Comparison of inhibition of murine leukaemia cell growth by 9-isothiocyanatoacridine and its cytosine adduct: involvement of thiols

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Cytotoxicity of two fluorescent acridine derivatives – 9-isothiocyanatoacridine (AcITC) and N-(9-acridinylthiocarbamoyl) cytosine (AcTCC) – a novel acridine compound, were investigated. Both substances have cytotoxic activity against the L1210 cellular line, IC_{50} values were in the micromolar range. Despite the high reactivity of AcITC towards thiols, its effects on leukemia cells were similar to naturally occurring isothiocyanates. AcITC changed the intracellular level of glutathione (GSH), and induced apoptosis. Arrest of cell cycle (G_2/M-phase) was also observed. AcITC primarily reacted with -SH groups on cellular surface, and the study of the interaction of the isotiocyanate with human erythrocyte ghosts confirmed that the plasma membrane was the first place where AcITC bound. AcTCC does not react with cellular thiols; images obtained with fluorescent microscopy confirmed interaction of AcTCC with chromatine. Although AcTCC induced cellular arrest in the G_2/M phase, apoptosis was not confirmed.

Key words: glutathione; thiols; isothiocyanate; growth inhibition; apoptosis

Several epidemiological studies have reported that dietary consumption of cruciferous vegetables is inversely correlated with a risk of cancer development [1]. The protective effect is associated with the isothiocyanates (ITCs). These organo-sulfur compounds are released with the hydrolysis of glucosinolates [2]. Natural ITCs are capable of both inhibiting the formation of cancer cells and eliminating existing cancer cells. These ITCs stimulate the expression of proteins, which have antioxidative/anticarcinogenic properties and induce apoptosis [3]. Studies of the cellular signalling pathways showed that modulation of ERK1/2, JNK, Akt, and p38 are involved in ITC-induced apoptosis [4, 5]. Isothiocyanates inhibit cell-cycle Cdk activity. More recently, up-regulation of p21/WAF1 and inhibition of histone deacetylases were observed in response to ITC exposure [6, 7, 8, 9].

In order to design more effective anticancer drugs, a large number of isothiocyanates have been synthesised and tested. The biological activities of synthetic heterocyclic isothiocyanates were initially investigated over thirty years ago [10]. The anticancer activity of acridine derivatives (naturally occurring ITC compounds) were first considered in 1920´s, and the best known of these compounds, amsacrine, is used in the treatment of acute leukemia [11]. Recently, this research group found out that 9-isothiocyanatoacridine (AcITC) inhibits proliferation of K562 leukemia cells [12]. However, the cytostatic activity of AcITC has not been investigated in detail. AcITC acts as a multitarget anticancer drug. It is thought that the intercalation property of the acridine chromophore confers to AcITC high affinity to DNA, as DNA is considered to be the target for acridine anticancer drugs. The strong electrophillic character of the NCS group on the ITC modulates the level of cellular thiols. Brousewitz et al. [13] showed thiocarbamoylation of the cysteinyl -SH group of GSH by isothiocyanate, however thiocarbamoylation of thiols can occur even at other sites [14]. Some -SH moieties on cysteine in proteins can ionise to thiolate anions in a high pH environment. These thiols have enhanced reactivity for isothiocyanate [10]. Recently, Hong et al. [15] obtained the first evidence of sulforaphane-Keap1 thionoacyl adduct formation. Considering the reactivity of ITCs, it is reasonable to suppose that some -SH rich-proteins on the plasma membrane could be an initial target for thiocarbamoylation.

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In order to obtain more information about the interaction of isothiocyanates with protein thiols and the role of the intracellular GSH pool in cytotoxicity, the biological effect of 9-isothiocyanatoacridine (AcITC) was investigated in the present study. GSH depletion by ITCs plays a critical role in the sensitization of cells to the induction of apoptosis. However, a loss of intracellular GSH is not sufficient in itself to initiate apoptosis [16, 17, 18]. Because of this, we synthesized N-(9-acridinylthiocarbamoyl) cytosine (AcTCC), a substance which does not react with thiols, and compared its activity to AcITC. The effects of both these compounds were studied on L1210 mice leukemia cells. Erythrocyte ghosts were used as a model for studying of the interaction of AcITC and AcTCC with cellular membranes.

Material and methods

Reagents: Propidium iodide (PI), Hoechst 33342, ethidium bromide, Triton X-100, reduced form of glutathione (GSH), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), phenethyl isothiocyanate (PEITC) and NADPH were obtained from Sigma-Aldrich Chemie (Germany). EDTA, RNase A and proteinase K were purchased from Serva (Germany). 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) was from Merck (Germany) and glutathione reductase was from Calbiochem (USA). Other chemicals were purchased from Lachema (Czech Republic).

Methods.

Synthesis of 9-isothiocyanatoacridine: 9-isothiocyanatoacridine was prepared according to Mazagova et al. [19] through the reaction of 9-chloracridine with AgSCN.

Synthesis of N-(9-acridinylthiocarbamoyl) cytosine hydrochloride: To an aqueous solution (10 ml) of 1.6 mmol cytosine we added 20 ml of 2 mmol 9-isothiocyanatoacridine in pyridine. The reaction mixture stirred while it was heated to 60 °C for 2 h. Excess of 9-isothiocyanatoacridine was removed by extraction with benzene. The aqueous phase was then separated and an equivalent amount of hydrochloric acid was added to the aqueous phase, which precipitated N-(9-acridinylthiocarbamoyl) cytosine hydrochloride, this was washed with methanol and dried. m.p. 304C, ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.58-7.62 (m, 4H, Acr); 7.94 (d, 1H, Cy, J=8.68 Hz); 8.051-8.013 (m, 4H, Acrl; 8.71 (d, 1H, Cy, J=8.60Hz); 10.09 (bs, 1H, NHCS; 13.90 (bs, 1H, Cy). Anal .calcd for $C_{18}H_{13}N_5O_3$.HCl : C, 56.32; H,3.67; N, 18.25. Found: C, 56.13; H,3.78; N, 18.39. ^{1}H NMR spectra were recorded on Variant Mercutry NMR 400 instrument using DMSO-d_6. The chemical shifts are reported in ppm (δ scale) and all coupling constants (j) values are in hertz (Hz). The splitting patterns are designated as follows: s (singlet), d (doublet), m (multiplet), bs (broad singlet).

Kinetic analysis of the reaction of ITC with thiols: Reactivity of the ITCs with thiols was investigated in system containing 50 mM Clark-Lubs buffer, pH=6.0-7.0 containing 30% of acetonitrile at 25 °C. The kinetic measurements were performed under conditions for a pseudomonomolecular reaction and the reaction rates were measured spectrophotometrically on a PHILIPS instrument (PU 8750 UV/VIS). Pseudo-first-order rate constants (k_{obs}) and rate constants of second-order reaction (k_2) were calculated.

Human erythrocyte ghosts preparation: Blood from healthy donors was purchased from the National Blood Transfusion Service (Bratislava, Slovakia). Erythrocytes were separated from plasma and leukocytes by centrifugation for 10 min at 4 °C at 600×g and washed three times with ice-cold phosphate-buffered saline (PBS: 0.15 M NaCl, 1.9 M NaH_2PO_4, 8.1 Na_2HPO_4 M, pH 7.4)**.** The erythrocyte ghosts were prepared from washed cells according to the method described in Dodge et al. [20]. The cells were haemolysed with 50 volumes of lysing medium (pH 3.6), containing 0.1 M $CaCl_2$, 4 M $MgSO_4$ and 1.3 M acetic acid and loaded with reconstitution medium containing KCl (150 mM), Tris (25 mM) at pH 9.2 and centrifuged for 20 min at 4 °C at 20 000×*g*. Then the ghosts were resuspended in ice-cold 5 mM phosphate buffer, pH 7.4, and centrifuged again. The process was repeated until the ghosts were free of residual haemoglobin. Protein determination in the erythrocyte ghosts was performed according to the method of Bradford [21] using bovine serum albumin as a standard.

Cell culture: Murine leukemia cell line (L1210) was obtained from the American Type Culture Collection (Rockville, MD, USA). The cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, NY, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. All *in vitro* experiments were performed during the exponential phase of cell growth.

Cytotoxicity analysis by direct cell counting: Cell proliferation, growth curves and cytotoxic potential of compounds were determined by the trypan blue dye exclusion test. For the 3-day experiments; cells were seeded at 1×10^5 cells/ml in Petri dishes. Cell proliferation was checked after 24, 48 and 72 h. Growth curves, cytotoxic effects and inhibition concentration IC_{50} were determined at the end of 3 days. All dye exclusion tests were performed three times.

Morphological changes: Apoptosis was evaluated by direct cell counting of cells with the apoptotical morphological features after Hoechst 33342 and PI staining. L1210 cells were stained with 10 μM of Hoechst 33342 and PI for 10 min then counted under a fluorescent microscope (JENA LUMAR, Carl Zeiss, Germany). Necrotic cells showed red-stained nuclei, whereas viable cells showed green and round nuclei, having been stained only with Hoechst 33342. Apoptotic cells exhibited, green or red nuclear fragmentation, apoptotic bodies and plasma membrane blebbing. AcITC and AcTCC are fluorescent substances and morphological changes of leukemia cells were examined without Hoechst 33342, using fluorescent microscopic examination with a 378-456 nm excitation block filter and 420/450 nm emission filter. Images of the

cells were captured using a digital camera (Olympus Camedia C-4000).

Apoptosis detection by DNA fragmentation and flow cytometry: The DNA fragmentation (intranucleosomal DNA cleavage) was detected from L1210 DNA extracts, using gel electrophoresis. Approximately 2×10^6 cells were washed with cold PBS twice and then lysed in 200 µl TE lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5% Triton X-100) for 15 min at 4 °C. After centrifugation at 10 000×g at 4 °C for 20 min, 10 µl RNase A (2.5 mg/ml) was added to the supernatant and incubated for 10 min at 50 °C and then 10 µl proteinase K (2.5 mg/ml) was added and the solution was incubated again for 30 min at 50 °C. 100 µl NaCl (1 mM) with 1 mM EDTA and 200 µl isoprophanol was added and the solution was held for 6 h at -20 °C. After centrifugation (20 min, 4 °C, 10 000×g) DNA precipitates were washed in 70% ethanol, then dissolved in TE buffer. Samples were loaded into 1.5% agarose gel, then stained with ethidium bromide (1 µg/ml), and subjected to electrophoresis (6 V/cm, 45 min). Separated DNA fragments (DNA ladders) were visualized on a UV transilluminator and their size was determined by comparison with DNA size markers (from 250 to 10000 bp).

Fluorescein diacetate (FDA)/PI staining: Apoptosis of the L1210 leukaemia cell line was detected by fluorescent staining using flow cytometry. Approximately 5×10^5 cells were washed twice in PBS and resuspended in 400 µl of PBS/0.2% bovine serum albumin (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, after 15 min the stained cells were analyzed using a Coulter Epics Altra flow cytometer.

Cell cycle analysis: This assay was based on the measurement of the DNA content of nuclei labelled with propidium iodide. For flow cytometry analyses of DNA cell cycle profile, approximately 5×10^5 cells were collected by centrifugation at 700×g for 3 min. The cells were washed twice with cold PBS and resuspended in 0.05% TritonX-100 and 15 ml of RNase A (10 mg/ml) for 20 min at 37 °C. The cells were then cooled and incubated on ice for at least 10 min before PI (50 mg/ml) was added. Finally, after 15 min the stained cells were analyzed using a Coulter Epics Altra flow cytometer.

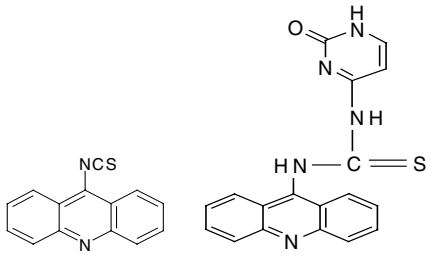
Flow cytometry measurements and data analysis: Coulter Epics Altra flow cytometer was equipped with 488 nm excitation laser and fluorescence emissions were measured using band-pass filter set at 525, 575, 610, and 675 nm. Forward/side light scatter characteristic was used to exclude the cell debris from the analysis. For each analysis, 1×10^4 cells were acquired for analysis. Data were analyzed with WinMDI version 2.7 software (J. Trotter, Scripps Research Institute, La Jolla, CA). The cell cycle calculations were performed with MULTI-CYCLE Software (Phoenix Flow System).

Determination of glutathione: Glutathione was measured according to the method described by Anderson [22]. To quantify the total molecular level of GSH (tGSH=GSH + GSSG), cells were washed with PBS twice (total amount of cells was 2×10^6) and then lysed by freezing and thawing (two cycles) in 450 µl of 1 mM EDTA, then was added 50 µl 16% (w/v) sulfosalicilic acid. This suspension was centrifuged (15 min, 4 °C, 10 000×g), then the acid supernatant was neutralized by 50 µl saturated $NaHCO_3$, 450 µl 150 mM sodium phosphate buffer (pH 7.6) and used for total GSH measurement. The pellet was used for protein determination. After 20 µl 5 mM NADPH, 20 µl 10 mM DTNB and 500 µl of 150 mM sodium phosphate buffer (pH 6.2) were added to 450 µl of neutralized supernatant, the assay was initiated by addition of 10 µl of glutathione reductase (470 U/ml). The absorption at 412 nm for 5 min at 25 °C was determined. tGSH concentration values were calculated from a standard curve and expressed in nmol/10^6 cells.

Determination of cell-surface SH groups: Cells after treatment were washed with PBS and DTNB was added to the cells (1×10^6/ml) to a final concentration 200 µM in PBS. After 20 min at room temperature, the supernatant was harvested and its absorbance read at 412 nm. Results were expressed in nanomoles of -SH per 10^6 cells calculated from standard curve of reduced GSH.

Statistical evaluation: All experiments were performed in triplicate and the results shown in the graphs represent the mean and standard deviation.

Table 1. Structure and fluorescence characteristics of AcITC and AcTCC



AcITC AcTCC

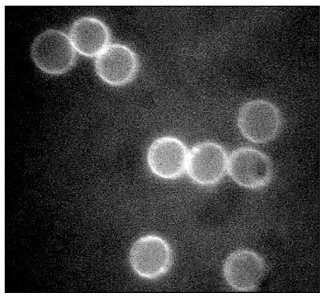
Compound	Ex_{max} [nm]	Em_{max}[nm]	Intensity	ϕ
AcITC	386	435	1.0	0.11
AcTCC	395	461	6.4	1.00

Fluorescence intensities were measured using 50 µM compounds 50 mM HEPES buffer (pH 7.4) containing 30% of dimethylformamide at 21 °C, ϕ : fluorescence quantum yield.

Results

Physical-chemical characteristics. Fluorescent spectra for AcITC and AcTCC were measured in system consisting of 50 mM Hepes solution buffer (pH 7.0) containing 30% dimethylformamide. The fluorescent characteristics of AcITC and AcTCC are given in Table 1. The fluorescent intensity of AcTCC is higher than the intensity of AcITC. The fluorescent parameters of both compounds allow the use of fluorescent techniques to determine their accumulation in cells.

a)



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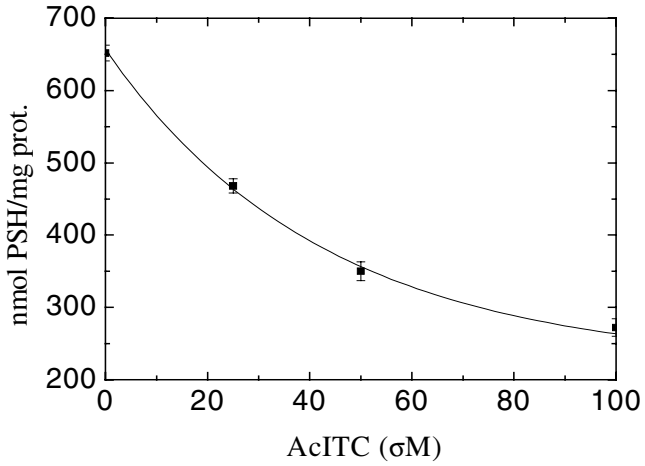


Figure 1. Localization of AcITC in membrane of human erythrocyte ghosts (a) and changes of PSH level of erythrocyte ghost cells (b) after one-hour incubation with AcITC. Representative fluorescent microscopy images of AcITC- treated ghosts were obtained by fluorescence microscope.

Table 2. Second order rate constants for reaction of ITCs with GSH and NAC

ITCs	k_2 [$M^{-1}.s^{-1}$]	
	GSH	NAC
PEITC	0.60±0.01	0.32±0.01
AcITC	14.16±0.07	3.36±0.06

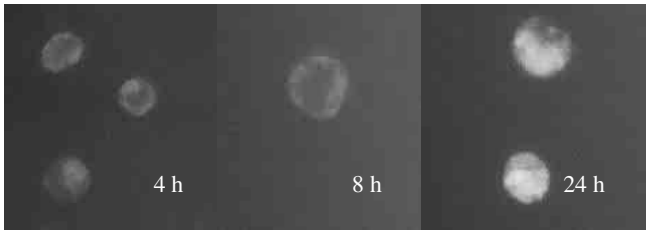
Starting concentrations were 0.05 mM ITC and 2.5 mM thiol in 100 mM Clark-Lubs buffer (pH 6.5) containing 30% ACN at 25 °C.

Table 3. Effect of the AcITC and AcTCC on L1210 growth and cell viability

		AcITC	AcTCC	PEITC
Direct cell counting	48 h	3.2±0.2	2.7±0.1	4.0±0.1
IC_{50} [μM]	72 h	3.7±0.2	2.4±0.1	3.8±0.1

Concentrations of the compounds were in the range 1.6 – 25 μM.

10 σM AcITC



10 σM AcTCC

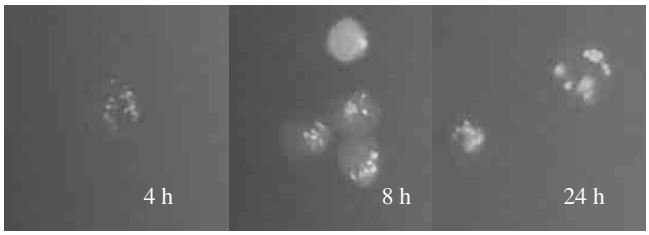


Figure 2. Cellular localization of AcITC and AcTCC. Treated cells were stained with PI and then observed by fluorescent microscopy (400×). Significantly induced blebbing of plasmatic membrane, condensed chromatin and fragmented nuclei. Images were obtained by fluorescence microscope.

Table 2 shows the estimated second-order rate constant, k_2, for the reaction of PEITC and AcITC with GSH and NAC in a system consisting of buffer and organic solvent. The reactivity of ITC depends on basicity of thiols and comparison of the reactivity of ITCs show that AcITC is 10-25 more reactive with respect to thiols than PEITC.

Interaction with plasmatic membrane: cell membrane binding. Human erythrocyte ghosts were incubated with 100 μM AcITC or 100 μM AcTCC at 37 °C, for 60 min than 3-times washed in PBS solution. Fluorescent microscopy observations showed that only AcITC had been accumulated particularly in plasmatic membrane (Fig. 1). We have supposed that AcITC reacts with cysteinyl residues from membrane proteins (PSH). To investigate the interaction of PSH with AcITC, ghosts were incubated with doses of AcITC ranging between 25 and 100 μM, at 37 °C, for 60 min. At the end of treatment, the ghosts were washed by centrifugation and the level of PSH was measured. As Figure 1a shows the typical fluorescent microscopic image of AcITC accumulating in the plasma membrane. Figure 1b shows a dose dependent decrease of sulfhydryl groups in human erythrocyte ghosts when the cells have been incubated with AcITC.

The results demonstrate that insertion of AcITC into the membrane may have important functional implications for the mechanism of cell death induced by the isothiocyanates

Antiproliferative activity. Both acridine compounds were evaluated *in vitro* for their inhibitory effects on proliferation of murine leukemia L1210 cells. Cells were harvested at 24, 48 and 72 h and the viable cells were counted by the trypan

blue exclusion test. The results, expressed as IC_{50} values (the concentration of tested compounds leading to 50% inhibition of cell growth), are reported in Table 3.

Direct cell counting indicated that antiproliferative activity of PEITC was comparable to AcITC and both isothiocyanates were less cytotoxic than AcTCC.

Uptake and cellular localization of substances. AcITC and AcTCC are fluorescent substances therefore their uptake and intracellular localization and the consequential morphological changes of cells could be observed by fluorescent microscopy without staining with specific dyes, with the exception of propidium iodide to determine cellular necrosis. AcITC and AcTCC were used in concentrations ranging from 2 μM to 50 μM. Intracellular distribution of AcITC and AcTCC and morphological signs of apoptosis (apoptotic blebbing, nuclear condensation and apoptotic bodies) were assessed during one-day incubation.

AcITC has been found mostly in plasma membranes after 4 h incubation with L1210 cells, after longer incubation AcITC was present in the cytosol. A typical cellular accumulation of 10 μM AcITC is shown in Figure 2. (images of cells treated with 2 μM AcITC are not presented because of low fluorescence intensity).

As seen in Figure 2, AcTCC was not accumulated in the plasma membrane. AcTCC was found in the nuclei right from the beginning of the incubation. The nuclei and the cytoplasm showed green fluorescence indicating the presence of AcTCC was shown at the beginning of incubation, and granular green fluorescence was observed in the nuclei in 8 h, suggesting increased concentration at 8 h.

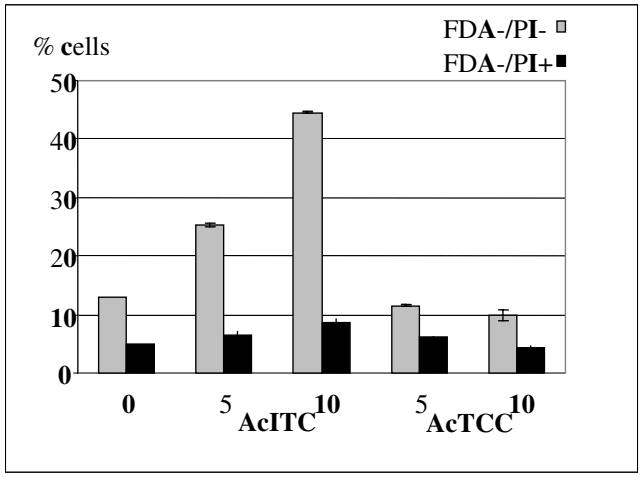
The morphological changes in the cells became evident after 4 h of incubation with AcITC. At 10 μM concentration numerous treated cells exhibited the characteristic morphological change of apoptosis – membrane blebbing and nuclear condensation, after 4 h of incubation (Fig. 2). In addition to morphological evaluation, the level of apoptosis induction by AcITC was ascertained by measuring DNA fragmentation, a biochemical hallmark of apoptosis. As illustrated in Figure 3b, agarose gel electrophoresis of DNA extracted from L1210 cells treated with AcITC (2 and 10 μM) for 24 h revealed a progressive increase in non-random fragmentation into a DNA ladder.

The different action of AcITC and AcTCC on leukemia cells was confirmed by apoptotic assay FDA/PI. As seen in Figure 3a, only AcITC has ability to induce apoptosis of L1210 cells in a concentration-dependent manner.

Cell cycle analysis. Flow cytometric analyses of cell cycle were performed after 6 h and 24 h incubation of L1210 cells with AcTCC or AcITC. As seen in Figure 4, 10 μM AcTCC induced cell-arrest in S-phase (about 56% cells) after short exposure. After one-day incubation the cell-cycle was entirely blocked in G_2/M-phase (about 73% cells) and 22% cells were arrested in S-phase.

The increase of G_2/M cells accompanied by decrease of G_0/G_1 cells in the AcITC treatment for 6 h were found, but substantial changes in G_2/M phase (about 63%) were observed after 24 h in 10 mM AcITC treated cells.

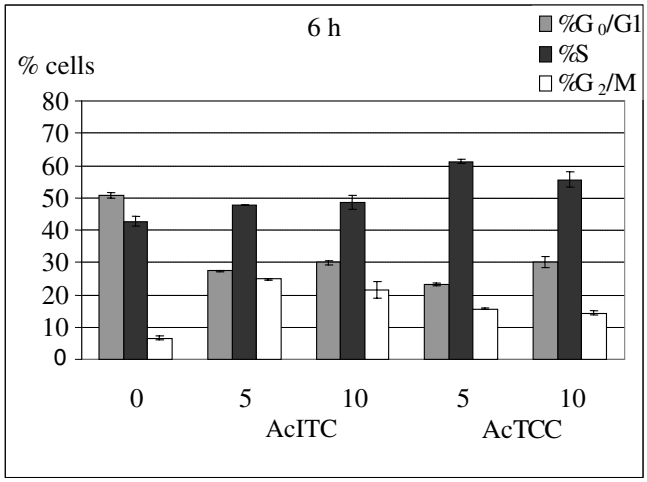
a)



b)

Figure 3. Mode of cell death induced by AcITC and AcTCC. Effect of AcITC and AcTCC treatment on apoptosis and necrosis induction in L1210 cells by flow cytometry (a). Exposure to either DMSO (control) cells or different concentrations (5 μM and 10 μM) of AcITC or AcTCC treated L1210 cells for 24 h. Percentage of apoptotic (FDA-/PI-) and necrotic (FDA-/PI+) cells were shown. The cells were analyzed using a coulter Epics Altra flow cytometer with photomultipliers FL1 required for fluorochrome FDA and FL2 for PI. The data presented are from two independent experiments; and the results are means ± S.D. Intranucleosomal fragmentation of DNA induced by AcITC (b). DNA extracts from AcITC-treated L1210 cells (24 h) were resolved on a 1.5% agarose gel (1-standard, 2-control, treatment with AcITC: 3 – 10 μM, 4 – 2μM).

Intracellular thiols. In order to assess the involvement of cellular thiols in cytostatic effects of AcITC and AcTCC the level of glutathione and cell surface -SH groups were estimated during cultivation of cells in the presence of the acridine derivatives. The cells (1×10^6 cells/ml) were incubated with the compounds in concentration of 2, 10 μM and



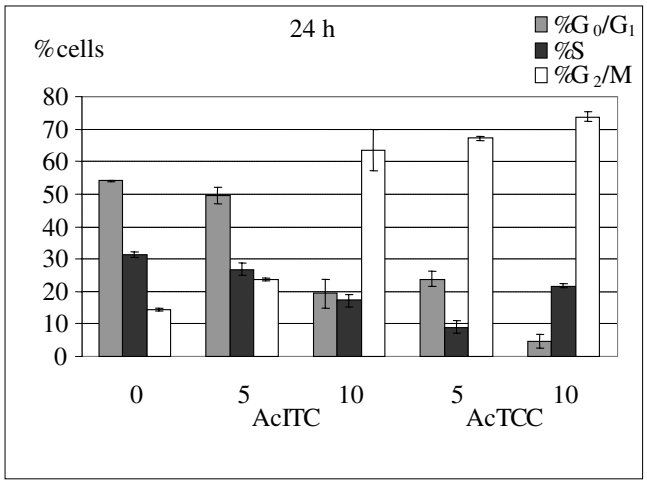
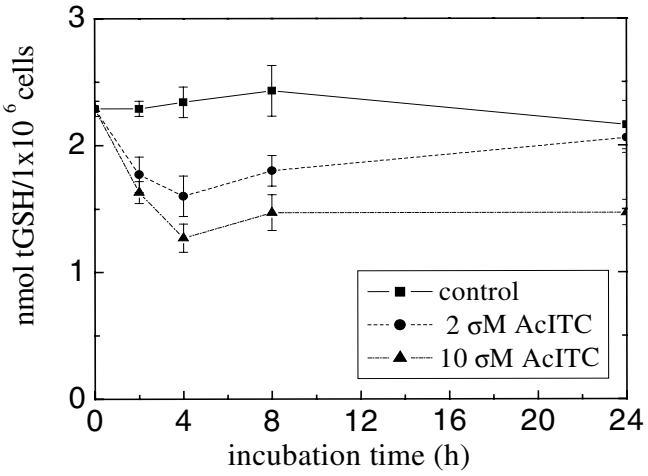


Figure 4. Cell cycle analysis of L1210 cells exposed for 6 h and 24 h to acridine drugs by flow cytometry. Distribution of cells in G_0/G_1, S and G_2/M phases were determined by Multi-cycle software of untreated (control) and AcITC or AcTCC-treated cells at 5 μM and 10 μM concentrations. Three independent experiments were performed and mean ± S.D are presented.



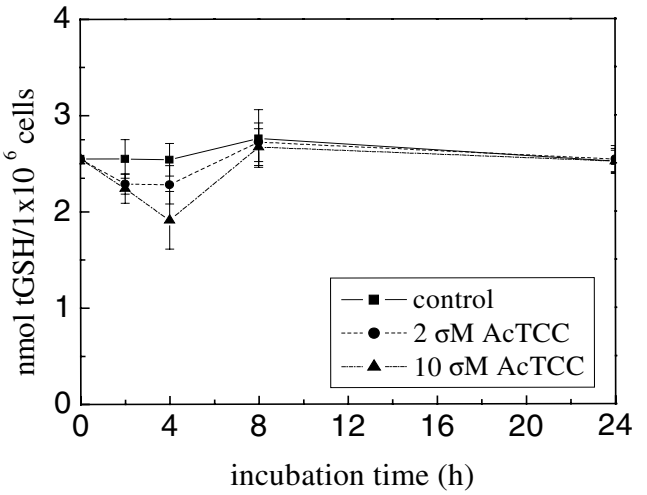


Figure 5. The effect of AcITC and AcTCC on intracellular glutathione. The data are expressed as nmol tGSH ± S.D per 1×10^6 cells of triplicate determinations from three separate experiments.

the concentration of intracellular glutathione (tGSH) was measured during one-day incubation. tGSH concentration (Figure 5) declined in the initial period of incubation. This decrease was dependent on concentration of both substances. AcITC produced greater decline in tGSH than AcTCC. tGSH levels returned to the initial level by 8 h when cells were treated with AcTCC. tGSH levels took 24 h to recover in cells treated with with 2 μM AcITC, while in cells treated with 10 μM AcITC the glutathione level remained suppressed.

Although both compounds reduced GSH levels, only AcITC was able to modify -SH groups on the cell-surface (csPSH) (Fig. 6). Approximately 60% of csPSH was modified by the 10 μM AcITC concentrations. AcTCC had no effect on csPSH at either low or high level concentrations (data not shown).

Discussion

The chemical basis of the biological activity of ITCs is their reactivity with cellular thiols. Exposing cells to ITCs leads to a transient change in redox state of glutathione [14]. Although dithiocarbamates resulting from conjugation with reduced GSH were thought to account for most of the activity of ITC [23, 24], thiocarbamoylation of -SH proteins was also theorised. Recently, Hong et al. [15] confirmed

sulforaphane-Keap1 thionoacyl adduct formation. As well as intracellular thiols, there are cellular membrane -SH groups that could be modified by isothiocyanates. This research involved the synthesis of AcITC and AcTCC and observation of the reaction of these compounds with cellular thiols. Biological effects have been observed which demonstrates activity of AcITC and AcTCC on GSH levels and cell surface thiols.

AcITC and AcTCC posses an interesting cytotoxic activity against L1210 cellular line and IC_{50} values are in micromolar range. However, cytotoxicity of the amsacrine, the best-known compound of acridines series, is about 50 times higher [25]. AcITC and AcTCC disturbed the cell cycle, with an accumulation of cells in the G_2/M phase but only AcITC induced apoptosis in the cell line. Almost all agents in the acridine series have DNA-intercalative properties. Consequently, it had been supposed that AcITC and AcTCC bind either reversibly or irreversibly to DNA. Indeed, AcTCC was accumulated directly in nucleus and probably its interaction with chromatine interrupted the cells in the S-phase after a short incubation time and in G_2/M phase of cell cycle after extended incubation. AcTCC, a compound without thiol reactivity temporarily decreased the level of GSH (25% reduction in a 10 μM AcTCC) and the level of GSH returned to baseline after 8 h of cellular incubation. No signs of apoptosis were observed in the presence of AcTCC. AcITC induced apoptosis and markedly reduced the level of cellular thiols. Despite the high reactivity of AcITC towards thiols, the cytotoxicity of AcITC was not higher than cytotoxicity of PEITC. The low cytotoxicity of AcITC is probably due to its interaction with -SH groups on cell surface. About 90% of the AcITC was bound to -SH proteins as demonstrated by a reduction in the level of csPSH. Only a small amount of AcITC entered the cells where it reacted with GSH. PEITC (10 μM) reduced of the level of csPSH (results are not shown) but its effect was only temporary and the level of csPSH returned to initial levels after a 4 h incubation period. GSH conjugates can be broken down and transported from cells. GSH transferases can catalyze not only conjugations of ITCs with GSH, but can also deconjugate GSH-ITC complexes [26].These conjugates undergo further enzymatic modifications and the by-products can be eliminated from the cells by the multidrug resistance associated protein-1 (MRP-1) or P-glycoprotein-1 (Pgp-1) [27, 28, 29]. It is possible that the degradation products of AcITC (acridone or 9-aminoacridine), were translocated to the nucleus where they bound to chromatine.

The reactions of csPSH with AcITC had reduced cytotoxicity of AcITC. Despite the high reactivity of AcITC towards thiols, the biological effects of PEITC and AcITC are similar, the level of intracellular GSH is reduced, cell-cycle is arrested and apoptosis is induced [4, 14, 30]. Our results have shown that although the biological action of ITCs depends on reactivity of NCS group, high reactivity may not be associated with high cytotoxicity.

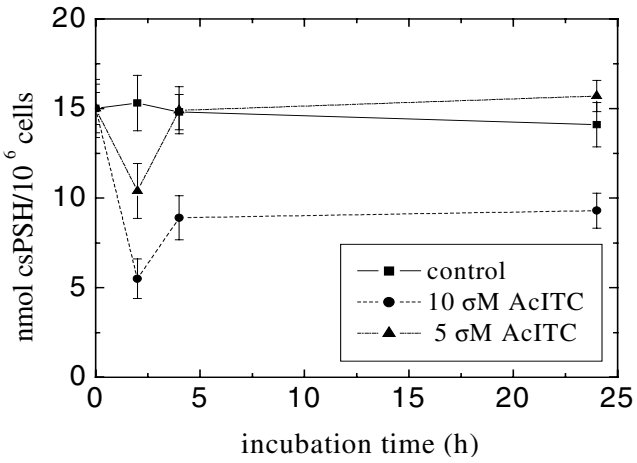


Figure 6. The effect of AcITC on cell-surface protein thiols (csPSH). L1210 cells (1×10⁶ cells/ml) were incubated with AcITC and csPSH were measured by using DTNB reagent. The data are expressed as a nmol csPSH ± S.D per 1×10⁶ cells of triplicate determinations from three separate experiments.

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