enzyme that drives immune escape in cancer. Anticancer activity of new derivatives of natural indole phytoalexin were studied [7] and new analogs of indole phytoalexin 1-methoxyspirobrassinol methyl ether were designed as potential anticancer and antimicrobial agents [16]. An interest in indole compound led to preparation of new analogs of phytoalexins, the indoles produced from the indole glucosinolates are studied, among them especially indole-3-carbinol, a hydrolytic product of glucobrassicin [4].

Indole-3-methyl isothiocyanate, an intermediate of metabolic pathway to brassinin, is generated at hydrolysis of glucobrassicin [17], but is unstable and gives rise to indole-3-carbinol. Its instability makes impossible to study of anticancer activity of this indole ITC, therefore more stable structural analog – indole-3-ethyl isothiocyanate (homoITC) has been investigated. It is known that sulfhydryl groups readily react with ITCs to

give rise to dithiocarbamates. The conjugation is generally regarded as a detoxification process. The conjugation of ITCs with glutathione is the first step leading to formation of N-acetylcysteine derivatives (urinary metabolites). The reactivity of homoITC (alkyl neutral ITC) towards thiols is similar to reactivity of PEITC (unpublished data), and it has been expected that homoITC may also act as an antiproliferative agent. Recently, homoITC and its analogs were screened to evaluate their cytotoxicity against neuroblastoma cell and human ovarian SKOV-3 and OVCAR-3 cells [18, 19]. 7-methyl-indole-3-ethyl isothiocyanate had stronger cytotoxic effect then homoITC. Adding a methyl and tert-butyl carbamate group increased lipophilicity and cytotoxicity of new indole compound [20], but it could modify also its reactivity. It is known that conjugations of ITCs with thiols, especially glutathione, resulted in rapid and high accumulation of ITCs in cells [21]. Conjugation reactions of GSH with electrophiles, including some cytostatic drugs and ITCs are catalyzed by glutathione transferases. The GS-conjugates are exported out of cells by multidrug-resistance protein 1 and 2 (MRP1, MRP2) [22].

Platinum compounds are used in treatment of many cancers including ovarian carcinomas, where they compose basic components of the first-line chemotherapy. However, frequent development of resistance to platinum-based chemotherapy limits its use and stimulates the search for combination therapy aimed at circumventing or decreasing of platinum resistance [10, 23]

Cellular resistance to platinum can arise by multiple mechanisms related to GSH-based detoxification and metallothionein content, ABC transporters overexpression, an increase in DNA repair capacity, but also because of aberrant properties of signal transduction pathways involved in cell cycle control and apoptosis (for example p53 alterations, Bcl-2 expression, ERK activation) [24].

In this study we show combined synergistic effect of synthetic homoITC and cisplatin treatment in human ovarian adenocarcinoma cell line A2780 and its cisplatin-resistant subline A2780/CP in growth inhibition and induction of apoptosis.

Materials and methods

Reagents. (Indol-3-yl) ethylisothiocyanate (homoITC) was kindly provided by P. Kutschy from P. J. Šafárik University, Košice and synthesized as described [25]. Dimethyl sulfoxide (DMSO), RNA-se A, Fluorescein diacetate (FDA), propidium iodide (PI) and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Cisplatin (cis-Pt) was acquired from LACHEMA (Czech Republic).

Cell culture and treatment. The human ovarian carcinoma cell line A2780 and its cisplatin-resistant variant A2780/CP were routinely cultured in RPMI supplemented with 10% fetal calf serum, 2mM L-glutamine, 100 μg/ml penicillin and 50 μg/ml streptomycin in 5% CO_2 at 37 °C. The cultures were passaged twice a week after reaching cell density of 0.8 – 1.0 x 10^6 cells/ml. Cells were plated at 3-6 x 10^4 cells/cm^2 density on day before treatment. Cells were exposed to various concentrations of homoITC or cis-Pt for the respective time indicated. In combination, homoITC was always added 1 hour before cis-Pt treatment (based on our previous experiments showing that this time is sufficient to activate ITC inducible signaling pathways). Stock solution of homoITC was originally dissolved in DMSO, and an equal volume of DMSO (final concentration <0.02%) was added to the control cells.

Flow cytometric analysis of apoptosis. Apoptotic cell enumeration was done according to the amount of cells with the cell membrane impermeable for PI with low amount of fluorescein, or cells with sub-G_1 amount of DNA after detergent permeabilization. As for FDA/PI staining [26], both cell lines were collected as described above. Pooled cells were washed twice with cold PBS. Approximately 5×10^5 cells were resuspended in 400 μl of PBS/0.2% BSA containing 10 nM of FDA (from a 5 mM stock in DMSO) for 30 min at room temperature. Then cells were cooled and 4 μl of PI (1 mg/ml) was added. Finally, cells were analyzed using a Coulter Epics Altra flow cytometer.

Caspase-3 activation. For detection of activated caspase-3, a direct staining with the PE-conjugated antibody which preferentially recognizes the p17 fragment of the active caspase-3 was used. Cells were harvested by trypsinization, adjusted to 10^6 cells/ml, washed in PBS and fixed in 0.5% paraformaldehyde in PBS for 5 min on ice. After permeabilization with saponin solution (0.2% saponin in PBS containing 0.2% BSA), cells were labeled for 40 min at room temperature with 1 mg/ml of the PE rabbit anti-active caspase-3 antibody (BD Pharmingen). Control samples were prepared with non-reactive PE labeled anti-CD45 monoclonal antibody (Immunotech). After washing with PBS/0.2% BSA, cells were resuspended in PBS and analyzed using a Coulter Epics Altra flow cytometer.

Cell cycle analysis. This determination was based on the measurement of the DNA content of nuclei labeled with propidium iodide. For the flow cytometry analyses of the DNA cell cycle profile, cells were washed twice with cold PBS and resuspended in 0.05% Triton X-100 and 15 μl RNA-se A (10 mg/ml) for 20 min at 37 °C. Then cells were cooled and incubated on ice for at least 5 min before propidium iodide (50 μg/ml) was added. Finally, after 15 min the stained cells were analysed using a Coulter Epics Altra flow cytometer.

Flow cytometry measurements and data analysis. Coulter Epics Altra flow cytometer was equipped with 488 nm excitation laser and fluorescence emission was measured using bandpass filter set 525, 575, 610, 675 nm with respective photomultipliers FL1-FL4 required for fluorochrome used as follows: PI (FL2), FDA and PI (FL1, FL2), PE (FL2), cell cycle (log FL2 - sub G_1, lin FL3 – DNA cell cycle histogram, lin/peak FL4 – discrimination of doublets). Forward/side light scatter characteristic was used to exclude the cell debris from the analysis. For each analysis, 1×10^4 cells were required. Data were analysed with WinMDI version 2.8 software (J. Trotter, Scripps Research

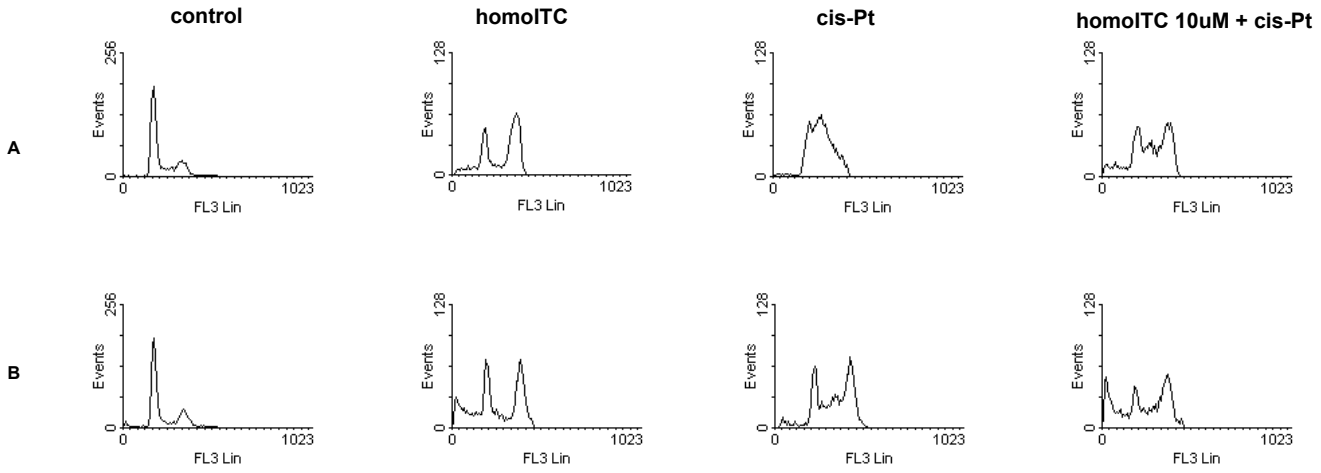


Fig. 1 Flow cytometry-based cell cycle analysis of homoITC, cis-Pt and their combination in A2780 (A) and A2780/CP (B) cells. Cells were exposed to DMSO (final concentration <0.02%) as a control and 10 μM homoITC or 10 μM cis-Pt for 24 h. For the combination effect, corresponding concentration of homoITC was added 1 h prior to administration of cis-Pt. The distribution of cells in G_0/G_1, S and G_2/M phase was evaluated with FCS Express de Novo software.

Institute, La Jolla, CA). The cell cycle calculations were performed with FCS Express (de Novo) software.

Cytotoxicity assay. Effect of homoITC, cis-Pt or their combination on survival of cells was determined by MTT assay [27]. Cells were seeded at (1-2) x 10^3 cell density in 96-well culture plates. Each dose of tested compounds (added it the volume of 50 μl) was tested in triplicate, and cytotoxicity curve was constructed from at least seven different concentrations. After 72 h, the cells were incubated with 50 μl of MTT (1 mg/ml) and left in the dark at 37 °C for an additional 4 h. Thereafter, medium was removed, the formazan crystals were dissolved in 200 μl of DMSO, and the absorbance was measured at 540 nm and 690 nm in Microplate reader (Dynatech Lab Inc., Chantilly, VA, USA). The concentration of drug that inhibited cell survival to 50% (IC_{50}) and combination index (CI) were determined by Calcusyn software (version 1.1, Biosoft, Ferguson, MO)

Calculation of combination effects. Combination effects of homoITC and cis-Pt on cell growth inhibition were calculated according to Chou et al. [28]. Briefly, for median-effect plots, log (fa/fu) was plotted against log (D), where D represents the concentration of each single compound alone or the mixture of both and fa and fu represent the affected and unaffected fraction, respectively, at each concentration D. Combination index (CI) was computed for every fraction affected. CI<1, CI=1 or CI>1 represent synergism, additivity or antagonism of combined drugs, respectively.

Results

Cell cycle changes. The effect of homoITC and cis-Pt on A2780 cell proliferation was analyzed based on the cell cycle distribution of PI stained cells. Fig. 1 shows DNA histograms of combined homoITC and cis-Pt treatment. HomoITC alone induced G2/M block in both A2780 and A2780/CP cell lines, while cis-Pt caused S-phase arrest, more advanced in parental cells (early S-phase). In combination, cells were exposed to homoITC (10 μM) 1 h prior to 24 h administration of cis-Pt (10 μM). In comparison to single cis-Pt treatment, decrease of proliferation in both tested cell lines was observed with concomitant increase of sub-G_1 population (apoptosis). We have found approximately 40% decrease of S-phase cells in A2780 and 25% decrease in A2780/CP cells. Interestingly, the chosen combination induced higher apoptosis in cisplatin-resistant A2780/CP cells.

HomoITC and cis-Pt induce apoptosis and necrosis. Three independent flow cytometry methods were used for evaluation of cell death induced by homoITC. The presence of apoptotic cells determined by the analysis of DNA fragmentation (sub-G_1 population) shown in Fig. 1 was confirmed by detection of activated caspase-3 (Fig. 2). This figure illustrates that the combination of homoITC and cis-Pt augmented activation of caspase-3 in both cell lines in comparison with single-compound treatment. This was more evident after 48h, where the percentage of cells positive on activated caspase-3 reached 36% for A2780 and 39% for A2780/CP cells.

The concentration-dependent effect of homoITC and cis-Pt on the apoptotic process in tested cell lines was quantified by FDA/PI staining. Fig. 3 depicts the percentage of apoptotic (FDA^-/PI^-) and necrotic (PI^+) cells after 24 h of treatment. In accordance with DNA analysis, these results demonstrate that homoITC alone induced increase of percentage of apoptotic cells and significantly increased cis-Pt – induced apoptosis after 24 h. The highest, more than additive accumulation

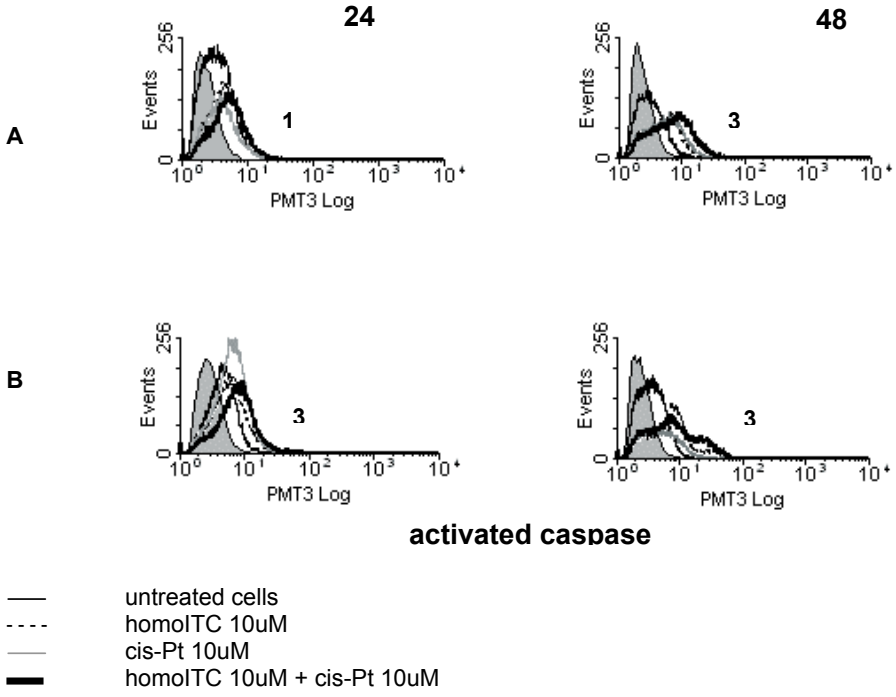


Fig. 2. Activation of caspase-3 by homoITC, cis-Pt and their combination in A2780 (A) and A2780/CP (B) cells. Cells were exposed to DMSO (final concentration <0.02%) as an untreated control (normal line) and 10 μM homoITC (dashed line) or 10 μM cis-Pt (grey line) for 24 h. For the combination effect(bold line), corresponding concentration of homoITC was added 1 h prior to administration of cis-Pt. Filled histograms represent negative controls prepared with non-reactive PE labeled anti-CD45 monoclonal antibody for each treatment.

of apoptotic population (approximately 45%) was found by combination of homoITC 15 μM and cis-Pt 10 μM for A2780 or cis-Pt 25 μM for A2780/CP cells (Fig. 3).

Quantification of combination effects. The method by Chou et al. [28] is a widely accepted approach to analyze synergistic, additive or antagonistic effects of two compounds. Important step of the procedure is to assess mutual exclusivity of the test compounds by generating median-effect plots, with log (fa/ fu) plotted against log (D). If the regression lines obtained for either compound alone and the mixture of both are parallel, those two drugs are mutually exclusive, i.e. they have the same target or mode of action. If the lines are not parallel, the tested compounds are mutually non-exclusive, i.e. they act independently or have different modes of action. As shown in Figure 4, the regression lines of homoITC, cis-Pt and their 1:1 mixture were not parallel, suggesting that homoITC and cis-Pt are mutually non-exclusive and inhibit cell proliferation by different mechanisms.

Anti-proliferative effects of homoITC and cis-Pt. Values of IC_{50} for homoITC and cis-Pt alone were determined from cell survival plots and were 6.2 ± 0.3 μM in A2780 and 8.5 ± 0.3 μM in A2780/CP for homoITC, and 1.4 ± 0.7 μM in A2780 and 9.1 ± 0.4 μM in A2780/CP for cis-Pt after 72 hour treatment.

To assess the effect of combination of homoITC and cis-Pt, cells were treated with different concentrations of homoITC and cis-Pt or both (Fig. 5). Combination index (CI) values (Calcusyn 1.1, Biosoft, 1996) were used to characterize the interactions as synergistic (<1), additive (=1), or antagonistic (>1). Significant synergistic effect of homoITC (5 – 15 μM) with cis-Pt (2.5 – 10 μM) on A2780 parental cell line (CI from 0.42 to 0.86) was observed also on A2780/CP resistant subline (CI from 0.18 to 0.73) for 10-50 μM cis-Pt concentrations. Dose reduction effects characterized by dose reduction index (DRI), which determines how many fold increase of each drug concentration (if used alone) is required for the same effect as is provided by the corresponding synergistic combination, have values as follows: 2.76 for homoITC and 2.07 for cis-Pt in A2780 cells and 4.3 for homoITC and 1.4 for cis-Pt in A2780/CP cells for affected fraction = 0.75.

Discussion

The clinical use of platinum compounds has influenced the chemotherapeutic management of many solid tumors and the prognosis for patients with ovarian cancer has improved significantly. Unfortunately, the effectiveness of platinum drugs in the treatment of cancer is hindered by intrinsic and acquired resistance and there is growing interest in searching for the means how this resistance phenotype could be circumvented [24, 29, 30]. One of the ways to enhance the efficacy of cisplatin-treatment in sensitive cancers and to overcome

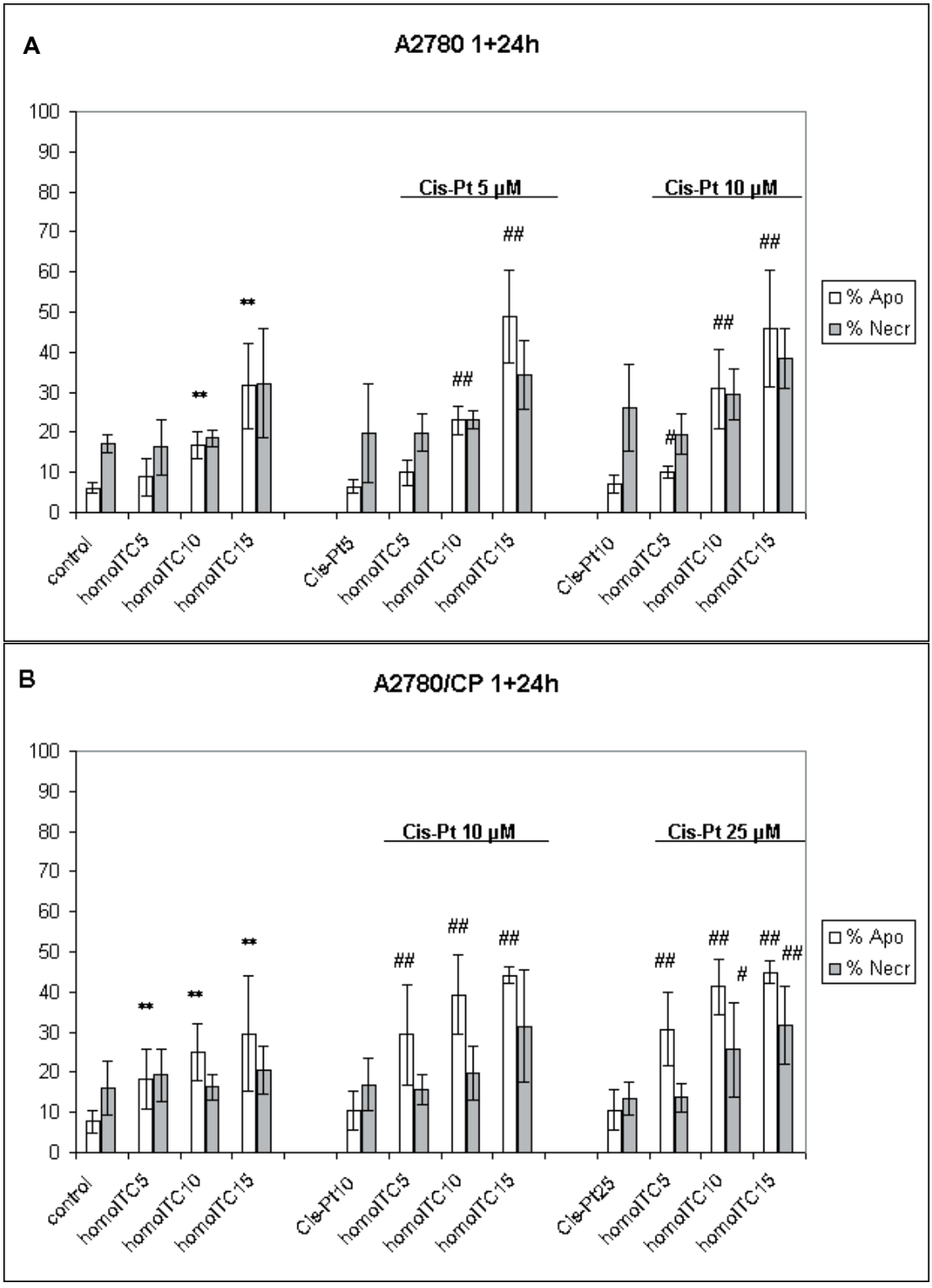


Fig. 3 Effect of homoITC, cis-Pt and their combination on apoptosis and necrosis induction in A2780 (A) and A2780/CP (B) cells. Cells were exposed to 5, 10 and 15 µM homoITC and two concentrations of cis-Pt: 5 or 10 µM (A), 10 or 25 µM (B) for 24 h. For the combination effect, corresponding concentrations of homoITC were added 1 h prior to administration of cis-Pt. Percentage of apoptotic (FDA^-/PI^-) and necrotic (PI^+) cells was depicted. The data represent results of 3 - 6 independent experiments. Statistical significance from the controls, *p<0.05, **p<0.01 and from corresponding cis-Pt – treated samples, #p<0.05, ##p<0.01.

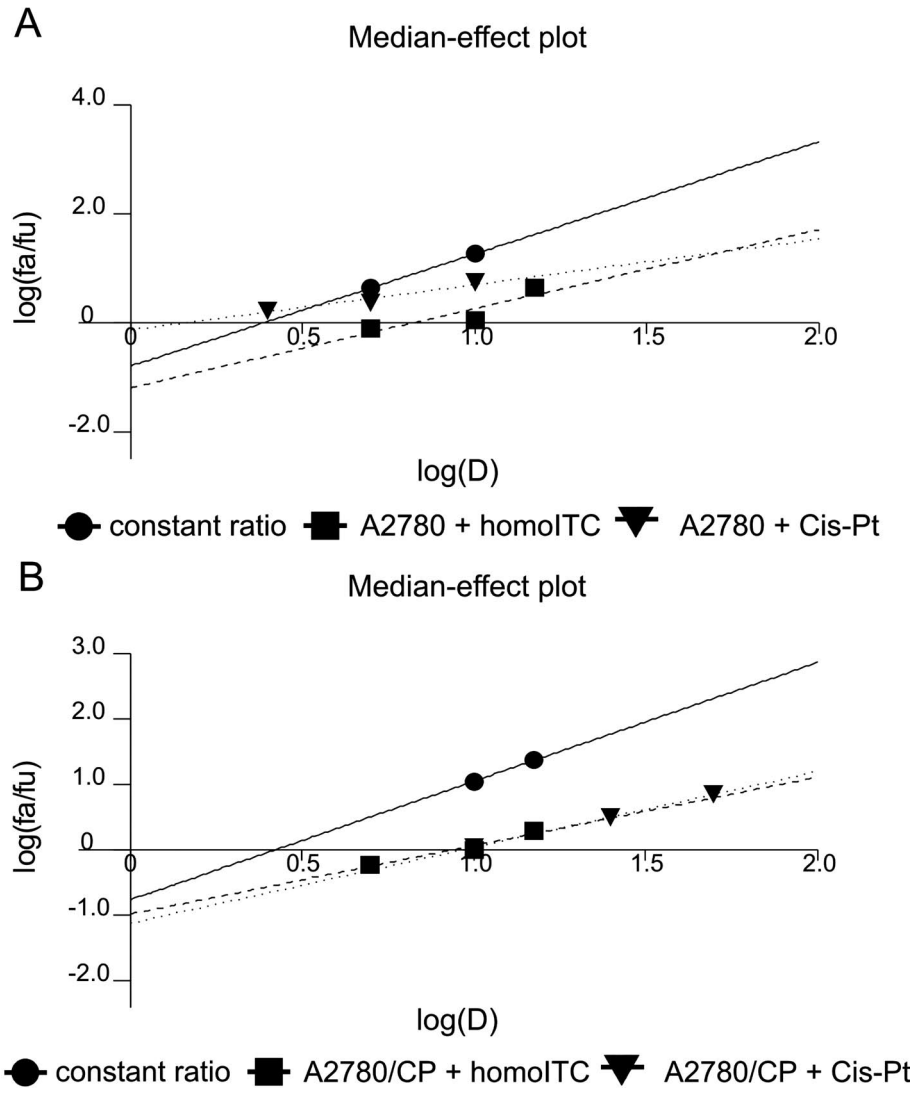


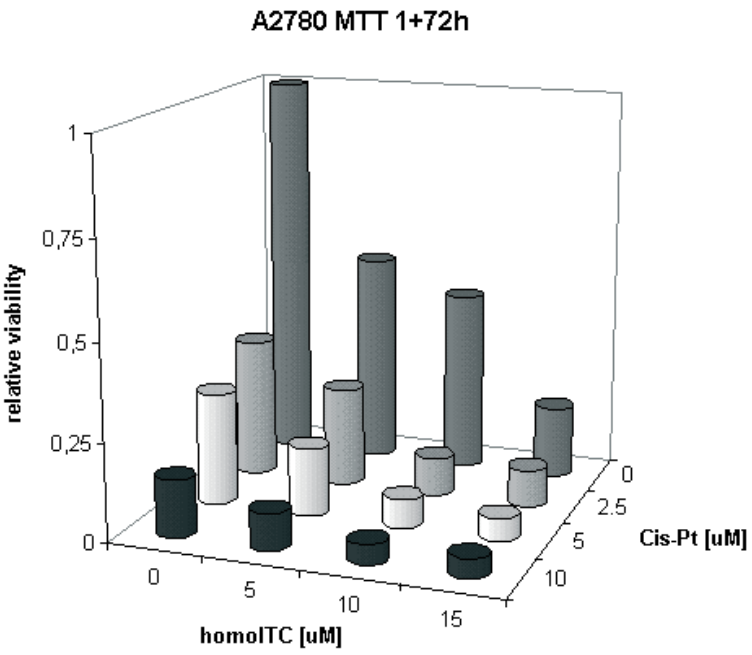
Fig. 4 Median-effect plot of A2780 (A) and A2780/CP (B) cells treated for 72 h with homoITC (▪),cis-Pt (▼), and a 1:1 mixture of homoITC and cis-Pt (■). Data were taken from one representative cell proliferation experiment. Log (fa/fu) was plotted against log (D), whereby fa and fu stand for affected fraction and unaffected fraction, respectively, and D represents the concentration.

drug resistance in unresponsive tumors, is to target involved signaling pathways with specific compounds in combination with cisplatin [31].

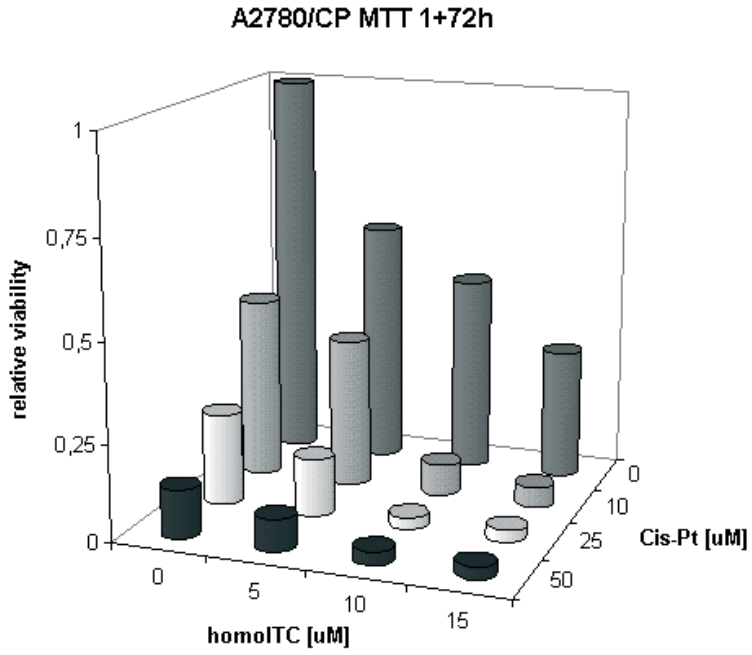
The antitumor activities of various ITCs have been reported recently including growth inhibiting properties in human ovarian [11, 19, 31, 32] and breast [33, 34] cancers, where also their anti-angiogenic and anti-metastatic effects have been described [35, 36].

Singh et al. [19] documented that methyl-indole-3-ethyl isothiocyanate had proapoptotic effects on human ovarian cancer cells SKOV-3 and OVCAR-3, which are resistant to several drugs, including cisplatin. This analog of indole ITC activates pro-apoptotic mitogen activated protein kinases (p38, SAP/JNK), downregulates pro-survival kinases and transcription factors (STAT-3, IKKα and NF-κB), promotes caspases-9, -8, -3 activation and regulates cell cycle progression.

This study presents effects of homoITC alone or in combination with cis-Pt on human ovarian carcinoma A2780 cell lines. Our data provide evidence that homoITC alone inhibited the cell growth of tested cell lines and augmented the cytotoxicity of cis-Pt in both A2780 and A2780/CP cells, inducing caspase-3 mediated apoptosis in vitro. In addition, the synergistic cytotoxic effect was observed in homoITC / cis-Pt -treated cell lines. In the combination experiments, the concentrations of homoITC and cis-Pt were chosen based on our data showing dose-dependent effects of homoITC



A2780	cis-Pt		
[mM]	2,5	5	10
homoITC	Combination index		
5	0,85	0,86	0,69
10	0,47	0,48	0,42
15	0,64	0,48	0,52



A2780/CP	cis-Pt		
[mM]	10	25	50
homoITC	Combination index		
5	1,09	0,73	0,73
10	0,25	0,18	0,41
15	0,19	0,20	0,34

Fig. 5 Relative viability (MTT assay) of cisplatin-sensitive A2780 cells (A) and cisplatin-resistant A2780/CP cells (B) after 1 hour exposition to 5 – 15 μM homoITC followed by corresponding concentration of cis-Pt. Cis-Pt at concentrations 2.5 – 10 μM (A) and 10 – 50 μM (B) was added for 72 hours. Relative viability of control cells = 1.0. The tables show the calculated combination indexes values.

and cis-Pt alone. The viability of homoITC /cisplatin treated cells was lower than viability in cis-Pt-treated cells, but if the concentration of homoITC was too low, no synergy was observed. Detailed analysis of mutual exclusivity of both tested compounds showed that homoITC and cis-Pt act independently, having different modes of action.

Molecular mechanisms responsible for the synergistic action of homoITC and cis-Pt have yet to be clarified. Theoretical rationale suggests that ITCs as HDAC inhibitors can demonstrate synergy in combination with cis-Pt due to the increased accessibility of DNA for the platinum binding [37]. It is also known that isothiocyanates [38] induce a decrease of

GSH level and that elevated levels of GSH are associated with cis-Pt resistance in A2780/CP cells [39], thus GSH depletion may be one of common links responsible for observed synergy in the combination treatment. Furthermore, in agreement with the others [19], our data suggest impact of other possible mechanisms, i.e. those participating in the cell cycle checkpoint control and apoptosis regulation, which might lead to acquisition of cisplatin resistance possibly modulated by ITCs. Therefore in our further studies we would like to explore the intracellular protein regulation elements (such as MAP kinases) in combination treatment with the aim of elucidating the mechanisms of the synergistic cellular toxicity in response to homoITC and cis-Pt.

To summarize, homoITC exerted cytotoxic effects and exhibited synergistic action in combination with cisplatin in A2780 and A2780/CP cells. Observed synergism was manifested over a wide range of concentrations suggesting that the dose of each reagent may be reduced in the combination treatment, which can lead to lowering of the side effect toxicity. Additional studies are necessary to determine the optimal way of cisplatin and homoITC combination, to explain mechanisms of their synergism and the efficacy of this combination remain to be tested in further preclinical models. Our data suggest that the combination of chemotherapeutic drug cisplatin and homoITC is promising in overcoming cisplatin-resistance in vitro and could be considered as a possible treatment modality of recurrent and resistant ovarian carcinomas.

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