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Differential response of human healthy lymphoblastoid and CCRF-SB leukemia cells to sulforaphane and its two analogues: 2-oxohexyl isothiocyanate and alyssin

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Abstract:
The chemopreventive effect of sulforaphane and two of its analogues on human B-lymphocytes derived cells was evaluated in this study. Two cell lines used in the experiments were: human lymphoblastoid cells and human B-leukemia CCRF-SB. Both cell lines were treated with three structurally related isothiocyanates: sulforaphane, 2-oxohexyl isothiocyanate and alyssin. The viability of cells, induction of a phase II enzyme-quinone reductase, apoptosis induction, GSH content and ROS formation were evaluated.
The results indicate the differences between the chemopreventive properties and apoptosis-inducing activity of three isothiocyanates. The significant differences in response to these compounds were observed between healthy lymphoblastoid and leukemia CCRF-SB cells.

Key words:
isothiocyanates, sulforaphane, 2-oxohexyl isothiocyanate, alyssin, apoptosis, chemoprevention, phase II enzymes

Abbreviations: CD – concentration required to double QR activity, CI – chemoprevention index, CMFDA – chloromethylfluorescein diacetate, DHR – dihydrorhodamine, GSH – glutathione, IC50 – inhibitory concentration 50%, ITC – isothiocyanate, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, QR – quinone reductase, ROS – reactive oxygen species, SFN – sulforaphane

Introduction

Isothiocyanates are the group of chemical compounds containing the strongest chemopreventive agents, both naturally occurring and synthetic. Natural isothiocyanates are dietary compounds of plant origin, which are synthesized and stored in plants as relatively stable precursors, known as glucosinolates, which are enzymatically hydrolyzed to yield isothiocyanates [9]. Recently several isothiocyanates has been shown to induce apoptosis in many cell lines, like in prostate cancer, T-lymphocytes, T-leukemia or colon carcinoma [4–6, 20]. Our previous study revealed that sulforaphane (SFN) and 2-oxohexyl isothiocyanate induce apoptosis in human melanoma and murine leukemia cell lines [11]. We have also shown that sulforaphane blocks the cell cycle in lymphoblas-

toid cells bearing various inherited BRCA1 mutations [10, 12].

Isothiocyanates were found to elevate phase II enzyme activities, like glutathione S-transferases and DT diaphorase in many cancer cell lines [2, 7, 24]. It was observed that SFN conjugation with glutathione (GSH) and accumulation of SFN inside a cell is GSH-dependent [25]. Recently, it has been confirmed that SFN enters *in vivo* the enterocytes in humans and elevates activity of phase II [13].

We have synthesized SFN analogues in order to search for the most efficient chemopreventive agents. The first compound was 2-oxohexyl ITC (CH_3CO-$(CH_2)_4$-NCS), and the second was alyssin (CH_3SO-$(CH_2)_5$-NCS). It was previously described that methyl sulfinyl group (CH_3SO-) could be replaced by an acetyl group (CH_3CO-) without significant changes in QR induction activity in murine hepatoma cells [22]. Elongation of SFN hydrocarbon chain by addition of one methyl group resulted in the formation of alyssin molecule. Alyssin was reported to be present in *Cruciferae* plants, especially from *Alyssum sp*., and to have similar chemopreventive activity as sulforaphane [23].

The aim of this study is to compare the chemopreventive effect of three structurally related isothiocyanates on healthy lymphoblastoid and on leukemia CCRF-SB cells. Our results show strong differences in response to each isothiocyanate between both cell lines, what could be useful in searching for potential chemopreventive dietary compounds.

Materials and Methods

Cells

Human B-lymphoblast acute leukemia cells CCRF-SB were maintained in RPMI-1640 medium, supplemented with 10% of heat inactivated fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin (250 ng/ml) and L-glutamine (2 mM). Human B-lymphoblastoid cells, established in the permanent cell culture by Epstein-Barr virus immortalization of B-lymphocytes originated from a healthy human, were grown in RPMI 1640 medium, supplemented with 19% heat inactivated fetal bovine serum, all other supplements were the same as for CCRF-SB cell line, which was purchased from ATCC. Cells were grown at 37°C in a humified atmosphere containing 5% CO_2 and the medium was changed every 48 h.

Chemicals

Sulforaphane, 2-oxohexyl ITC and alyssin were synthesized as described previously by Schmidt and Karrer [16]. The purity of all ITC was 99.8%, as determined by gas chromatography. MitoLight™ Apoptosis Detection Kit was purchased from Chemicon International, Inc. (Temecula, CA, USA), while Annexin V Kit was form Caltag Laboratories (Burlingame, CA, USA). CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) and dihydrorhodamine 123 were obtained from Molecular Probes. All other chemicals were purchased from Sigma-Aldrich.

General protocol for cell treatment

Cells were incubated in 48-well plates at a density of 2.5×10^5 cells/ml with a series of concentrations of isothiocyanates for 48 h.

Cell viability assay

The culture medium was removed by centrifugation and 200 μl of 5 mg/ml solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] in RPMI 1640 medium without serum and phenol red was added to each of them. After 3 h of incubation, cells were centrifuged the MTT solution was removed and acidic-isopropanol was added. The absorption was measured using microplate scanning spectrophotometer PowerWave X (Biotek Instruments) at 570 nm test and 690 nm reference wavelengths. The final result was the mean of eight experimental data. Experiments were made in duplicate.

NAD(P)H:quinone reductase activity assay

NAD(P)H: (quinone-acceptor) oxidoreductase (EC 1.6.99.2) (QR) activity was estimated in a direct assay, measuring the NADPH-dependent menadiol-mediated reduction of MTT. The reaction mixture was prepared as Prochaska and Santamaria previously described [14].

The QR activity was normalized per mg of total protein in each well and expressed in mU/mg protein. The absorption coefficient of reduced MTT was set at the level of 11300 M^{-1} cm^{-1} at $\lambda = 610$ nm according to Prochaska and Santamaria. Total protein content

was assessed by the Bradford assay. Experiments were made in duplicate.

Determination of ROS formation

The evaluation of ROS production was performed by the dihydrorhodamine 123 (DHR) assay. Dihydrorhodamine 123, which is uncharged, passes freely through the plasma membrane into the cell. Inside the cell DHR is oxidized upon the action of cellular ROS into positively charged rhodamine 123, which accumulates preferentially in mitochondria [15]. Analysis was performed using 96-well black plates. Cells were incubated with 5 μg/ml of DHR 123 in PBS at 37°C for 15 min, and then the cellular fluorescence of formed rhodamine 123 was measured using Fluoroscan (LabSystems) fluorescence plate reader. Excitation and emission wavelengths were 485 and 538 nm, respectively. The results were normalized per mg of total protein in the sample, determined by the Bradford method. Experiments were made in duplicate and each result is the mean of eight measurements.

Measurement of mitochondrial membrane potential changes ($\Delta\Psi$mt)

The changes in the mitochondrial membrane potential were measured using MitoLight^{TM} Apoptosis Detection Kit (Chemicon International, Inc.). Cells, after 48 h of treatment with 2.5 and 5 μM ITCs, were resuspended at the concentration of 1×10^6 cells/ml in MitoLight^{TM} solution according to the vendor's protocol.

The mitochondrial indicator in the kit is 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine chloride. This dye belongs to a group of chemical substances that form J-aggregates [18]. The aggregation leads to a large shift in its absorption and fluorescence spectra to a longer wavelength.

In healthy cells, the dye accumulates forming aggregates and emits red light (λ_{em}= 585/590 nm). When cells have depolarized mitochondria, the dye remains in cytoplasm and its fluorescence is green (λ_{em}= 527/530 nm).

The fluorescence was measured with a confocal microscope (Olympus Fluo View 500 system equipped with Olympus IX70 microscope). A 40× objective lens (UPlan APO) was used. Argon laser (488 nm) and He-Ne (543 nm) were employed to excite sequentially monomers and aggregates, respectively. The fluorescence of monomers was collected through a 505–525 nm bandpass emission filter. The fluorescence of aggregates was collected using a 560 dichroic mirror and a 560–610 nm bandpass filter.

Evaluation of GSH content

The evaluation of GSH content was performed using Cell Tracker green 5-chloromethylfluorescein diacetate (CMFDA) assay. The reagent passes freely through cell membranes. Inside the cells, the dye is conjugated to thiols by glutathione-S-transferases. To become fluorescent, the dye needs to be transformed by cellular esterases. The transformation can occur either before or after the conjugation with thiols [17].

Cells were loaded with 1 μM of CMFDA in PBS for 30 min at 37°C, then the staining solution was removed by centrifugation and replaced by fresh medium and incubated for 15 min at 37°C. The analysis was performed using 96-well black plates using Fluoroscan (LabSystems) fluorescence plate reader. The excitation and emission wavelengths were 485 nm and 538 nm, respectively. The results were normalized per mg of total protein in the sample, measured by the Bradford method. The experiments were made in duplicate and each result is the mean of eight measurements.

Results

The effect of isothiocyanates on cells viability

The effects of sulforaphane, 2-oxohexyl ITC and alyssin on viability of B-lymphoblastoid and CCRF-SB leukemia cells measured by MTT test at 48 h are presented as IC_{50} values and shown in Table 1.

All isothiocyanates revealed similar cytotoxic effect on lymphoblastoid cells with IC_{50} values (concentration that inhibits viability by 50%) approximating 6 μM. Alyssin had the highest IC_{50} value which was significantly different ($p < 0.05$) from IC_{50} value of two other ITCs. IC_{50} values of sulforaphane and 2-oxohexyl ITC were not significantly different in this cell line.

SFN had the highest IC_{50} value in CCRF-SB cells, and it was the highest value obtained in the study on both cell lines. Lower IC_{50} values were obtained for alyssin and 2-oxohexyl ITC, and the value for 2-oxohexyl ITC was the lowest observed in the study.

Tab. 1. The IC_{50}, CD and CI values of isothiocyanates

Isothiocyanate	B-lymphoblastoid			CCRF-SB		
	IC_{50} [μM]	CD [μM]	CI	IC_{50} [μM]	CD[μM]	CI
Sulforaphane	5.9 ± 0.16	0.42 ± 0.07	14.04 ± 2.0	7.75 ± 0.25	3.65 ± 0.44	2.12 ± 0.36
2-Oxoheksyl ITC	6.1 ± 0.22	0.5 ± 0.08	12.2 ± 2.1	3.3 ± 0.66	0.44 ± 0.03	8.25 ± 1.8
Alyssin	6.8 ± 0.16	1.6 ± 0.02	4.25 ± 0.5	4.0 ± 0.13	–	–

Cells were treated with sulforaphane, 2-oxohexyl isothiocyanate and alyssin at increasing concentrations for 48 h and IC_{50} values (μM) were estimated from the MTT survival curves, CD values (μM) were estimated from QR activity curves and CI values were estimated from IC_{50} / CD. Data are shown as the mean ± SE obtained from two independent experiments

All IC_{50} values obtained for lymphoblastoid cells were significantly different in comparison with respective data observed in CCRF-SB.

The effect of isothiocyanates on NAD(P)H: quinone reductase activity

The effect of sulforaphane, 2-oxohexyl ITC and alyssin treatment on QR activity in lymphoblastoid and CCRF-SB cells was evaluated by the method of Prohaska and Santamaria [17] at 48 h. The CD values were estimated from the ITC dose *vs*. QR activity curve as the concentrations required to double QR activity. The CD values are presented in Table 1.

In lymphoblastoid cells, the highest CD value was obtained for alyssin and the lowest for sulforaphane. The inductive potency of 2-oxohexyl ITC was similar to sulforaphane, and both values were significantly different from alyssin ($p < 0.05$).

The highest CD value for CCRF-SB cells was obtained after sulforaphane treatment and this value was the highest observed in the whole study. The CD values observed after 2-oxohexyl ITC treatment in both CCRF-SB cells and lymphoblastoid cells were similar. The QR activity was not doubled after any alyssin concentration tested, therefore, no CD value was obtained.

The chemopreventive indexes

The chemopreventive index (CI) was evaluated from IC_{50} and CD values, according to the equation:

$$CI \quad \frac{IC_{50}}{CD}$$

[19]. The CI values for sulforaphane, 2-oxohexyl ITC and alyssin for lymphoblastoid and CCRF-SB cells are presented in Table 1. The highest value was obtained for sulforaphane and 2-oxohexyl ITC (12.2) in lymphoblastoid cells. In CCRF-SB cells those values were lower. When cells were treated with alyssin, the evaluation of CI was possible only for lymphoblastoid cells, since in CCRF-SB QR activity was not doubled.

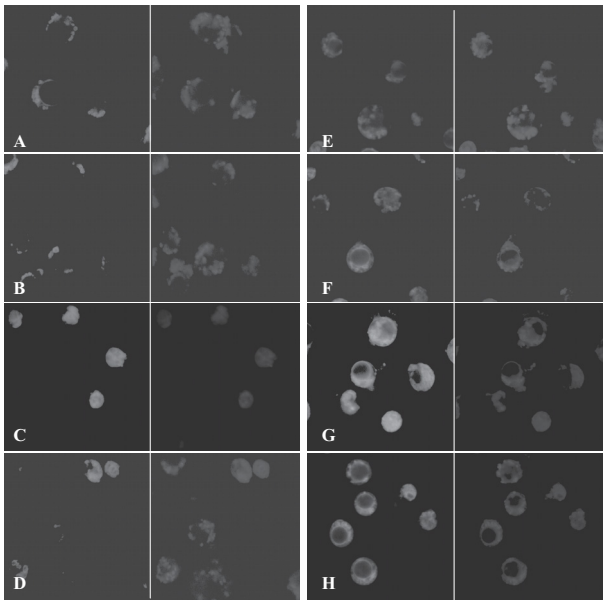

Fig. 1. Microscopic images of cells stained with MitoLight™ Apoptosis Detection Kit. Human lymphoblastoid cells: control (**A**), treated with 5 μM of sulforaphane (**B**), 2-oxohexyl isothiocyanate (**C**) and alyssin (**D**), CCRF-SB cells: control (**E**), treated with 5 μM of sulforaphane (**F**), 2-oxohexyl isothiocyanate (**G**) and alyssin (**H**) for 48 h, were loaded with the MitoLight™ dye and examined with the confocal microscope Olympus FV 500 to detect the changes in the mitochondrial membrane potential. Right image of each pair presents aggregates (red fluorescence) and the left image shows monomers (green fluorescence)

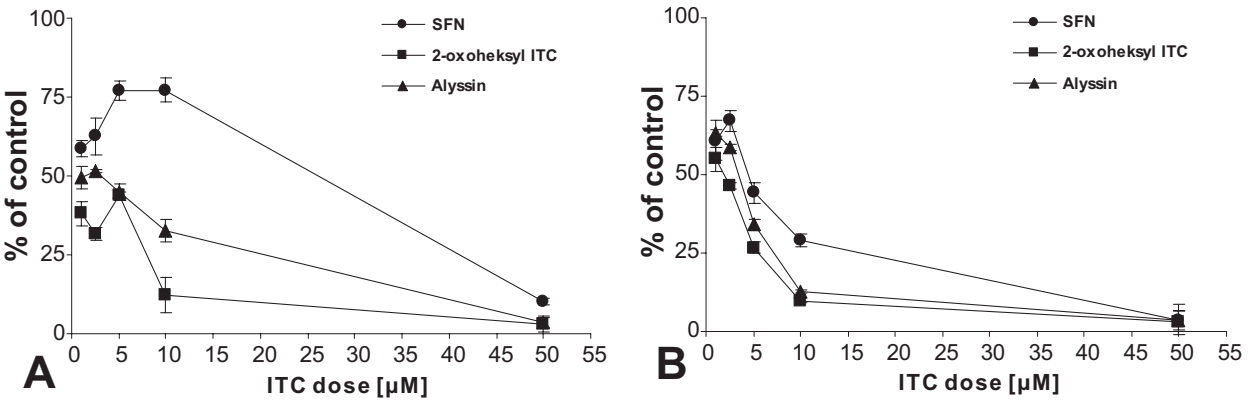

Fig. 2. The changes in GSH level in lymphoblastoid cells (**A**) and CCRF-SB (**B**) after 48 h treatment with sulforaphane, 2-oxohexyl isothiocyanate and alyssin. Cells treated with increasing concentrations of isothiocyanates for 48 h were loaded with CMFDA and examined as described in Materials and Methods. Data are shown as the mean of two independent experiments

The changes in mitochondrial membrane potential

The mitochondrial membrane potential was evaluated using potential-sensitive dye-5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine chloride – Mitolight™. Cells, previously treated for 48 h with 5 μM sulforaphane, 2-oxohexyl ITC and alyssin, were loaded with the dye and tested with the confocal microscope. As shown in the Figure 1, the image analysis revealed high polarization of control cells from both cell lines tested. Sulforaphane caused an increase in green fluorescence with a concomitant decrease in red fluorescence in both cell lines. For both cell lines, the highest depolarization was detected after 2-oxohexyl ITC treatment. Alyssin caused an increase in green with decrease in red fluorescence signals similarly as sulforaphane in lymphoblastoid cells, in contrary to CCFR-SB cells in which it caused stronger depolarization than sulforaphane.

The detection of changes in cellular GSH level

The GSH content was evaluated by fluorescence assay using Cell Tracker Green-CMFDA as indicator. Cells, previously treated with each of three ITC, were loaded with the dye and the fluorescence intensity was measured. As shown in Figure 2, sulforaphane at lower concentrations (1–2.5 μM) caused similar effect in both cell lines, i.e. the decrease in GSH level to 40%, since at higher concentrations used (5–10 μM) the effect in each cell line was significantly different. In CCRF-SB cells, the GSH level decreased further in a dose-dependent manner to 30% at 10 μM. In lymphoblastoid cells the GSH content was elevated to the level of 80% of control at 5 μM and was at the same level for 10 μM. The CCRF-SB cells exposed to 2-oxohexyl ITC revealed the dose-dependent decrease in GSH level, while in lymphoblastoid cells the fall of GSH level was observed at lower concentrations (1–2.5 μM). The significant rise was shown at 5 μM, which was still below the control level, and at 10 μM the significant decrease was observed. After alyssin treatment the dose-dependent drop in GSH level was observed for both cell lines, however, the fall was stronger in CCRF-SB cells. The strongest decrease was observed after 2-oxohexyl ITC, and the lowest was seen after sulforaphane treatment for both cell lines.

The detection of ROS formation

The change in ROS formation was estimated by measurement of H_2O_2 level using dihydrorhodamine 123 assay. The results shown in Figure 3. proved that in lymphoblastoid cells 2-oxohexyl ITC and alyssin treatment resulted in similar profile of cellular H_2O_2 decrease to about 50% of control cells at lower concentrations (1 μM). Higher concentrations caused the dose-dependent rise, with the highest level of 80% in control at 10 μM. Sulforaphane-treated lymphoblastoid cells responded with no changes in H_2O_2 level at lower concentration (1 μM) and after treatment with 2.5 and 5 μM it decreased to 20%, however the higher dose caused the elevation of H_2O_2 level to the control.

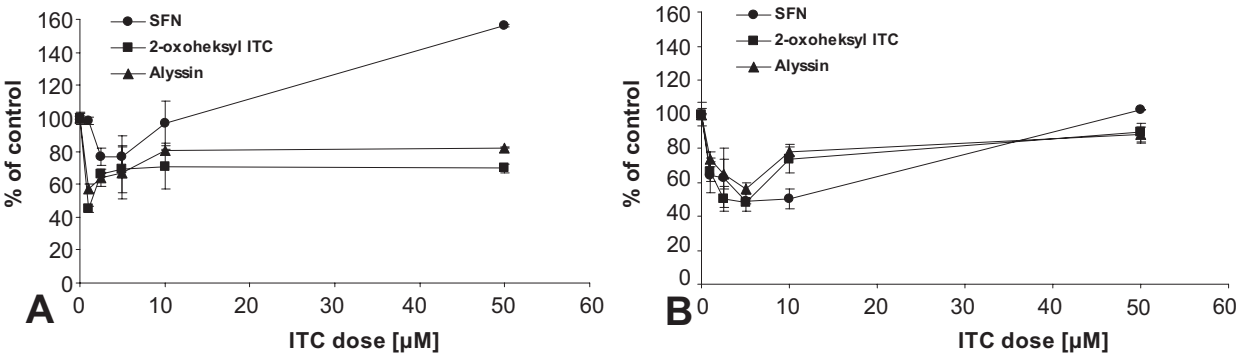

Fig. 3. The changes in ROS measured by DHR 123 assay in lymphoblastoid cells (**A**) and CCRF-SB (**B**) treated with increasing concentrations of sulforaphane, 2-oxohexyl isothiocyanate and alyssin for 48 h and loaded with DHR 123 as described in Materials and Methods. Results are the mean of two independent experiments

In CCRF-SB cells, all three isothiocyanates elicited similar action on H_2O_2 content. The decrease to the level of 50–60% of control was observed in the range of concentrations between 1–2.5 μM of each ITC. At 5 μM no significant differences in H_2O_2 level compared to 2.5 μM were observed, while 10 μM caused an increase for 2-oxohexyl ITC and alyssin. The increase in sulforaphane dose did not significantly change the H_2O_2 content, which remained at similar level of 2.5–10 μM.

Discussion

In the present study, we evaluated the chemopreventive effect of sulforaphane and its two analogues on human B-lymphocyte derived cells. Two cell lines used in the experiments were: human lymphoblastoid cells, obtained by EBV immortalization of B-lymphocytes from a healthy volunteer and human B-leukemia CCRF-SB. The cell lines were treated with the series of concentrations of three isothiocyanates: sulforaphane, 2-oxohexyl ITC and alyssin and the viability of the cells, induction of a phase II enzyme – QR, apoptosis induction, GSH content and ROS formation were evaluated.

Sulforaphane was shown to induce phase II enzymes in many cell lines, like hepatoma HepG2, breast cancer MCF-7, prostate cancer LNCaP or cervical cancer HeLa cells [2, 7, 24] and to induce cell death in DU-145 prostate cancer, human non-transformed T lymphocytes, human T leukemia or BRCA1 mutated lymphoblastoid cells [4, 5, 12].

We found that sulforaphane was more cytotoxic for lymphoblastoid than for leukemia cells, however, it induced QR activity at lower dose in lymphoblastoid cells than in CCRF-SB line. The dose of sulforaphane needed to induce phase II enzymes in healthy cells was almost 9 times lower than in CCRF-SB cells. It was proven, that in people after ingestion of 200 μmol of broccoli sprout the concentration of isothiocyanates (most of which was sulforaphane) in blood reached 1 h after ingestion nearly 1 μM [21]. In accordance with those results, our experiments show that sulforaphane in that concentration range cannot induce phase II enzymes in CCRF-SB leukemia cells. Those concentrations induce QR activity in healthy cells. Higher concentrations caused cell death through apoptosis, what was proven by the detection of mitochondrial membrane depolarization. In healthy cells, sulforaphane slightly decreased GSH content what is in accordance with the known mechanism of sulforaphane accumulation in cells by GSH conjugation [25]. Once sulforaphane is conjugated with GSH, the level of free thiols decreases, what regulates genes containing ARE and encoding detoxificating enzymes [3]. In CCRF-SB cells, the decrease in GSH content was observed too, however, this was mostly the effect of apoptosis, since the decrease was rapid and more dramatic than in healthy cells. The level of cellular ROS was found to be reduced in both cell lines. We consider that the decrease observed in CCRF-SB cells is more important in this experimental model. It was shown that elevated cellular ROS level could be responsible for neoplastic cell proliferation by promotion of cell division and blocking of cell death signaling pathways [1].

2-Oxohexyl ITC was shown to be almost twice more cytotoxic for leukemia than for healthy cells, however, it induced QR activity at the similar concentrations in both cell lines. It was previously described that methyl sulfinyl group (CH_3SO-) can be replaced by an acetyl group (CH_3CO-) without significant changes in QR induction activity in murine hepatoma cells [22]. Our observations made on healthy cells are in accordance with those results, however, we observed significant differences between sulforaphane and 2-oxohexyl ITC in potency of induction of phase II enzymes in leukemia cells. The latter was more potent, what could not be necessarily desired in leukemia therapy, since it could cause resistance of cancer cells to therapy. However, almost twice higher cytotoxicity against leukemia than against healthy cells could be promising in using this isothiocyanate as apoptosis inducing agent. It was previously observed by us that these isothiocyanates caused cell growth cessation and induced apoptosis in murine leukemia L1210 and in human melanoma ME-18 cells [11]. The strongest GSH decrease was caused by this isothiocyanate in CCRF-SB cells in a dose-dependent manner, while in healthy cells the rise was observed after the first decrease. It was shown that, like for sulforaphane, the 2-oxohexyl ITC decreased H_2O_2 level. We observed that the same mechanisms as for sulforaphane were responsible for these effects.

In healthy cells, alyssin was found to be the least cytotoxic among all tested isothiocyanates and its CD value was the highest one, but still in the range of possible blood concentration, it was non-toxic for healthy cells. This isothiocyanate was found to be more cytotoxic for leukemia cells and the activity of QR was not doubled, what can speak for this isothiocyante as a possible chemopreventive agent during chemotherapy. It seems not to induce protective mechanisms in neoplastic, but to don't in healthy cells. These properties of alyssin are extremely important, when considering side-effects of chemotherapy in healthy cells of the organism. Alyssin induces apoptosis in both cell lines, however, the stronger apoptotic effect was observed in leukemia cells at 5 μM. The GSH decrease was observed in both cell lines, however, the decrease was stronger in CCRF-SB cells. It was shown that, like for two other isothiocyanates, alyssin decreased the H_2O_2 level. We propose that the same mechanisms underlay alyssin action as that of sulforaphane, while alyssin is reported to be structurally related and produced by similar mechanism in *Cruciferae* plants [8].

In conclusion, we have shown that sulforaphane and its analogues have different potencies in inducing apoptosis and QR activity in healthy and leukemia cells. 2-Oxohexyl isothiocyanate, which was previously shown to be effective in killing human melanoma cells was the most potent in killing leukemia cells [11]. Alyssin, which was described by Kjær in 1960 to be present in *Alyssum* plants seems to be the most promising isothiocyanate to use in chemoprevention [8]. All three isothiocyanates induce apoptosis in both cell lines, however, the stronger induction was detected in leukemia cells, what indicates that the elimination of neoplastic cells from the organism can include apoptotic cell death.

We assume that further studies should be conducted to elucidate the mechanisms activated by each isothiocyanate, however, our results strongly indicate the utility of those compounds in cancer prevention and chemotherapy assistance.

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