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Intracellular distribution of 3,6-bis(3-alkylguanidino)acridines determines their cytotoxicity

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Cytotoxicity of two derivatives of 3,6-bis(3-alkylguanidino)acridines (GNDAs; pentyl- and hexyl-GNDA) was determined against three cell lines: a murine immortalized fibroblast cell line NIH-3T3, a human ovarian carcinoma cell line A2780, and a human neuroblastoma cell line SH-SY5Y. We found out that these GNDAs were cytotoxic against A2780 and NIH-3T3 cells but they showed only a marginal cytotoxicity against neuroblastoma cells SH-SY5Y. To explain differences in cytotoxicity, intracellular distribution of GNDAs was monitored. GNDAs were accumulated in A2780 and NIH-3T3 cells in the nuclei (fluorescence microscopy). In contrast to these cell lines, in SH-SY5Y cells, GNDAs were localized outside of the nuclei, at the plasma membrane and surroundings, extending also to the cytosol. This distribution of GNDAs was confirmed by an ImageStream Flow Cytometer. Acetylcholinesterase (AChE) activity in the SH-SY5Y cells decreased upon incubation with GNDAs. Kinetic studies showed that GNDAs were able to inhibit AChE by the same mode as tacrine (9-amino-1,2,3,4-tetrahydroacridine), a known inhibitor of AChE. A low cytotocity of GNDAs against SH-SY5Y cells could be caused by their affinity to AChE (the enzyme is localized mainly at the plasma membrane). The interaction of GNDAs with AChE may affect their intracellular distribution and consequently the cytotoxicity.

Key words: acetylcholinesterase, acridine, neuroblastoma cell line SH-SY5Y

DNA represents an important target for some of the established and new anticancer drugs [1-6]. Among these, derivatives of acridine have shown a high anticancer activity. Amsacrine and its analogues belong among the most tested acridine drugs used in antitumor therapy [7-9]. Cytotoxicity of these drugs is associated with their DNA binding activity, whereby most of them act as inhibitors of topoisomerases or DNA intercalators [10]. The search for novel anticancer drugs encouraged the preparation of many sets of new acridine derivatives [11-15]. A number of novel acridine derivatives have been prepared in our laboratory and their interaction with DNA and *in vitro* anticancer activity have been studied [9, 10]. Recently, Plsikova et al. [16] studied 3,6-bis(3-alkylguanidino) acridines (GNDAs), the new guanidine derivatives based on proflavine, which possess symmetrical, extending alkyl chains (alkyl-GNDA, Fig. 1). These substances have a strong DNA binding activity and these with penty- and hexyl- alkyl chains also a high cytotoxicity, leading to apoptosis of HL-60 leukemia cells [16].

The purpose of this study was to investigate cytotoxicity of the GNDAs to other neoplastic cell lines and the factors which could influence effects of these compounds. In particular, an intracellular distribution of GNDAs in the cells was a main object of our interest.

Materials and methods

Chemicals. All chemicals and reagents were purchased and used without further purification. $Na_2HPO_4.12H_2O$, KH_2PO_4, and NaCl were obtained from Lachema (Czech Republic), FBS (fetal bovine serum), DMEM (Dulbecco's Modified Eagle's Medium), HAM F12 (Ham's Nutrient Mixture F12), RPMI 1640, propidium iodide, PBS (Phosphate Buffered Saline), trypsin, DMSO (dimethyl sulfoxide), tacrine, and

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide) from Sigma-Aldrich Chemie (Germany), paraformaldehyde from Merck KGaA (Germany), and Triton X-100 (100%) from Serva (Germany).

3,6-Bis(3-alkylguanidino)acridine hydrochlorides (Fig. 1, GNDAs) were synthesized in the Department of Organic Chemistry, Faculty of Science, University in Kosice as hydrochloride salts [16].

Cell cultures and treatments. The murine immortalized fibroblast cell line NIH-3T3 was obtained from the American Type Culture Collection, Rockville, MD (USA). The cells were routinely cultured in DMEM supplemented with 10% FBS, 2 mM *L*-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). The human ovarian carcinoma cells A2780 were grown in the RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), and with (2 mM) *L*-glutamine. The human neuroblastoma cell line SH-SY5Y was routinely cultured in a DMEM : Ham F12 medium (1:1) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), penicillin (100 U/mL), 1% *L*-glutamine, and 0.1% non-essential amino acids (all obtained from Sigma-Aldrich Chemie).

The cells were seeded into the tissue culture flasks (75 mL) containing the supplemented medium and kept in a humidified atmosphere of 5% CO2 and 95% air at 37 °C.

Cytotoxic studies. To evaluate the cytotoxic effect of GNDAs, the cells were seeded in 96-well plates (2.5×10^4 cells/well, MTT assay) and treated with different concentrations of GNDAs for 24, 48, or 72 h. All experiments were carried out 24 h (NIH-3T3 and A2780 cells) or 48 h (SH-SY5Y cells) after the cells were seeded. Control experiments with equivalent volumes of DMSO were carried out.

MTT assay. A cell viability in the presence or absence of the experimental agent was determined using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide) microculture tetrazolium assay as described previously [17]. After 48 h exposure to the compound, MTT (50 μl, 1 mg/mL) was added to each well. After 3 h, the cell cultures were centrifuged, the supernatant discarded, and the resulting pellets thoroughly extracted into 200 μL of DMSO. Absorption at 540 nm was recorded using a MicroPlate Reader (Labsystem Multiscan, Multisoft, Finland).

Intracellular accumulation of GNDA. To investigate the intracellular distribution of GNDAs, the cells were seeded onto a cover slip in 40 mm Petri dishes. The NIH-3T3, A2780 (1.5×10^5/mL) and SH-SY5Y (2.5×10^5/mL) cells were treated with 5 μM hexyl-GNDA for 30 min or 48 h, then washed with sterile PBS, and observed using a fluorescence microscope (Axio Zeiss Imager A1, camera AxioCamMrc, Germany).

Analysis of localization of hexyl-GNDA in the cell nucleus. SH-SY5Y cells were treated with 10 μM GNDAs (1 h or 48 h, 37 °C) and then the cells were incubated with 100 nM Syto62Red (35 min, 37°C), a nucleic acids dye. After incubation, the cells were rinsed twice in PBS and immediately visualized using an Amnis ImageStream Imaging Flow Cytometer: hexyl-GNDA (channel 8), Syto62Red (channel 11), VIS (channel 1). Bright Detail Similarity Features were calculated for double positive, single, and focused cell population.

Preparation of human erythrocytes $AChE_E$. Erythrocytes were isolated from a fresh blood of healthy donors of both sexes (provided by the Hematologic Clinics Ruzinov in Bratislava). The blood was centrifuged (2500×g, 10 min), the erythrocyte sediment was washed, suspended in the solution (165 mM NaCl), centrifuged at 2500×g for 10 min, and the washing was repeated until the supernatant became completely clear. Distilled water was added in the ratio 1:5 and the mixture was incubated (25 °C, 90 min). The suspension was centrifuged at 12000×g (4 °C, 25 min). The sediment was washed three times with distilled water and centrifuged after each washing under the same conditions as above. The Sörensen solution (2 mL, 0.1 M, pH 7.2), 1 mL of 1 mM EDTA, and 1% Triton-X 100 were added to the sediment. After 5 min centrifugation at 20800×g, the sediment was removed and the supernatant was stored at -20 °C as a source membrane bound $AChE_E$ for further use up to 2 weeks [18].

AChE activity. Activity of AChE was determined by an Ellman's method [19] modified by Alhomida et al. [20]. Hydrolysis of acetylthiocholine (ATCh) was monitored at 436 nm by formation of a 5-thio-2-nitrobenzoate anion resulting from the reaction of 5,5´-dithio-bis(2-nitrobenzonic acid) (DTNB) with thiocholine that is released from enzymatic hydrolysis of ATCh. The hydrolysis rate was measured in 2 mL assay solutions with 100 mM phosphate buffer, pH 7.4, 0.1 mM ATCh, and 0.5 mM DTNB at 37 °C. Isolated $AChE_E$ or cell lysate (the SH-SY5Y cells were harvested with a plastic spatula and disintegrated by sonication in 300 μL PBS) was added to the reaction mixture and pre-incubated with DTNB for 5 min at 37 °C. Before measuring an absorbance change (a Specord 250 spectrophotometer, Analytic Jena, Germany), the ATCh was added. The hydrolysis of ATCh was monitored for 10 min and the enzyme activity was calculated from a slope of the obtained linear dependence.

The activities were calculated using a molar absorption coefficient of 11280 $M^{-1}cm^{-1}$ [21]. Data were normalized to the amount of protein. Results were presented as a percentage of the activity in untreated cells (as 100%). The protein content was determined according to a method of Bradford [22].

To study an effect of GNDAs or tacrine on the AChE activity, the enzyme suspension ($AChE_E$ or cell lysate) was pre-incubated with GNDAs or tacrine for 5 min prior to addition of the substrate. To determine that effect in SH-SY5Y cells (the cells were seeded in 75 mm Petri dishes, 2×10^6 cells/dish), the cells were incubated with the substances for 48 h, then were scraped and collected in the phosphate buffer, pH 7.4 (2 mL), after that, the cells were disrupted by freezing and thawing and the cell lysate was and kept on ice and used for determination of the AChE activity.

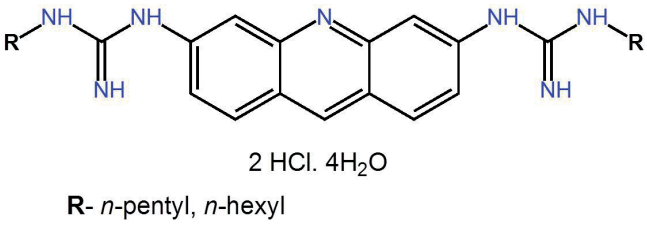

Figure 1. Chemical structures of tested 3,6-bis(3-alkylguanidino)acridine hydrochlorides.

Kinetic studies. Kinetic studies were performed using an isolated $AChE_E$. Enzyme activities were determined at 37 °C with growing acetylthiocholine concentrations (0.02 – 0.1 mM) in the presence or absence of inhibitors (0.5 – 1.5 μM). Lineweaver-Burk plots were used to reveal a mechanism of inhibition. Replots of the slopes versus the inhibitor concentrations gave estimates of K*i*. Graphs were plotted in an Origin Pro 8 program.

Statistical analysis. Results were calculated as a mean ± standard deviation (S.D.) of at least three independent experiments. Statistical significance was determined by the Student´s *t* test and $p \leq 0.05$ was taken as the limit of statistical significance.

Table 1. Cytotoxicity of GNDAs against A2780, NIH-3T3 and SH-SY5Y cells

Cell line	Compound	*c* [μM]	Viability[%][a]		
			24 h	48 h	72 h
A2780	Pentyl-GNDA	10	62±9.8	76±10.3	66±12.3
		50	51±10.5	57±9.5	39±8.7
	Hexyl-GNDA	10	46±9.8	60±10.8	60±11.3
		50	37±12.7	38±8.6	39±9.6
NIH-3T3	Pentyl-GNDA	10	58.3±14.2	57±11.4	46±10.0
		50	35.3±8.5	46±9.3	40±9.8
	Hexyl-GNDA	10	64±12.6	54±11.0	26±8.0
		50	25.5±14.8	8± 5.7	6±5.0
SH-SY5Y	Pentyl-GNDA	10	88.0±13.2	84.2±15.4	84.9±10.5
		50	84.0±8.7	80.7±10.8	77.7±9.4
	Hexyl-GNDA	10	84.8±9.7	80.7±14.2	86.2±9.8
		50	79.5±9.0	74.9±9.1	74.9±7.7

[a] The results are presented as a mean ± SD (n = 3).

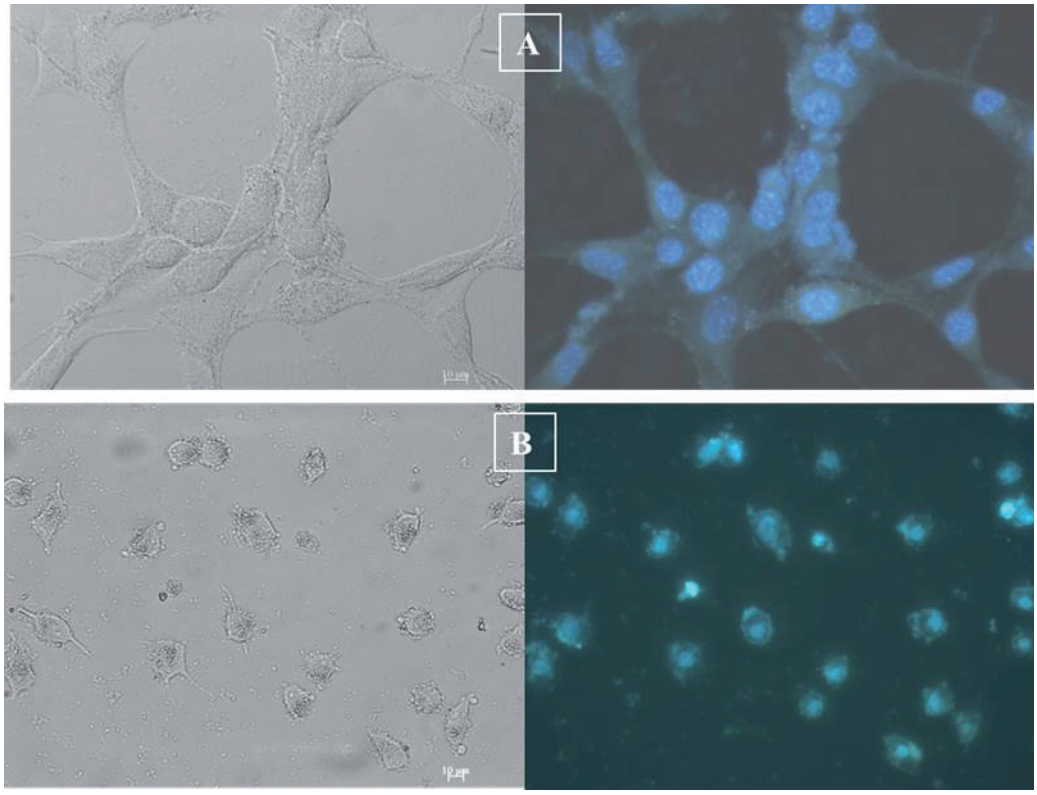

Figure 2. Intracellular accumulation of hexyl-GNDA in NIH-3T3 (A) and A2780 (B) cells visualized by fluorescence microscopy. The cells were incubated with hexyl-GNDA (5 μM) for 30 min and then washed with PBS. Images were obtained by the fluorescence microscope Axio Zeiss Imager A1, camera AxioCam MRc, magnification 630x or 400x. The cells were visualized using a filter with an emission wavelength of 420 nm. Representative images are shown from three independent experiments.

Results

Cytotoxicity. We explored the toxicity of two GNDA derivatives, pentyl-GNDA and hexyl-GNDA, against the A2780, NIH-3T3 and SH-SY5Y cells. As can be seen in Table 1, GNDAs markedly decreased viability of A2780 and NIH-3T3 cells. For example the hexyl-GNDA derivative reduced the viability of these cells by 40 and 74%, respectively, after 72 h treatment in 10 μM concentration. However, the toxicity of the both GNDAs against SH-SY5Y cells in the same conditions was very low, when the viability decreased only by 15%.

Intracellular distribution. The cytotoxicity of GNDAs against NIH-3T3 and A2780 cells was much higher than against the neuroblastoma cells SH-SY5Y. Such differences in toxicity may result from a different intracellular distribution of GNDAs. As the both tested analogs showed the same distribution in all cell lines, we will further show the hexyl-GNDA as an example. As shown in Fig. 2 (A, B), the hexyl-GNDA was accumulated in the nucleus and cytosol of the NIH-3T3 or A2780 cells after 30 min incubation. However, in the SH-SY5Y cells, we did not observe any nuclear accumulation of GNDAs after short-time (30 min) incubation (Fig. 3A). We checked also their long-term (48 h) distribution in SH-SY5Y cells by fluorescence microscopy, but with the same negative result (Fig. 3B). The cellular sequestration of the hexyl-GNDA on microphotographs (Fig. 3) shows a strong fluorescence signal observed at the plasma membrane of SH-SY5Y cells and surroundings, extending also to the cytosol, but remaining outside of the nucleus.

To confirm localization of the GNDAs outside of the nucleus, an Image stream imaging technique for colocalisation using the Syto62Red nucleic acid dye was applied. As shown in Fig. 4, the fluorescence of hexyl-GNDA and Syto62Red were not overlaid even after long-term incubation. The values of Bright Detail Similarity R3 Feature were below 1.0 and 1.7 after 1 h and 48 h incubation, respectively (Fig. 4). The obtained results proved that hexyl-GNDA were not present in the nuclei of SH-SY5Y cells.

Interaction with AChE in SH-SY5Y. Intracellular localization of the hexyl-GNDA outside of the nucleus (Fig. 3 and 4) indicates that this substance has a high affinity to other targets in the SH-SY5Y cells. Considering localization of the GNDA at the plasma membrane, proteins of the plasma membrane could interact with these substances. Based on the literature reports [23] we supposed that acetylcholinesterase (EC 3.1.1.7; AChE) among others could be a cellular target of GNDAs in the SH-SY5Y cells, therefore effects of GNDAs on the enzyme activity in SH-SY5Y cells were investigated and compared to a tacrine (9-amino-1,2,3,4-tetrahydroacridine)

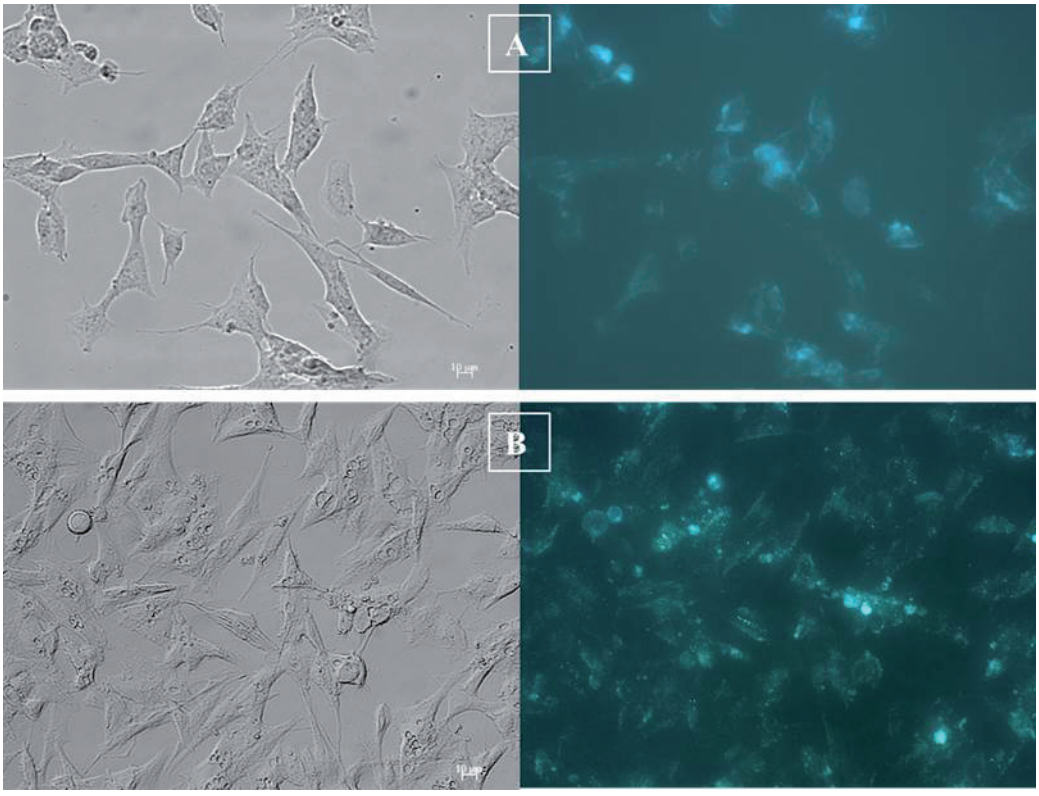

Figure 3. Intracellular distribution of hexyl-GNDA in SH-SY5Y cells. The cells were incubated with the hexyl-GNDA for 30 min (A) or 48 h (B), and then washed with PBS. The images were obtained using the same instrument as above in magnification 400x.

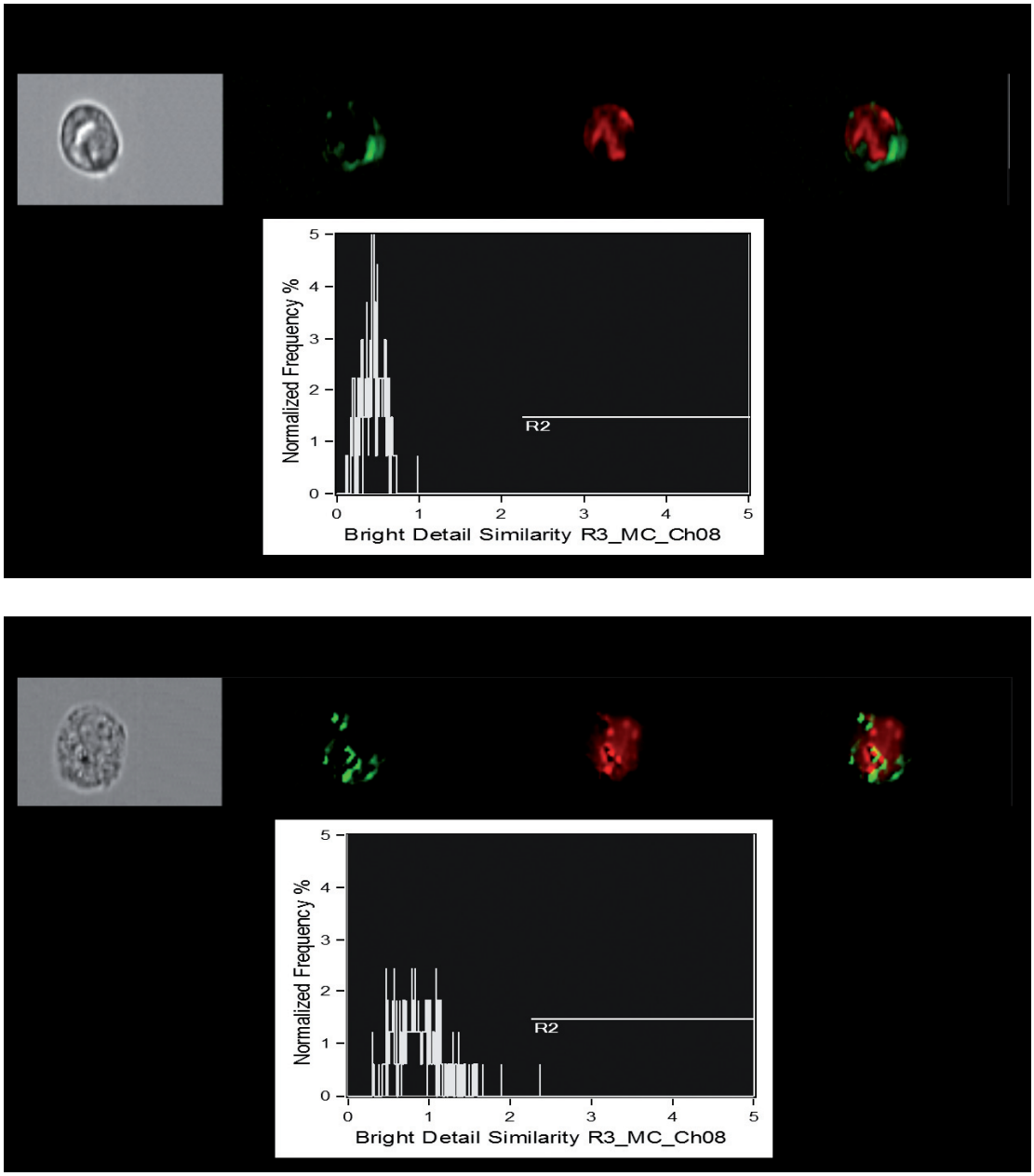

Figure 4. Colocalization of fluorescence of the hexyl-GNDA and Syto62Red nucleic acid dye. SH-SY5Y cells were incubated with the hexyl-GNDA (10 μM, green) for 1 h (A) or 48 h (B). After labeling with Syto62Red (100 nM), the cells were analyzed using an Amnis ImageStream Imaging Flow Cytometer. The parameter reflecting the colocalization of two probes, Bright Detail Similarity R3 Feature, was calculated. The resulting values around 1 mean no colocalization, the values close to 3 mean a perfect colocalization. Representative images are shown from three independent experiments.

standard, a known inhibitor of acetylcholinesterase [24]. After short 5 min incubation of the cell lysate with GNDAs (0.5 μM), a remaining 52 – 72% antiAChE activity was found (Fig. 5). Tacrine proved to be a more potent AChE inhibitor (only 30% remaining enzyme activity) than GNDAs but its inhibitory effect significantly decreased after extended 48 h incubation. Such a recovery of the AChE activity has not been observed after 48 h incubation of the cells with GNDAs.

Mechanism of the ACHE inhibition. To compare inhibitory effects of GNDAs and tacrine, kinetic data were analyzed using a double-reciprocal Lineweaver-Burk plot. Human erythrocyte acetylcholinesterase ($AChE_E$) was used and a dose-dependent inhibition of the enzyme with GNDAs was

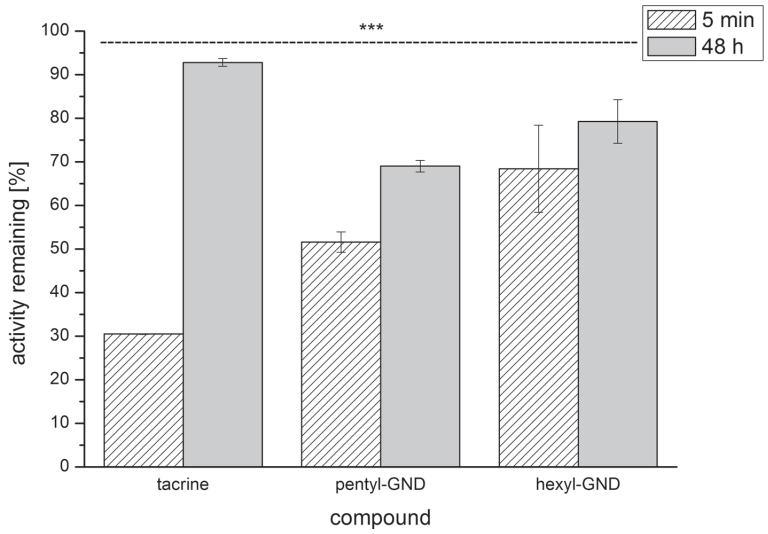

Figure 5. The effect of GNDAs on AChE activity in SH-SY5Y cells. SH-SY5Y cells were incubated with 0.5 μM GNDAs or 0.5 μM tacrine for 48 h (non-cytotoxic concentrations), then the cells were harvested and ACHE activity was determined in the cell lysate as described in Methods. The inhibition effect was evaluated after short-time incubation; the substances were incubated with the cell lysate (1x10^6 cells/0.1 mL) for 5 min and the enzyme activity was assayed. The results are presented as the mean ± SD (n = 3), statistical significance $p < 0.001$(***) for particular experimental group compared to untreated control.

estimated first. The concentration-response curves showed that these compounds inhibited AChE in a dose-dependent manner. Determined IC_{50} values proved that GNDA derivatives were less potent than tacrine (Table 2).

As can be seen in Fig. 6A, the double-reciprocal Lineweaver-Burk plot revealed that both slopes and intercepts increased with a higher pentyl-GNDA concentration, demonstrating thus a mixed-type competitive and non-competitive inhibition. The inhibition constant K_i = 0.4 μM was obtained from a secondary plot of the slope versus the concentration of pentyl-GNDA (Fig. 6A; inset). Tacrine behaved as a mixed-type inhibitor (Fig. 6B) as indicated in articles [25, 26].

Table 2. *In vitro* inhibition effects of GNDAs and tacrine on $AChE_E$

Compound	IC_{50} [μM][a]
pentyl-GNDA	0.60±0.09
hexyl-GNDA	1.60±0.19
tacrine	0.23±0.02

[a] The results are presented as a mean ± SD (n = 3).

Discussion

Most of acridine compounds are cytotoxic and their toxicity is caused by their DNA binding activity and some of them thus can serve as probes for mutagenesis examination [15, 27-32]. In spite of that, cytotoxicity of some acridines may not be connected with their interaction with nuclear DNA but they could induce an ER stress or oxidative stress in cells. Such types of acridines were prepared in our laboratory [33-35]. It was proven that 3,6-bis((1-*n*-alkyl-5-oxo-imidazolidin-2-yliden) imino)acridine hydrochlorides were localized in mitochondria [36]. Likewise, unusual non-nuclear accumulation of 3-amino-4-hydroxymethyl-acridine derivative in the form of aggregates in the cytoplasm accompanied by its localization also in lysosomes was found out by Peixoto et al. in 2009 [37].

Recently Plsikova et al. [16] has documented that novel derivatives of acridine, 3,6-bis(3-alkylguanidino)acridines are effective DNA-intercalating agents, whose cytotoxic action is dependent mainly on their intracellular accumulation in nuclei of leukemia HL-60 cells. We have proceeded further with investigation of the cytotoxicity of GNDAs. Surprisingly, considerable cytotoxicity of the pentyl- and hexyl-GNDA (the most cytotoxic were GNDAs derivatives against HL-60 cells) was confirmed only against A2780 and NIH-3T3 cells, but not against SH-SY5Y neuroblastoma cells. It was shown that derivatives of GNDA were localized in nuclei of the A2780 and NIH3-T3 cells. But, the presence of the GNDA derivative in nuclei of SH-SY5Y cells was not proved. We have observed that GNDAs were accumulated at the plasma membrane of SH-SY5Y cells and surroundings.

We have searched for a potential cellular target for GNDAs in the SH-SY5Y cell line that is being used as a cellular model of cholinergic phenotype [38] with expression of acetylcholinesterase [39-41]. AChE is responsible for the termination of cholinergic transmission (breakdown of acetylcholine) [42, 43]. Two isoforms of AChE are synthesized in neuroblastoma cells, AChE-T (AChE-T, a tailed form of AChE) is linked to the

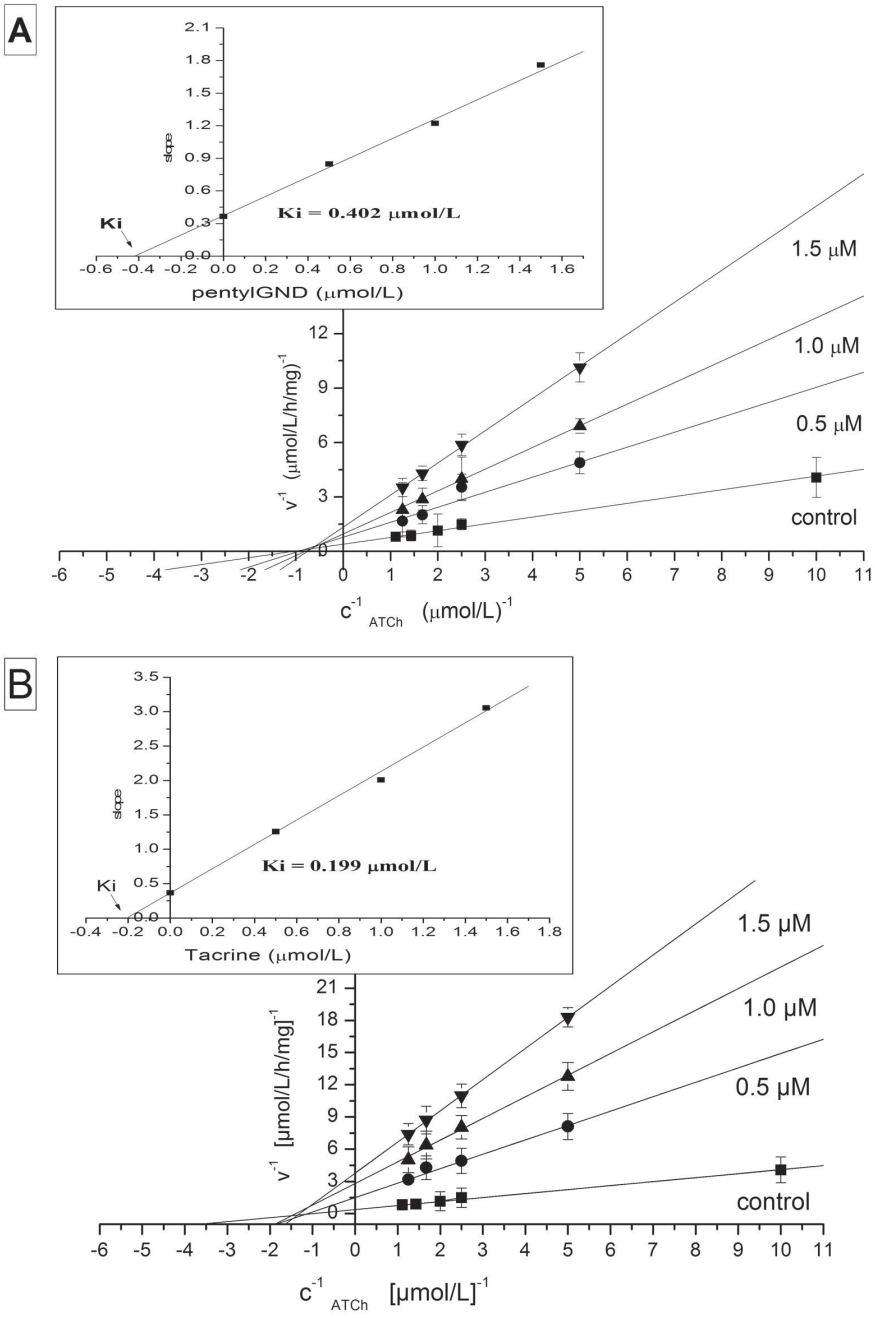

Figure 6. The inhibition of acetylcholinesterase by pentyl-GNDA and tacrine. Lineweaver–Burk plots representing reciprocals of initial enzyme velocity versus ATCh concentration in the absence and presence of different concentrations of pentyl-GNDA (A) and tacrine (B). Inset: Secondary replots of Lineweaver–Burk plot slope versus tacrine or GNDA concentrations. The data are expressed as the mean ± SD (n = 3).

cellular membrane and its soluble splice variant, AChE-R, is realeased into the cell media [44-49]. Thus acetylcholinesterase has been proposed as a potential target.

Our study of the interaction between GNDAs and AChE confirmed that these derivatives of acridine were able to inhibit the AChE activity in SH-SY5Y cells. Kinetic analysis (AChE from human erythrocytes was used for this study) revealed that GNDAs were acting as the mixed-type AChE inhibitors as well as tacrine (9-amino-1,2,3,4-tetrahydroacridine), a known inhibitor of AChE that was the first AChEI introduced in the therapy of the Alzheimer's disease [50, 51]. The effect of GNDAs on AChE in the SH-SY5Y cells was not the same

as the action of tacrine. Although tacrine was more potent AChE inhibitor than GNDAs, its effect on AChE activity in the SH-SY5Y cells was only temporal. The inhibition of AChE by tacrine is known to elicit a feedback process that leads to expression of AChE [52-54]. The effect of GNDAs had longer duration and the AChE activity in SH-SY5Y cells remained reduced also after 48 h.

Our results showed that explored 3,6-bis(3-alkylguanidino) acridines, the substances with high affinity to DNA, were cytotoxic against the cells A2780 and NIH-3T3. But, their cytotoxicity against neuroblastoma SH-Y5Y cells was very low probably due to their interaction with AChE, which influenced their intracellular distribution, preventing GNDAs to reach the nucleus.

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