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DRUG BIOCHEMISTRY

COMBINATION TREATMENT WITH 5-FLUOROURACIL AND ISOTHIOCYANATES SHOWS AN ANTAGONISTIC EFFECT IN CHINESE HAMSTER FIBROBLAST CELLS LINE-V79

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Abstract: The isothiocyanates present in the cruciferous plants were proved to have the antiproliferative and cytotoxic effect on cancer cell lines. Natural compounds in combination with chemotherapy agents enhance anticancer activities of drugs and reduce their toxicity. The aim of the presented study was to determine an effect of isothiocyanates and 5-fluorouracil used alone or in combination (in sequential or co-administrative treatments) on normal cell lines-V79. There were compared abilities of three isothiocyanates to interact with 5-fluorouracil. There was also investigated the mechanism of interaction and influence of isothiocyanates on 5-fluorouracil.The cell survival was evaluated with MTT assay. Combination effects between isothiocyanates and 5-fluorouracil were estimated in the way described by Chou and Talalay. The cycle progression and the living cells number were determined with flow cytometry. The type of cell death was detected with a confocal microscope. There was observed an antagonistic effect which was mainly dependent on the cell cycle distribution e.g., sulforaphane increased the cell number in the G2/M phase, whereas 5-fluorouracil and combination of these two compounds increased the cell number in the S phase. If each compound blocked the S phase of the cell cycle, their combination increased the cell number in the S phase, but the increase was not statistically significant when compared with single substance treatments. The highest antagonistic effect in normal cells was obtained for co-administrated 5-fluorouracil and 2-oxoheptyl isothiocyanate at the fraction affected at 0.5 and 0.75. Isothiocyanates did not affect 5-fluorouracil cytotoxicity in normal cell lines-V79.

Keywords: isothiocyanates, 5-fluorouracil, Chinese hamster lung fibroblast cell line, combination index, antagonism

Sulforaphane is the most potent isothiocyanate. It is a naturally derived compound that is present in the tissues of Cruciferae plants in the form of glucosinolates. Sulforaphane exhibits multiple activities: inhibition of the procarcinogen metabolism (e.g. it inhibits CYP1A1- and CYP1A2-mediated activation of polycyclic aromatic hydrocarbons (1, 2)), activation of the phase II enzymes in many types of cells (e.g., in hepatic cells (3), prostate cancer cells (4), lymphocytes and lymphoblastoid cells (5, 6)), and alteration of the phase III metabolism (it alters activity of ABC-transporters such as Pgp or MRP1 proteins (7)). At higher doses, it exhibits cytostatic and cytotoxic activities through its influence on the cell cycle and its induction of apoptosis, which has been proven in many types of tissues, mostly in cancer cell lines (8-10). This multi-pathway activity of sulforaphane makes it one of the most potent naturally-derived chemopreventive compounds. The results of *in vivo* studies also showed that sulforaphane inhibits chemically-induced carcinogenesis through the mechanism described before. There are currently registered three clinical trials using sulforaphane in the form of broccoli extracts (11-13). This form is easily available as a dietary supplement.

Two analogs of sulforaphane were designed and synthesized: 2-oxohexyl isothiocyanate (ITC) and 2-oxoheptyl isothiocyanate (ITC). To receive 2-oxohexyl ITC, the S=O group in sulforaphane was replaced with the C=O group. To receive 2-oxoheptyl ITC, the hydrocarbon chain in 2-oxohexyl ITC was elongated through addition of the CH_2 group. The structures of the three isothiocyanates are presented in Figure 1.

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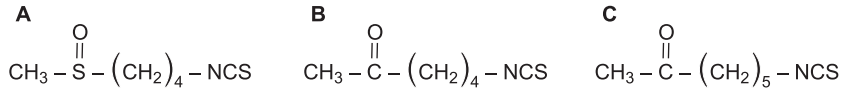

Figure 1. Structures of isothiocyanates: (a) sulforaphane, (b) 2-oxohexyl ITC, (c) 2-oxoheptyl ITC

Table 1. The values of each concentration (uM) used in research of interaction mechanisms.

simultaneous treatment				sequential treatment			
	fa	2-oxoheptyl ITC	5-Fu		fa	2-oxoheptyl ITC	5-Fu
	0.25	1.68	2.69		0.25	1.25	0.84
	0.5	3.55	5.68		0.5	2.24	1.5
	0.75	7.5	12.02		0.75	4.02	2.68
		2-oxohexyl ITC	5-Fu		2-oxohexyl	ITC	5-Fu
	0.25	0.88	1.77		0.25	1.66	1.51
	0.5	2.28	4.55		0.5	2.81	2.56
	0.75	5.86	11,73		0.75	4.77	4.34
		sulforaphane	5-Fu			sulforaphane	5-Fu
	0.25	1.84	2.01		0.25	5.14	2.86
	0.5	4.01	4.37		0.5	9.18	3.57
	0.75	8.72	9.51		0.75	13.06	7.25

As the isothiocyanates have an influence on the metabolizing enzymes, the question arises if there are possible interactions with other substances. It has already been shown that sulforaphane can act synergistically with doxorubicin, which results in lower toxicity of doxorubicin (16). Sulforaphane was also proven to act synergistically with 5-fluorouracil in human adenocystic carcinoma in two types of cell lines (17). Interactions between naturally occurring compounds and anticancer drugs have been a subject of many publications. Studies of the interactions done on normal cells (18) and on animals (19, 20) have shown a protective effect of natural compounds, whereas studies on cancer cells or tissues have presented an enhancing effect on drugs efficacy (16, 17, 21, 22).

5-Fluorouracil is used to treat many types of cancers such as colorectal (23), breast (24) and prostate (4) cancer. Its common usage was an inspiration to conduct research to verify if sulforaphane and its two analogs can influence 5-fluorouracil cytotoxicity. There were examined the interactions between sulforaphane and its two analogs and 5-fluorouracil in a normal cell line, the-V79 cell line. This cell line is traditionally used in cytotoxicity and toxological studies (25).

The interactions were evaluated in two schemes of administration: (1) co-administration of 5-fluorouracil and isothiocyanates and (2) pre-treatment with isothiocyanates and subsequent treatment with 5-fluorouracil. The aim of the study was to determine the types of interaction between isothiocyanates and 5-fluorouracil and their mechanisms. There was also verified the influence of isothiocyanates on 5-fluorouracil cytotoxicity in the normal cell line.

EXPERIMENTAL

Chemicals

Sulforaphane, 2-oxohexyl ITC and 2-oxoheptyl ITC were synthesized as described by Schmidt and Karrer (26). 5-Fuorouracil, MTT (3,-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), RNA-ase, Triton, FDA (fluorescein diacetate) and PI (propidium iodide) were obtained from Sigma Aldrich, Steinheim. Isopropanol, ethanol and PBS were purchased from POCH, Gliwice. FITC Annexin V Apoptosis Detection Kit I was obtained from BD Biosciences, New Jersey. All the media constituents were purchased from Sigma Aldrich, Steinheim.

Cells

V79 cells were obtained from American Type Culture Collection (ATCC). The cells were grown

in RPMI 1640 medium, supplemented with 10% FBS, penicillin (100 IU/mL), streptomycin (100 μg/mL), amphotericin (250 ng/mL) and L-glutamine (2 mM). They were maintained at 37°C in humidified atmosphere containing 5% of CO_2. The growth medium was changed every 72 h. On average, eight passages of cell culture were used during the experiments.

Cell viability assay

The MTT assay was used to test cell viability (27). Cell suspensions of 100 μL (density of 4×10^4 cells/mL) were placed in every well of a 96-well plate. The plated cells were pre-incubated for 24 h. Different dilutions of the tested compounds included in 100 μL culture medium were added to the wells to obtain the final volume of 200 μL. After the treatment period (24 h or 48 h) the plates were centrifuged. The culture medium was removed and the cells were washed twice with 150 mL PBS. After centrifugation, there was added MTT (20 mg/mL) to nd the samples were incubated for 3 h at 37°C. Isopropanol (200 μL) was added to dissolve the insoluble product of MTT into a colorful solution. The absorbance of converted MTT was measured with the microplate scanning spectrophotometer (Power Wave X) at the wavelength of 570 nm with background subtraction at 690 nm. The experiment was repeated three times for each dilution of tested compounds. IC_{50} parameters were evaluated on the basis of the dose / survival plots.

Quantitative analysis of interactions

The combined effects of two tested agents were determined through the median effect analysis as described by Chou and Talalay (28). In the interaction studies, the tested substances were added at constant ratios which were approximately their IC_{50} ratios (17) (concentration which inhibits cell viability by 50%).

Concurrent and sequential treatments were also assessed. In the concurrent schedule the cells were treated with graded concentrations of each agent and their mixtures, and then left for 48 h. In a sequential schedule, the cells were treated with isothiocyanates and left for 24 h, and then they were subsequently incubated with 5-fluorouracil for 48 h. In addition, in the same experiments the cells were also incubated with each of the substances alone at concentrations corresponding to the concentrations used in combinations.

In the first place, the median-effect plot was determined (the plot: x = log (substance concentration) whereas y = log (the fraction affected / the fraction unaffected at the concentration)). If the lines in the plot were parallel for the compound used alone and for its combination with other substance, it indicated that the substances had acted in a mutually exclusive manner and they possibly had the same molecular target. If the lines in the plot were non-parallel, it indicated that they had acted in a mutually non-exclusive manner and they had different molecular targets (29).

The calculations were conducted only for $r > 0.95$, where r was the value of linear correlation coefficient. To generate Combination Index (CI) for each fraction of the affected level there was used the equation:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{(D)_1 \cdot (D)_2}{(D_x)_1 \cdot (D_x)_2} \quad (1)$$

where: $(D)_1$, $(D)_2$ = the concentration of the tested substance 1 (which inhibited cell viability by y%) and the tested substance 2 (which inhibited cell viability by z%); $(D_x)_1$, $(D_x)_2$ = the concentration of the tested substance 1 in combination with a dose of the tested substance 2, which together inhibited cell viability by n% (when n = 25%, then the level of the fraction affected is 0.25).

If CI > 1, CI < 1 or CI = 1, it indicated: antagonism, synergism or additive effect, respectively. The experimental data were calculated with a computer program Microsoft Office Excel 2003. The procedures were repeated three times.

Research for the mechanism of interaction

The cells were seeded in 24-well plates and treated with combination of 5-fluorouracil and ITC or with each of the compounds alone. The doses of substances used in combination treatment inhibit cell viability by 25% (the affected fraction (fa) 0.25), 50% (fa 0.5) and 75% (fa 0.75).

The values at each concentration, which are presented in Table 1, were estimated with ratios of concentration of combined substances and the median effect equation:

$$D_x = D_m [f_a/(1 - f_a)]^{1/m} \quad (2)$$

where D_x = concentration of the tested substance 1 in combination with a dose of the tested substance 2 which was required to inhibit the cell viability by x%; D_m = the median-effect dose; m = the shape parameter of the dose-effect curve.

Cells were also incubated with each of the substances alone at concentrations corresponding to the concentrations used in the combinations. After incubation, the cells were trypsinized and rinsed with PBS and there were performed the subsequent tests.

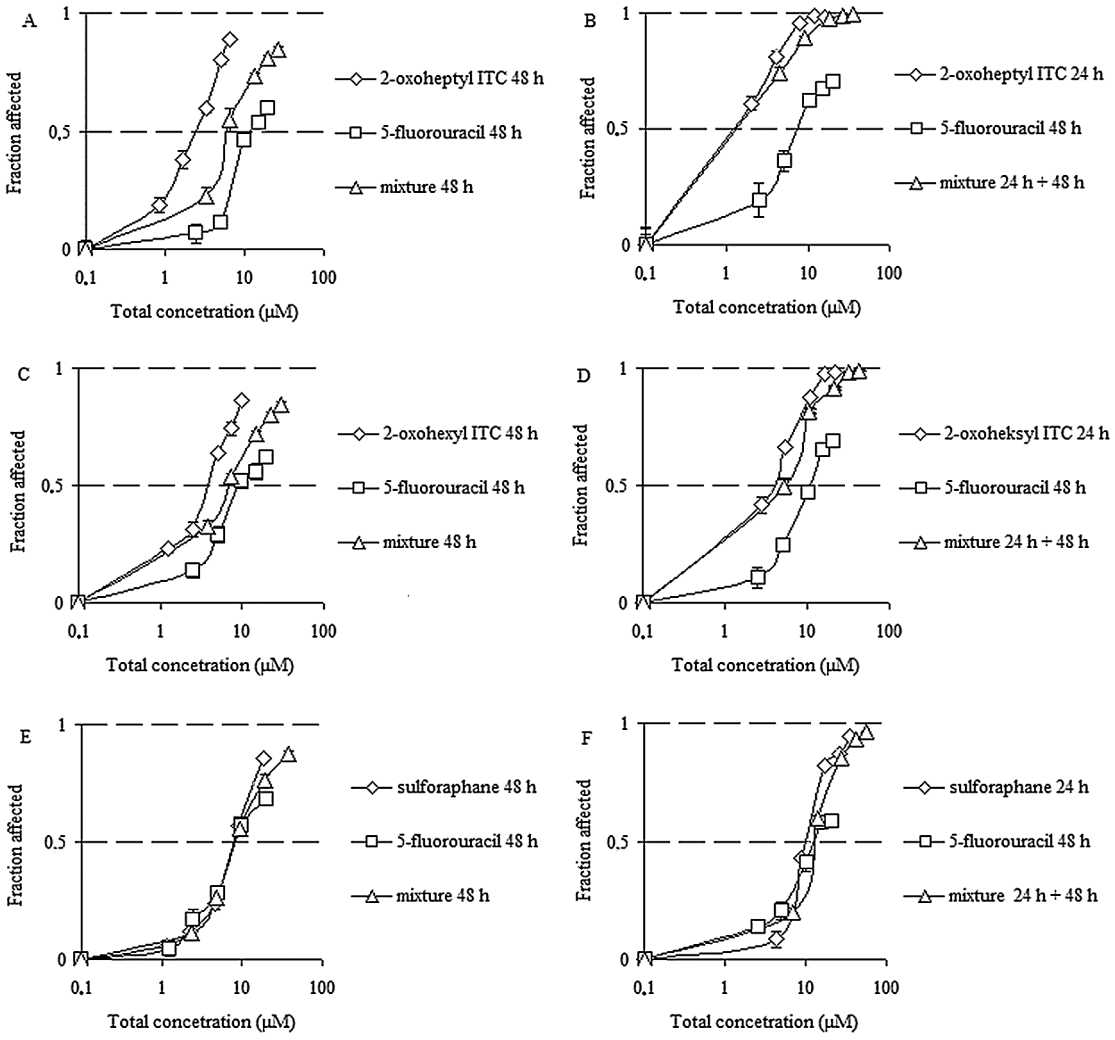

Figure 2. Cell growth inhibition after incubation with co-administered 5-Fu and ITC (A, C, E) and after incubation with sequentially administered ITC (for 24 h) and 5-Fu (for next 48 h) (B, D, F). Cell growth inhibition is expressed as fa (the affected fraction) when compared to the untreated control. The procedure was repeated three times

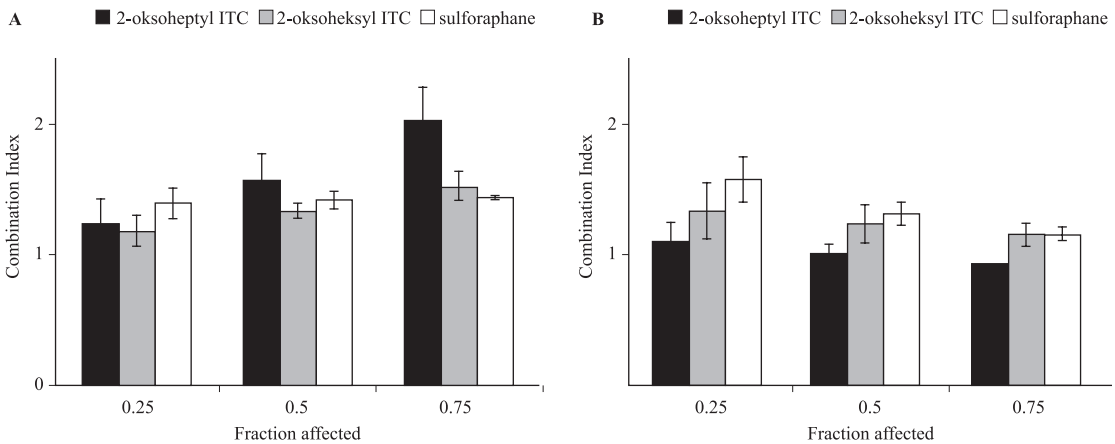

Figure 3. Mean values of Combination Index after treatment with 5-Fu in combination with ITC at fa 0.25, 0.5 and 0.75; A – co-administration; B – sequential administration

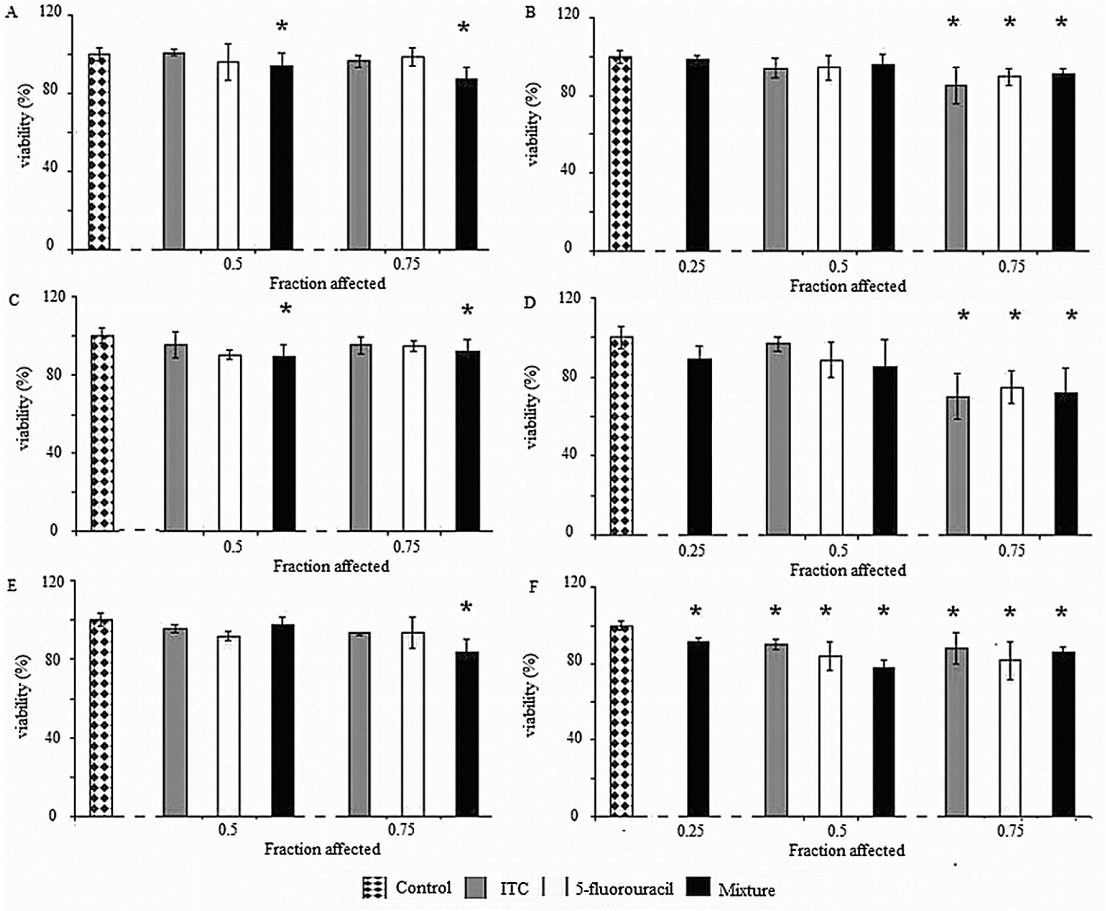

Figure 4. The number of viable cells was evaluated with flow cytometry and FDA/PI staining. The number was checked after incubation with co-administered substances (A, C, E), sequentially administrated substances (B, D, F) and incubation with each substance alone at concentrations corresponding to those used in the combinations. The doses of substances used in combination treatment inhibit cell viability by 25% (fa 0.25), 50% (fa 0.5) and 75% (fa 0.75) (MTT assay). The procedure was repeated three times. *Stages for a significant difference from the untreated control, $p < 0.05$

Cell cycle analysis

After centrifugation, the cells were suspended in 75% ice cold ethanol and stored at –20°C. For the purposes of analysis, each sample was centrifuged and suspended in 50 µL staining solution containing 50 µg/mL PI. After 10 s, there were added 500 µL of solution containing 100 µg/mL RNA-ase and 0.1% Triton. The cells were incubated for 30 min at room temperature. Cell cycle distribution was examined with EPICSXL flow cytometry (14). The cell cycle was analyzed with Multi Cycle Analysis ™ Software (Phoenix Flow System). The procedure was repeated three times.

Determination of living cell number

Cells were trypsinized and rinsed with PBS. Each 100 µL sample in PBS was treated with 10 µL of 6.25 µg/mL FDA and 10 µL of 50 µg/mL PI solution prepared in PBS. After 5 min of incubation in ice, 0.5 mL of PBS was added and the sample was analyzed with flow cytometry (30). The cell viability was calculated using WIN MDI. The procedure was repeated three times.

Cell numbers

The cell number was estimated with the cell counter – Beckman Coulter Z1 (Beckman Dickinson). The procedure was repeated three times and the result is a mean of three measurements.

Identification of apoptosis or necrosis

For the purposes of microscopy analyses, the cells were stained with the FITC Annexin V Apoptosis Detection Kit I by the BD Biosciences Company (San Diego, USA). There was used Annexin V Binding Buffer Annexin, which was dilut-

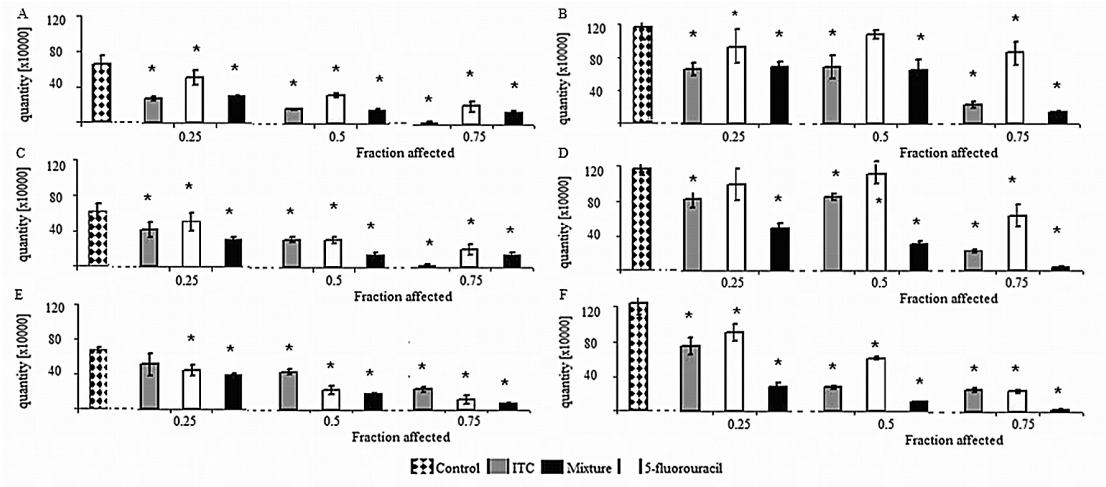

Figure 5. The number of cells after incubation with co-administered 5-fluorouracil and isothiocyanates (A) and after incubation with sequentially administered isothiocyanates (for 24 h) and 5-Fu (for next 48 h). The procedure was repeated three times. *Stages for a significant difference from the untreated control, $p < 0.05$. Cell viability was calculated using the median-effect equation

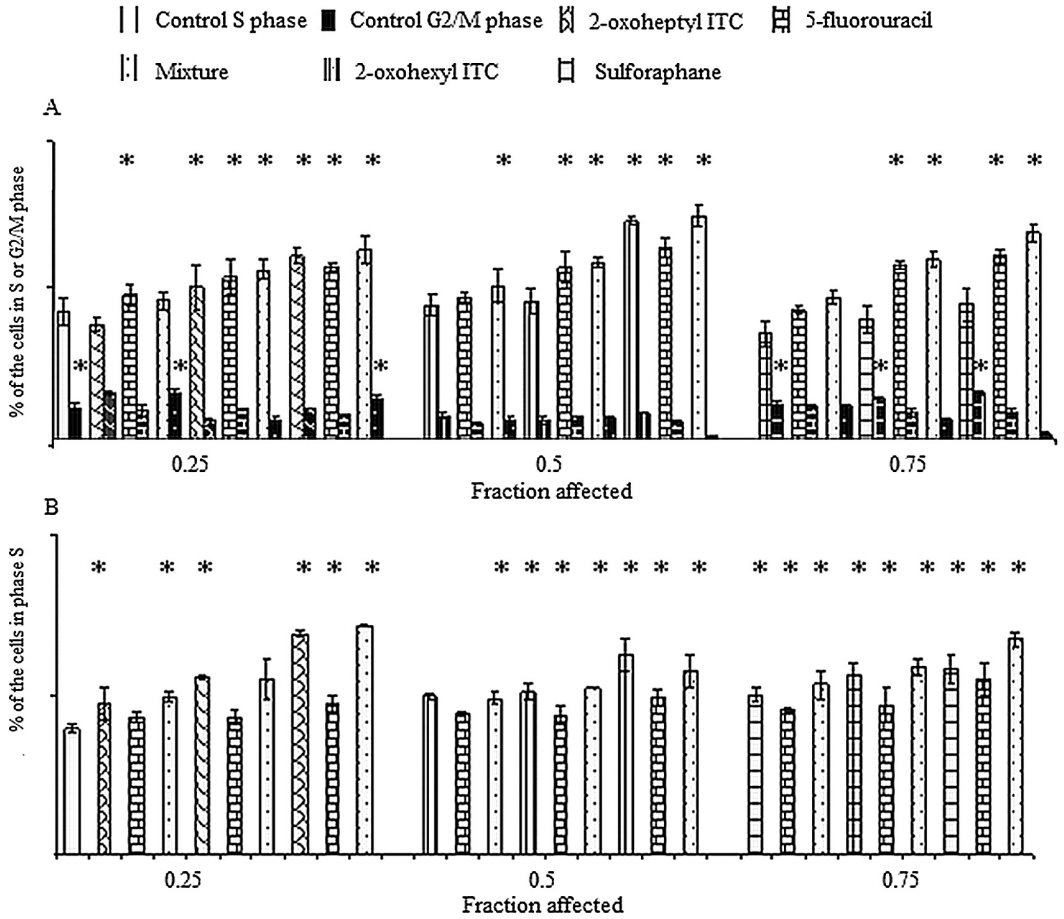

Figure 6. Distribution of the cells in the S phase (A, B) and G2/M phase (A), analyzed with flow cytometry as described in Materials and Methods. The cells were incubated with 5-fluorouracil and isothiocyanates in combination treatment (A) and in sequential treatment (B) at the levels of fa 0.25, 0.5 and 0.75. The procedure was repeated three times. *Stages for a significant difference from the untreated control, $p < 0.05$

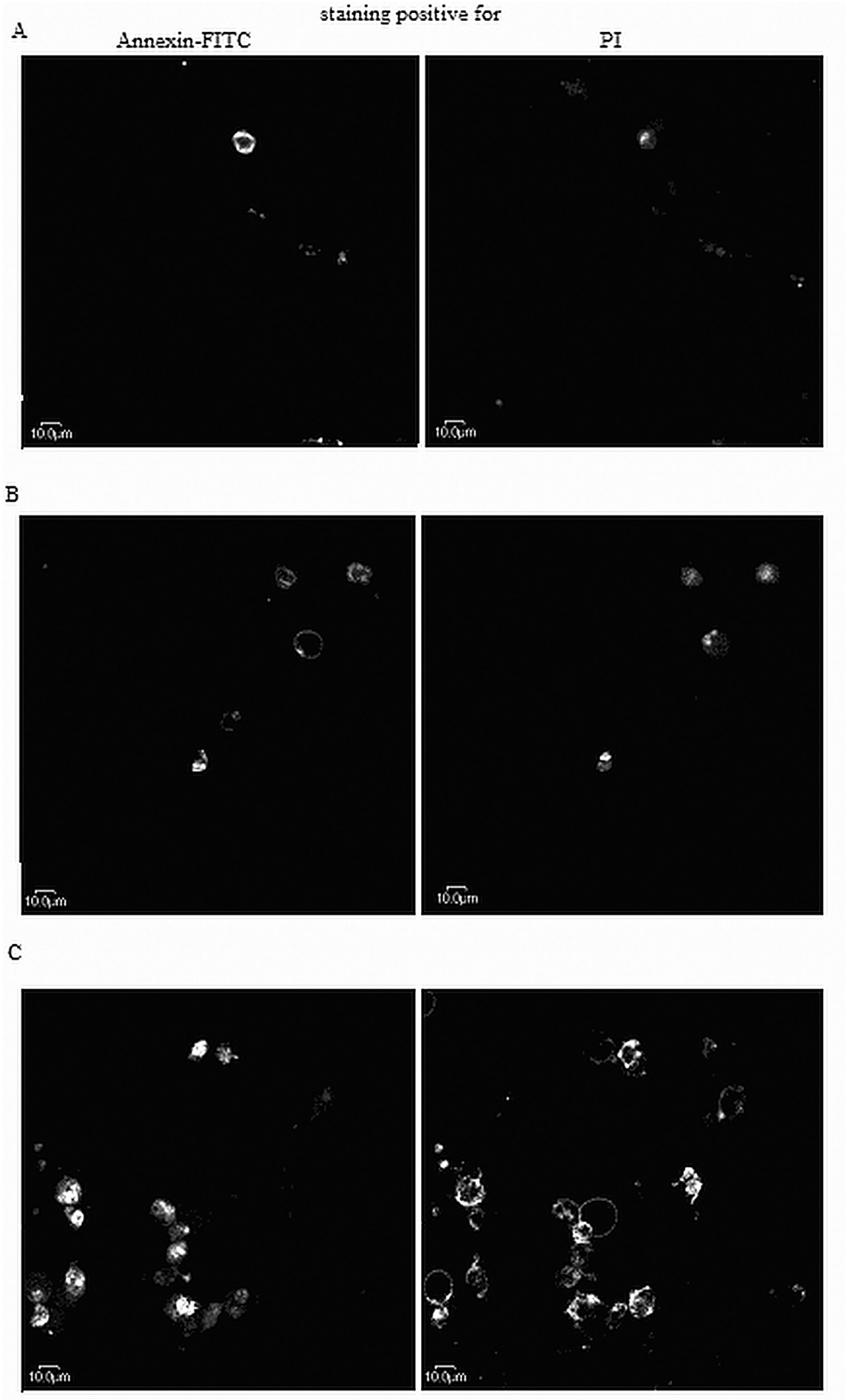

Figure 7. Microscopic images of necrotic or late apoptotic cells stained with Annexin V FITC/PI. A. Co-administration of 5-fluorouracil and sulforaphane at the level of fa 0.75. B. Sequential treatment with 5-fluorouracil and 2-oxoheptyl ITC at the level of fa 0.5. C. Sequential treatment with 5-fluorouracil and 2-oxohexyl ITC at the level of fa 0.75

ed with distilled water and there were further added FITC Annexin V (5 μL per 1 mL buffer) and propidium iodide (5 μL per 1 mL buffer). After 15 min of incubation, there was performed identification of the type of cell death with the confocal Olympus IX 70FV500 microscope. Fluorescein isothiocyanate (FITC) was excited with 488 nm and PI 543 nm lasers. The FITC and PI fluorescence were collected using 520 nm and 600 nm filters, respectively. There were distinguished: living cells ($AnnV^-/PI^-$), early apoptotic cells ($AnnV^+/PI^-$) and necrotic or late apoptotic cells ($AnnV^+/PI^+$) (31). On average, ten pictures were taken and the most representative ones are presented in Figure 7. The numbers of cells observed in the field of view were similar. There were only investigated the cases in which a decrease in the number of living cell was statistically significant.

Statistical analysis

Only the mean results (± SD) were presented. Statistical analyses were carried out using the one-way analysis of variance (ANOVA) followed by the *post-hoc* Dunnett's test for separate comparisons with the untreated control. When the comparisons involved two groups, the data were analyzed with Student's *t*-test. When $p < 0.05$, the differences were considered statistically significant.

RESULTS

Antiproliferative and cytotoxic effect of 5-fluorouracil and isothiocyanates

The viability of cells after treatment with ITC and 5-fluorouracil was evaluated after 24-h and 48-h incubation. The rate of cell survival after treatment with isothiocyanates was related to their dose and the time of treatment. One way analysis of variance (ANOVA) revealed that the type of isothiocyanate was a significant factor that had influenced cell survival ($p < 0.05$).

After 24 h it was impossible to determine IC_{50} of 5-fluorouracil, because the cell survival decreased only to 60% for the tested concentration range up to 1 mM. Prolonged treatment of cells with 5-fluorouracil resulted in higher cytotoxicity and IC_{50} was at the level of 10 μM. Among the given three isothiocyanates, 2-oxoheptyl ITC was the most cytotoxic – it had the lowest IC_{50} values for both times of incubation. IC_{50} of 2-oxoheptyl ITC was 7.96 ± 1.82 μM for 24-h incubation and 3.32 ± 0.55 μM for 48-h incubation. IC_{50} of 2-oxohexyl ITC was 11 ± 1.0 after 24 h and 5 ± 0.4 after 48 h, and IC_{50} of sulforaphane was 17.5 ± 4.8 μM after 24 h and 9.15 ± 0.84 μM after 48 h.

Quantitative analysis of interactions

The level of fa after treatment of the cells with combination of 5-fluorouracil and 2-oxohepytyl ITC or 5- fluorouracil and 2-oxohexyl ITC was higher than after treatment with just one of them (Fig. 2). This effect was observed in the whole tested concentration range. A similar tendency was observed for the combination of sulforaphane and 5-fluorouracil only at higher concentrations and the effect was stronger for sequence administration than for co-administration.

In all cases the lines at the median-effect plot were not parallel (data not shown), which indicated that 5-fluorouracil and isothiocyanates had acted independently regardless of the scheme of administration. CI values were calculated with Chou's method at the three levels of fa (0.25, 0.5 and 0.75) (Fig. 3).

After co-administrative treatments the CI values decreased, while the fraction affected increased. The strongest antagonistic effect was observed for 2-oxoheptyl ITC and 5-fluorouracil at the level of fa 0.5 (CI = 1.6) and at the level of fa 0.75 (CI = 2.0). After sulforaphane and 5-fluorouracil co-administrative treatment the CI values were changed, but not significantly (CI ~ 1.4) – this type of interaction is called moderate antagonism. After 2-oxohexyl ITC and 5-fluorouracil co-administrative treatments at the levels of fa 0.25 and 0.5 there were observed moderate antagonism (fa 0.25 CI 1.2–1.3) and antagonism (fa 0.75 CI = 1.5).

The CI values and the level of fa increased after sequential treatments. 5-Fluorouracil and 2-oxoheptyl ITC treatment indicated a nearly additive effect (CI = 1.1–0.9). After sulforaphane and 5-fluorouracil treatment there was observed moderate antagonism for all the levels of fa. The CI varied from 1.6 to 1.2. After 2-oxohexyl ITC and 5-fluorouracil sequential treatments, there were noticed: moderate antagonism (fa 0.25, CI = 1.3), slight antagonism (fa 0.5, C I = 1.2) and a nearly additive effect (fa 0.75, CI = 1.1).

Research of interactions

Effects of isothiocyanates, 5-fluorouracil and their combinations on the living cell number

The living cell number was evaluated with flow cytometry and by staining living cells with FDA and PI (Fig. 4.). The biggest decreases in the living cell number occurred at the level of fa 0.75 for co-administrative or sequential treatments with isothiocyanates and 5-fluorouracil when compared with the untreated control. The changes, however, were small e.g., the number of living cells decreased just by 12%, 13%, and 17%, after co-administrative

treatments with 5-fluorouracil and 2-oxohexyl ITC, 5-fluorouracil and 2-oxoheptyl ITC, and 5-fluorouracil and sulforaphane, respectively, when compared to the untreated control.

Student's *t*-test showed no statistically significant differences in the living cell number between combination treatments and treatments with a single substance.

Effects of isothiocyanates, 5-fluorouracil and their combinations on the cell number

The numbers of the treated cells were compared with the numbers of the cells in untreated control. There were observed a decrease in cell numbers and an increase in the fractions of affected cells (Fig. 5).

Differences in a decrease in cell numbers after co-administration were larger than after treatment with each isothiocyanate alone at levels of fa 0.25 and 0.5; except for sulforaphane – then the differences were bigger just at the level of fa 0.75. Treatments with 2-oxoheptyl ITC or 2-oxohexyl ITC alone at the level of fa 0.75 resulted in a bigger decrease in cell number than their simultaneous treatments with 5-fluorouracil.

The greatest significant decrease in the number of cells was detected after sequential exposures. Sequential treatment of sulforaphane and 5-fluorouracil caused the largest changes in cell numbers: a decrease by 78%, 91%, and 98% at the levels of fa 0.25, 0.5 and 0.75, respectively. Sulforaphane in the concentrations corresponding to the concentrations used in sequential exposure decreased cell numbers more than any other of the three isothiocyanates.

Effects of isothiocyanates, 5-fluorouracil and their combinations on the cell cycle

Statistical analyses showed mainly a significant increase in the numbers of cells in the S phase when compared to the untreated control (Fig. 6.). However, in some cases an increase in the cell number was observed in the G2/M phase.

The greatest S phase accumulation at the level of fa 0.75 was seen after simultaneous treatment of the cells with combination of 5-fluorouracil and 2-oxohexyl ITC (79%) or with combination of 5-fluorouracil and 2-oxoheptyl ITC (52%) or after treatment with each of two isothiocyanates alone (74 and 47%, respectively) when compared to the untreated control. The differences in cell numbers in the S phase between combined treatments with 5-fluorouracil and 2-oxohexsyl ITC or 5-fluorouracil and 2-oxoheptyl ITC and treatments with 2-oxoheptyl ITC or 2-oxohexyl ITC alone were not significant for all levels of fa.

In addition, after co-administration there was observed a significant increase in the fraction of cells in the G2/M phase when compared to the untreated control. Sulforaphane treatment alone increased the cell number in the G2/M phase by 14% (fa 0.25), 33.5% (fa 0.5), and 47.8% (0.75) when compared to the untreated control.

After sequential exposure, there were observed increases in the cell number only in the S phase when compared to the untreated control. There were no statistically significant differences between the cell number in the S phase after treatments with each isothiocyanates alone and after combination treatments when the used concentrations were the same. Only at the level of fa 0.75, 2-oxohexyl ITC alone treatment increased the number of cells in the S phase by 14% when compared to combination treatment. All single isothiocyanate treatments were more effective than 5-fluorouracil alone treatments.

Effects of isothiocyanates, 5-fluorouracil and their combination on cell death

The cells were stained with AnnexinV-FITC/PI and observed with a confocal microscope. When the cells were stained with AnnV/PI there was no effect in the untreated control. In all other cases, necrotic or late apoptotic cells were observed. After 2-oxohexyl ITC treatment at the level of fa 0.75 and at concentrations corresponding to the concentrations used in sequential treatment there were noticed early apoptotic cells (Fig. 7). The largest number of necrotic or late apoptotic cells was noticed after sequential treatment of 5-fluorouracil with 2-oxohexyl ITC at the level of fa 0.75. The strongest inductor of late apoptosis or necrosis was 2-oxohexyl ITC, then 2-oxoheptyl ITC, the weakest one was sulforaphane. Sequential treatment was more effective than co-administration.

DISCUSSION AND CONCLUSION

Isothiocyanates, and especially sulforaphane, are more and more frequently an object of interest of many studies about *in vitro* cancer treatments. They were investigated not only as chemopreventive anticancer agents, but as candidates to be used in combination treatments with anticancer drugs. It was proven recently that sulforaphane sensitized Caco-2 colon cancer cell line to oxoliplatin (32), EOC epithelial ovarian cancer cells to paclitaxel (33), and ACC salivary gland adenoid cystic carcinoma cell lines to 5-fluorouracil (17). Isothiocyanates not only reduce the drugs doses used in treatments, but also

they cannot cause development of the chemioresistant phenotype.

Unlike other publications, the aim of the presented study was to investigate an interaction of 5-fluorouracil and isothiocyanates and its mechanism in the normal cell line. The results showed an antagonistic interaction after simultaneous exposure to 5-fluorouracil and one of the isothiocyanates and an antagonistic or nearly additive effect after sequential exposure to the substances. In case of normal cells, antagonism is a beneficial interaction. As it was presented in the publications by Khafif et al. (34) and Kano et al. (35), the antagonistic interaction is considered to be protective for the normal cells. Khafif et al. noticed antagonism betwenn (–)-epigallocatechin-3-gallate and curcumin in normal cells and their synergism in cancer cells. These results are important for the clinical use of the investigated drugs, because they prove their protective effect on normal cells. In the presented study, there was also obtained an antagonistic interaction, however, there was no protective effect. The statistical analysis showed that there were no statistically significant differences in living cells numbers after exposure to 5-fluorouracil in the concentrations corresponding to the ones used in combination with isothiocyantes and after exposure to 5-fluorouracil and isothiocyanates (in sequential and simultaneous treatment). In Adeyemo's study on colorectal carcinoma cell lines, it was noticed that treatment with 5-fluorouracil and antioxidant (N-acetylcysteine or vitamin E) was more cytotoxic than exposure to 5-fluorouracil alone (20), and only simultaneous treatment induced apoptosis. A similar effect was observed after co-administration of 5-fluorouracil with one of ITC at the level of fa 0.5 or 0.75 and after sequential treatment with 5-fluorouracil and sulforaphane at the level of fa 0.5. The difference between Adeyemo's research and the presented study was in the number of apoptotic cells and the type of cell lines. In Adeyemo's study the number of apoptotic cells in the carcinoma cell increased several times when compared to the untreated control. However, in the presented study the observed change in cell viability was very small.

Antagonistic interactions between substances were interpreted by many researchers as a result of their influence on different phases of a cell cycle e.g.,: between sulforaphane and 3.3'-diindolylmethane in the colon cancer cell line (39) and between 5-fluorouracil and paclitaxel in non-small-cell lung cancer A549, breast cancer MCF7 and ovarian cancer Pa1 cell lines (36). In the presented study interactions can be explained in the same way. As it has been documented in many publications (36) and also observed in the present study, 5-fluorouracil blocks the S phase of a cell cycle. After treatment with sulforaphane in the concentrations corresponding to the concentrations used in co-administration of sulforaphane and 5-fluorouracil at the levels of fa 0.25, 0.5, and 0.75, there were detected blocks in G2/M phase. In a few cases of isothiocyanates and 5-fluorouracil co-administrative treatments and many cases of sequential treatments with them, there were noticed blocks in the S phase of the cell cycle. The statistical analysis showed that there were no statistically significant differences between the percentage of cells in the S phase after sequential exposure to isothiocyanates and 5-fluorouracil and after treatment with each isothiocyanate alone. It shall be emphasized that after sequential exposure to 5-fluorouracil and 2-oxoheptyl isothiocyanate or to 5-fluorouracil and 2-oxohexyl isothiocyanate, there were observed slight antagonism and a nearly additive effect. It was further proved that the mechanism of interaction depended mainly on cell cycle distribution. The differences between the type of blocks observed in the cell cycle in treatments with sulforaphane in the concentrations corresponding to the concentrations used in co-administration and sequential administration of sulforaphane and 5-fluorouracil can be explained in the way it was done in the previous publications. Cell cycle arrests by isothiocyanates depend on their dose, on the cell line and on the time of exposure (6). Isothiocyanates can cause a temporary arrest of a cell cycle phase, as it was noticed after exposure of B-lymphocytes 4153 Del A mutations cell line to alyssin in the G1 phase (15). Isothiocyanates can also cause the permanent arrest of a phase of a cell cycle after exposure of the PC-3 cell line to sulforaphane in the G2/M phase (37). The sulforaphane inhibitory effect on G2/M phase has already been observed in MCF7, F311 Sarcomatoid Mammary Carcinoma (37), Jurkat T-lymphocyte (38) and PC-3 (8) cell lines.

There were also compared abilities of three isothiocyanates to interact with 5-fluorouracil. There was observed stronger antagonistic effect after co-administration of 2-oxoheptyl ITC and 5-fluorouracil than after co-administration of other isothiocyanates and 5-fluorouracil at the levels of fa 0.5 and 0.75. The presented study is the first one in which 2-oxoheptyl ITC was used, because it is a new sulforaphane analog. For many cell lines elongation of the methylene chain causes an increase in the cytotoxicity of ITC (6, 14, 15). As it was said before, 2-oxoheptyl ITC is more cytotoxic than 2-oxoheptyl ITC. The CI values

did not differ significantly after sequential treatment with each of the analogs and 5-fluorouracil. The only exception was for treatment with sulforaphane and 5-fluorouracil at the level of fa 0.25.

In conclusion, it was noticed that the exposure to 5-fluorouracil and isothiocyanates had an antagonistic or nearly additive effect in the normal cell line. The strongest antagonistic interactions with regard to cell viability and CI values, and in comparison to other schemes of administration, were observed after co-administration of 5-fluorouracil and 2-oxoheptyl ITC. The isothiocyanates did not enhance 5-fluorouracil cytotoxicity. The mechanism of the antagonistic interaction was consisted by blocking different phases of the cell cycle with the investigated compounds.

While other publications had proved synergic effect of anticancer drugs and isothiocyanates on cancer cell line, this study showed the antagonistic effect of 5-fluorouracil and isothiocyanates on normal cell line. These results should encourage starting new research on cell lines and on animals considering the influence of isothiocyanates on cytotoxicity of anticancer drugs.

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