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Identification of spiro-fused pyrrolo[3,4-*a*]-pyrrolizines and tryptanthrines as potential antitumor agents: synthesis and initial *in vitro* evaluation

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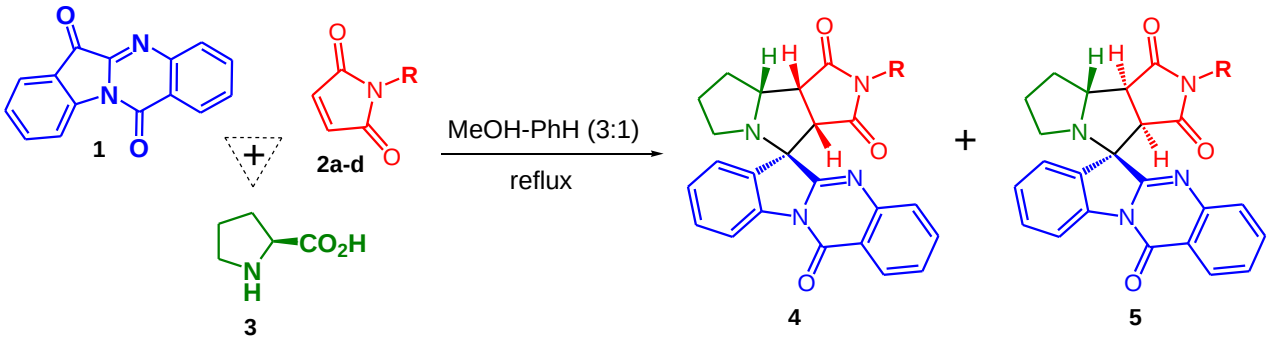
Identification of spiro-fused pyrrolo[3,4-*a*]-pyrrolizines and tryptanthrines as potential antitumor agents: synthesis and initial in vitro evaluation



		K562		
	4a (R=H)	1.9±0.2 μg/mL	**5a**	14.9±0.5 μg/mL
	4b (R=Et)	7.8±0.4 μg/mL	**5b**	47.0±0.7 μg/mL
		HeLa		
	4a (R=H)	6.9±0.4 μg/mL	**5a**	16.2±0.5 μg/mL
	4b (R=Et)	29.7±0.7 μg/mL	**5b**	46.8±1.1 μg/mL

Abstract: A series of heterocyclic compounds containing spiro-fused pyrrolo[3,4-*a*]-pyrrolizine and tryptanthrin framework have been synthesized and studied as potential antitumor agents. Antiproliferative activity of synthesized products was screened against human erythroleukemia (K562) and human cervical carcinoma (HeLa) cell lines. Spiroadducts with all cis bridge-protons of pyrrolo[3,4-*a*]pyrrolizine moiety were more active in all cases. Replacement of hydrogen atom of pyrrole moiety by either alkyl or aryl group leads to significant decreasement in activity of both formed cycloadducts. In agreement with the DNA cytometry studies, the tested compounds have achieved significant cell-cycle perturbation with higher accumulation of cells in G2/M phase and induce apoptosis. Using confocal microscopy, we found that with synthesized products treatment of HeLa cells, actin filaments disappeared, and granular actin was distributed diffusely in the cytoplasm in 76-91% of cells. We discovered that HeLa cells after treatment with screened compounds significantly reduced the number of cells with filopodium-like membrane protrusions (from 63 % in control cells to 29% after treatment) and decrease in cell motility.

Keywords: antiproliferative activity, morphological changes (cytoskeleton), cell cycle, cell death, cell motility.

Introduction

Oncological diseases are one of the most common public health problems and the second leading cause of death after cardiovascular disease. Increased drug resistance and the emergence of tumor resistance as well as severe side-effects of chemotherapeutic agents reduce the clinical efficacy of currently used anticancer drugs and treatments. Despite the increasing use of targeted drugs and methods of immunotherapy of oncological diseases, the development of cytostatic agents remains an important challenge for the treatment of cancer. At the same time, the emergence of tumor resistance requires the creation of cytostatics that are not just derivatives of "classical" drugs, but originating from compounds of a new nature.

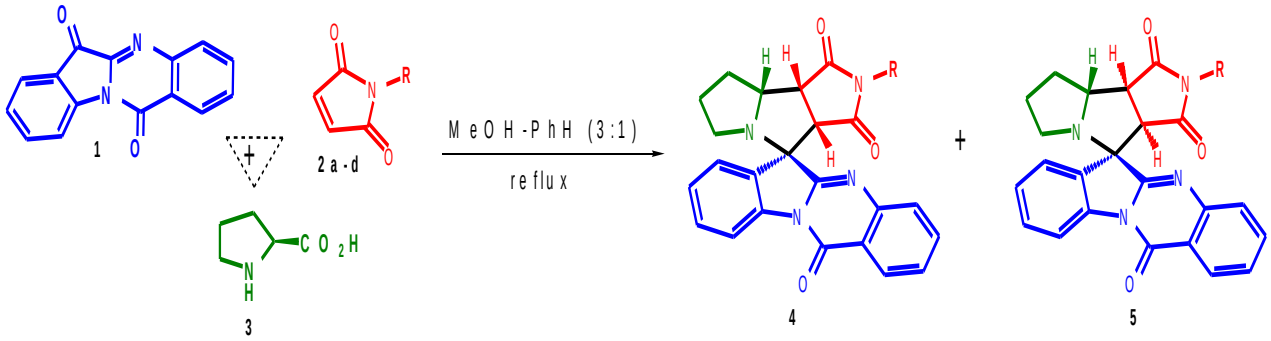
Natural products or synthetic compounds inspired from natural products continue to be excellent sources for new drug candidates. Many of the most currently applicable (or that under clinical trials) anticancer drugs are either themselves compounds of natural origin, or designed based on naturally occurring compounds.

We here report that one-pot three-component 1,3-dipolar cycloaddition reactions of various maleimides with in situ generated tryptanthrin-derived azomethine ylide lead to the formation of two diastereomeric products with pyrrolo[3,4-*a*]pyrrolizine moiety. All the compounds were evaluated for their antiproliferative activity as well as cell motility, morphological changes, cell cycle, cell death were evaluated for the most active products.

Results and discussion

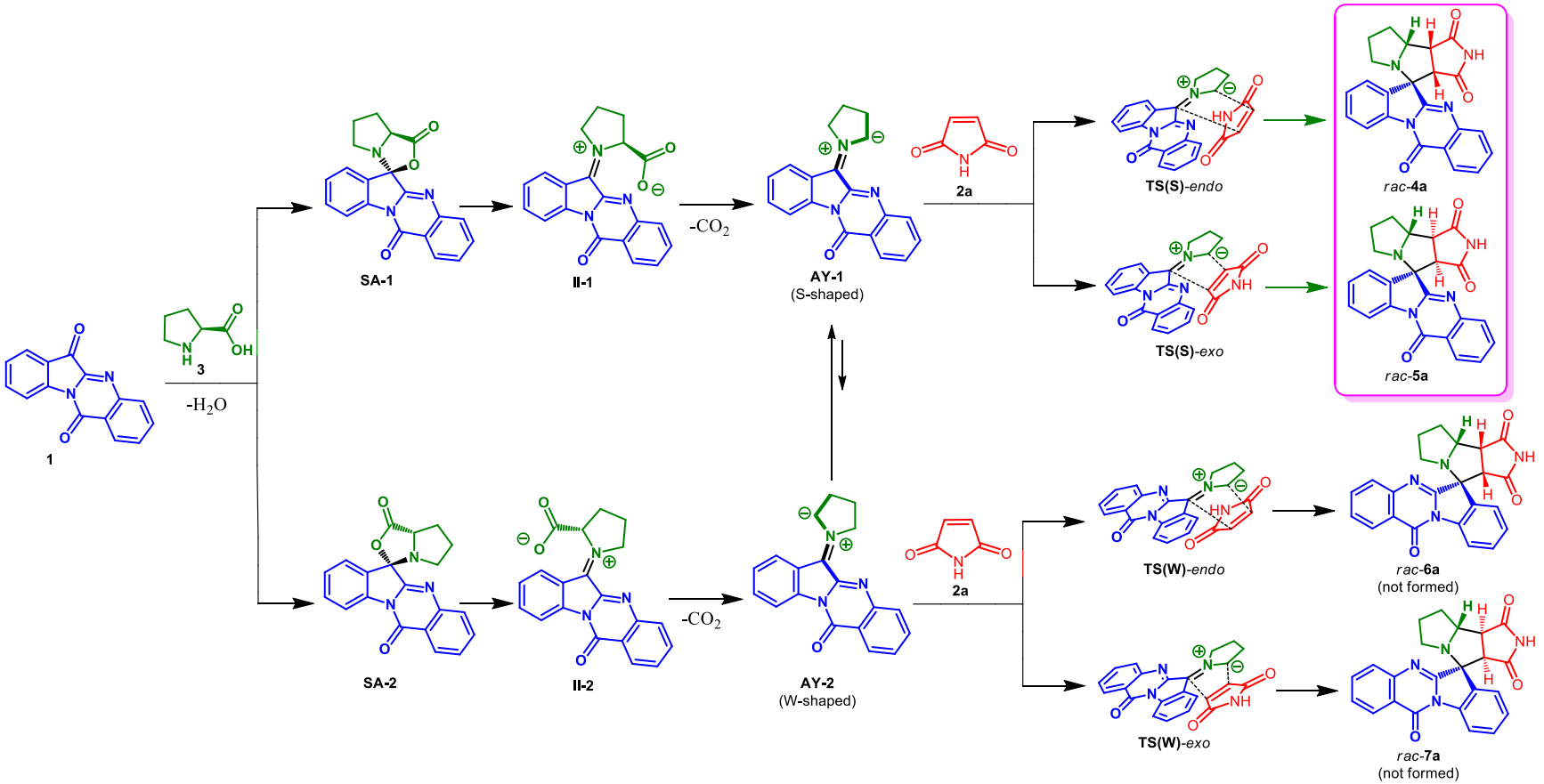
Synthesis and structure elucidation

One-pot three-component 1,3-dipolar cycloaddition reactions of various maleimides with *in situ* generated tryptanthrin-derived azomethine ylide lead to the formation of two diastereomeric products with up to 64% overall isolation yield. The ratio of products **4** and **5** was found to be 1.78-1.0 to 1 correspondingly. The major diastereomer in all cases was product **4**, the structures of both isomers were assigned on the basis of NMR spectra analysis and unequivocally verified by X-ray crystal analysis.



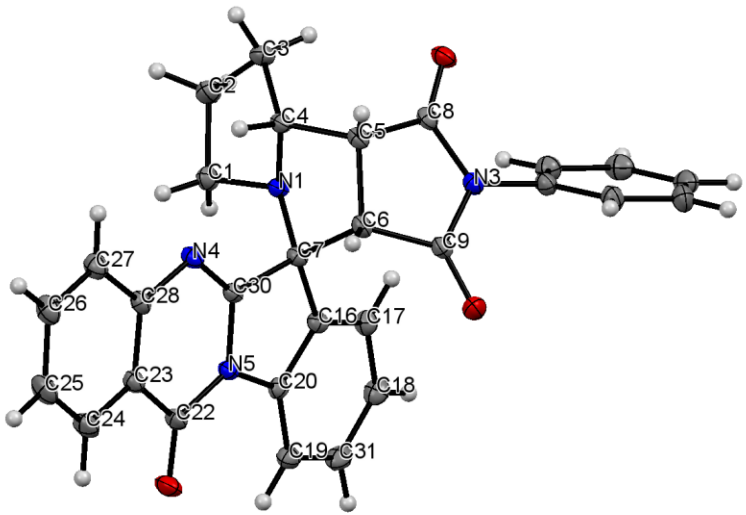
Entry	Maleimide	Substituent R	Ratio 4 : 5	Yield, %	
				Product 4	Product 5
1	2a	H	1.15 : 1.0	34	30
2	2b	Et	1.25 : 1.0	29	23
3	2c	Ph	1.0 : 1.0	7	7
4	2d	CH_2CH_2Ph	1.78 : 1.0	20	11

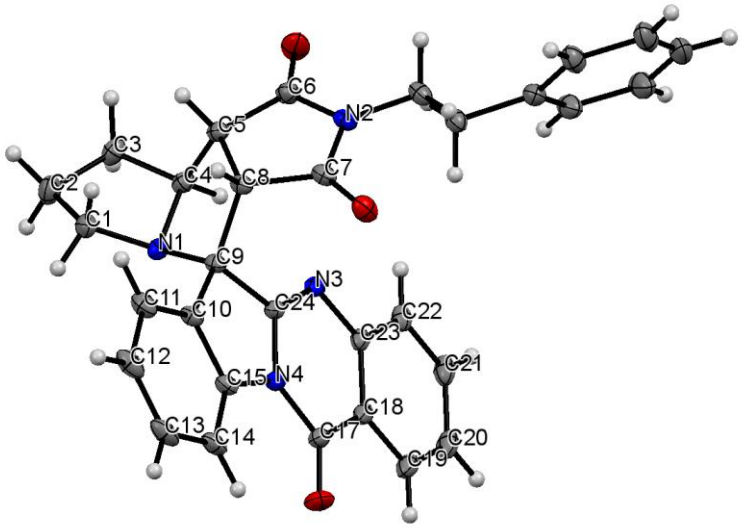
Synthesis and structure elucidation



Plausible reaction mechanism for the formation of compounds **4** and **5**

Synthesis and structure elucidation





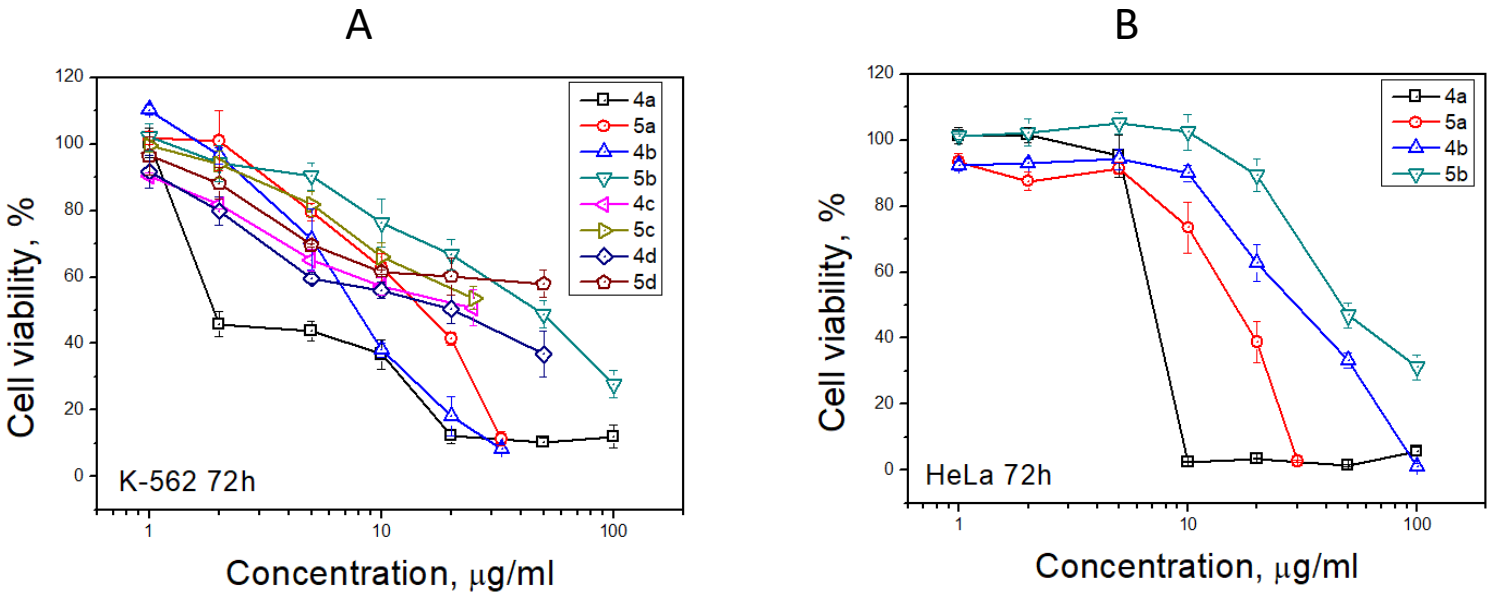
4c (CCDC 2105205)	5d (CCDC 2105206)

ORTEP representation of the molecular structure of **4c** and **5d**

Antiproliferative effect of synthesized compounds against cancer cell lines

Antiproliferative activity of synthesized spiro-fused tryptanthrines and pyrrolo[3,4-*a*]-pyrrolizines **4** and **5** against human erythroleukemia (K562) and cervical carcinoma (HeLa) cell line was evaluated *in vitro* by the standard MTS assay for 24 and 72 h. All substances showed no significant cytotoxicity after 24 h of exposure. It was found that spiroadducts with all *cis* bridge-protons of pyrrolo[3,4-*a*]pyrrolizine moiety were more active in all cases (compare 4 *vs* 5) after treatment for 72 h. Replacement of hydrogen atom of pyrrole moiety by either alkyl or aryl group leads to significant decrease in activity of both formed cycloadducts. It was found that among target compounds with spiro-fused tryptanthrin and pyrrolo[3,4-*a*]pyrrolizine moiety only unsubstituted at pyrrole ring cycloadduct **4a** demonstrates significant activity with IC_{50} 1.9±0.2 µg/mL (K562, 72 h), while its diastereomer, **5a**, demonstrates nearly 7 fold lower activity with IC_{50} 14.9±0.5 µg/mL (K562, 72 h). Third most potent ethyl substituted at pyrrole ring cycloadduct **4b** demonstrates intermediate activity with IC_{50} 7.8 ± 0.9 µg/mL (K562, 72 h), while its diastereomer, **5b**, was nearly 6 times weaker with IC_{50} 47.0 ± 0.7µg/mL (K562, 72 h). Since similar results were also found and for HeLa cell lines, all followed tests were performed for cycloadducts **4a** and **5a** only.

Antiproliferative effect of synthesized compounds against cancer cell lines



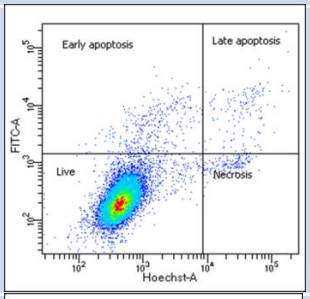
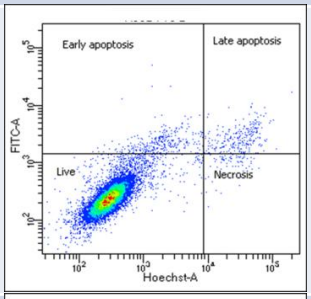
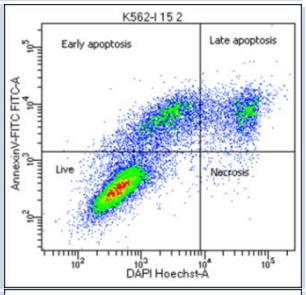
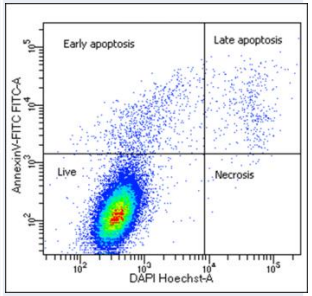
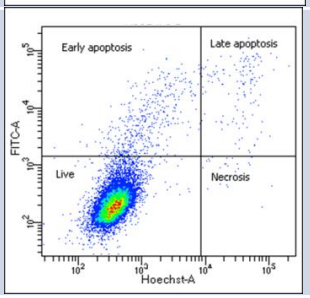
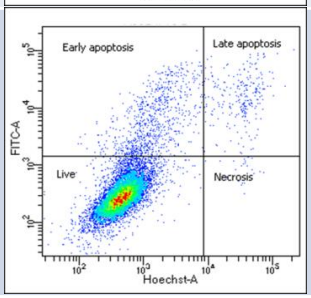
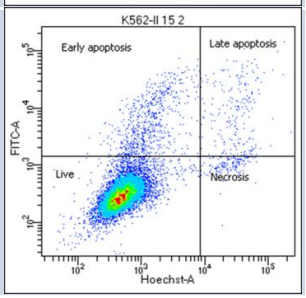
Antiproliferative activity of spiro-fused pyrrolo[3,4-*a*]pyrrolizines and tryptanthrines against K562 (A) and HeLa (B) cell lines for 72 h.

Cell death analysis

The apoptotic effect of compounds **4a** and **5a** was evaluated by Annexin V-FITC/propidium iodide (AV/PI) dual staining assay to examine the occurrence of phosphatidylserine externalization, which facilitated the detection of live cells (lower left quadrant; AV-/PI-), early apoptotic cells (upper left quadrant; AV+/PI-), late apoptotic cells (upper right quadrant; AV+/PI+) and necrotic cells (lower right quadrant; AV-/PI+). The percentage of early apoptotic cells after treatment with compounds **4a** and **5a** increased, respectively, from 5.0% (control) to 23.0 and 9.4% for K562 cell line and from 4.5% (control) to 20.4 and 26.7% for HeLa cell line. The percentage of late apoptotic cells after treatment increased from 1.9% (control) to 13.6 and 2.4% for K562 cell line and from 5.9% (control) to 32.9 and 23.6% for HeLa cell line for compounds **4a** and **5a**, respectively. So the total percentage of apoptotic cells increased for K562 cell line from 6.9% (control) to 36.6 and 11.8% and for HeLa cell line from 10.4% (control) to 53.3 and 43.1% (for compounds **4a** and **5a,** respectively). The percentage of necrotic cells was not significantly changed for both cell lines.

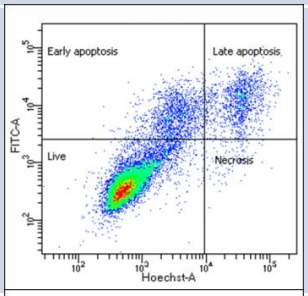
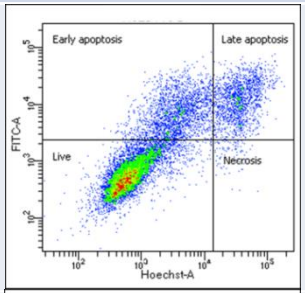
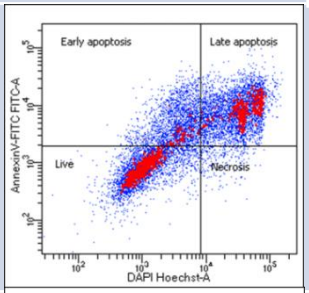
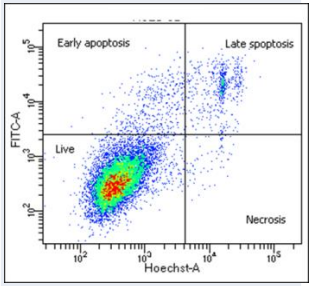
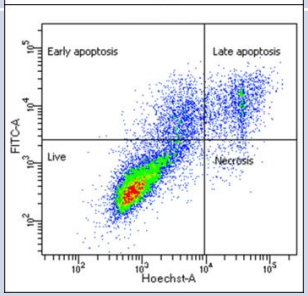
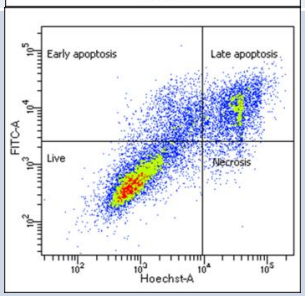
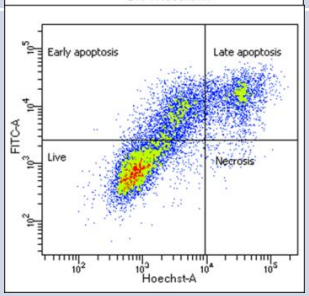
It was indicated that compounds **4a** and **5a** could induce apoptosis of both HeLa and K562 cells. At the same time, we have demonstrated that compounds **4a** and **5a** lead to inhibition of the growth rate of the cells.

Cell death analysis

Compound	Concentration, μg/mL			Control
	5	10	20	
4a				
5a				

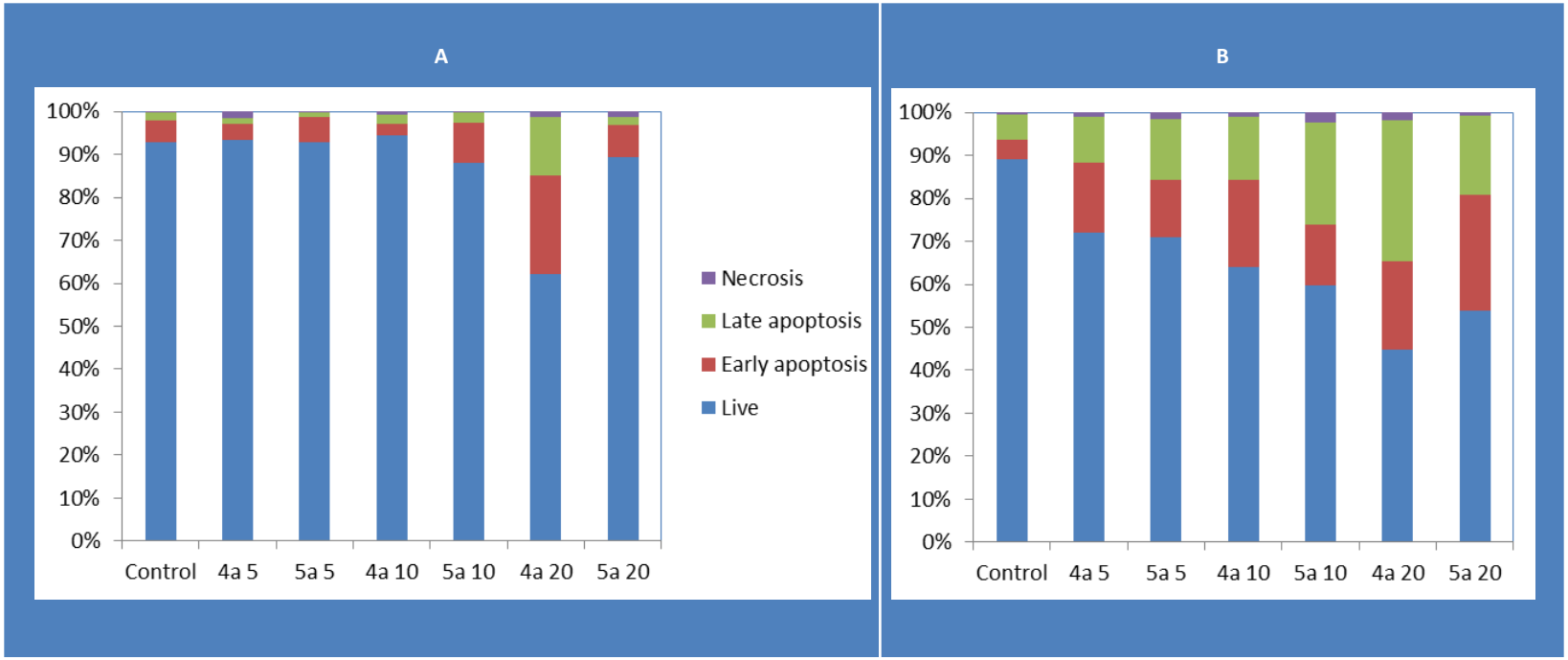
Annexin V-FITC/Propidium iodide (PI) dual staining assay of K562 cells treated with cycloadducts **4a** and **5a** at concentrations 5, 10 and 20 μg/mL using flow cytometry

Cell death analysis

Compound	Concentration, μg/mL			Control
	5	10	20	
4a				
5a				

Annexin V-FITC/Propidium iodide (PI) dual staining assay of HeLa cells treated with cycloadducts **4a** and **5a** at concentrations 5, 10 and 20 μg/mL using flow cytometry

Cell death analysis



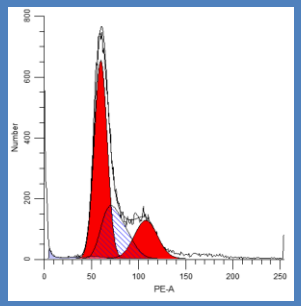
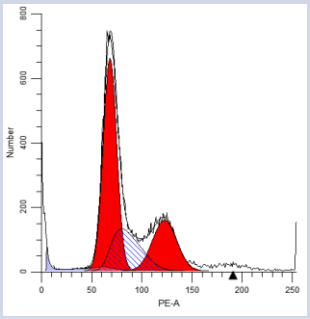
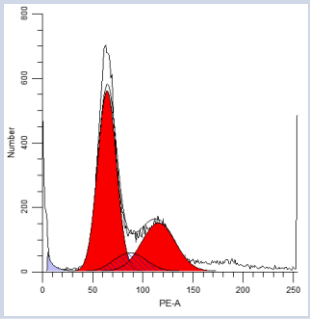
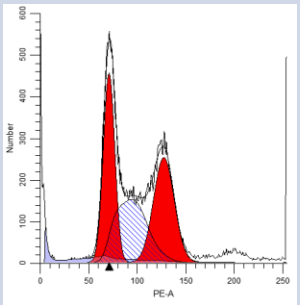
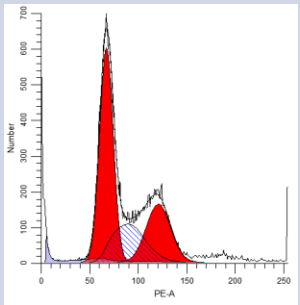
Annexin V-FITC/Propidium iodide (PI) dual staining assay of K562 (A) and HeLa (B) cells treated with cycloadducts **4a** and **5a** at concentrations 5, 10 and 20 μg/mL using flow cytometry

Cell cycle analysis

Analysis of the experimental results showed that both compounds stop the HeLa cell cycle in G2/M phase. So, after the impact of compounds **4a** and **5a** at concentration 10 μg/mL for 24h, the percentage of cells in G2/M phase of cycle is 31.73% for compound **4a** and 22.34% for compound **5a** (compared to 18.85% for control). The percentage of cells in the synthetic phase (S) of the cycle is lower for treated with **5a** (21.59%) then untreated cells (26.38%), while for **4a** it was increased (to 30.83%). After 24h of incubation of K562 cells, both compounds showed an increase in S-phase from 8.93% in the control cells to 16.60 and 15.35% in the treated cells (for **4a** and **5a**, respectively), and they increased sub-G1 phase from 3.62% to 14.36 and 10.31% (for **4a** and **5a**, respectively). Meanwhile, the percentage of cells in G2/M-phase was nearly the same, while it was decreased in G0/G1 phase to 50.62% for **4a** and to 57.78% for **5a** compared to 67.51% in the untreated cells. These findings indicate that the compounds **4a** and **5a** prevent cancer cells from starting DNA division. The strongest cytostatic effect was observed after treatment with compound **4a**. One of the most studied mechanisms of cell arrest in G2/M phase is the p53 upregulated cell arrest that prevents the transition of cells from G2 to mitosis. Western blot analysis shows an increase in p53 expression up to 310% compared to control under the treatment with **4a** compound. A significant dependence of the efficiency of inhibition of the cell cycle depending on the conformation of the spiro-fused heterocycle has been shown.

Cell cycle analysis

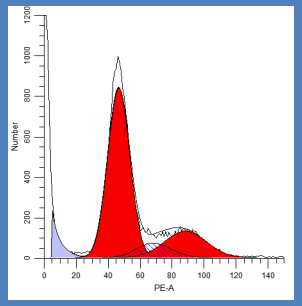
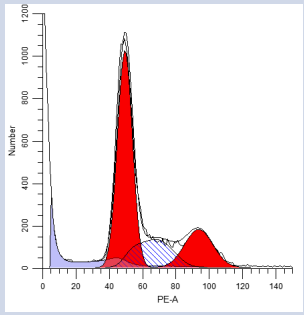
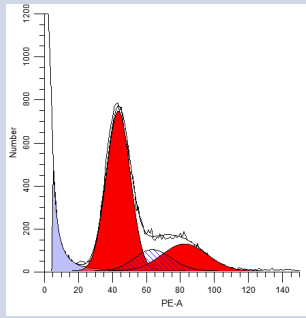
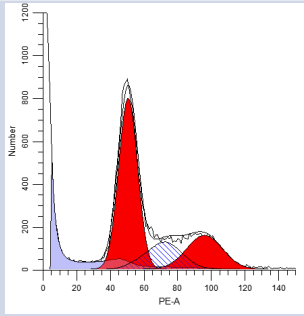
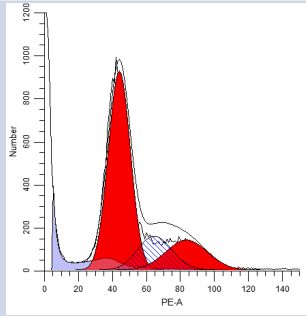
Effect of compounds **4a** and **5a** at 5 and 10 μg/mL concentration on the distribution of **HeLa** cells in the cell cycle after 24h treatment

Control	4a, 5 μg/mL	5a, 5 μg/mL
		
	4a, 10 μg/mL	5a, 10 μg/mL
		

	Control	4a (5 μg/mL)	4a (10 μg/mL)	5a (5 μg/mL)	5a (10 μg/mL)
SubG1 (%)	2.41±0.19	4.22±0.25	6.07±0.83	1.08±0.12	4.21±0.51
G0/G1 (%)	53.03±1.67	49.75±1.33	31.82±3.18	59.91±1.45	51.86±2.13
S (%)	26.38±1.05	21.73±1.36	30.83±2.79	10.22±0.62	21.59±1.46
G2/M (%)	18.85±0.93	24.30±1.25	31.73±3.02	28.79±1.48	22.34±1.67

Cell cycle analysis

Effect of compounds **4a** and **5a** at 5 and 10 μg/mL concentration on the distribution of **K562** cells in the cell cycle after 24h treatment

Control	4a, 5 μg/mL	5a, 5 μg/mL
		
	4a, 10 μg/mL	5a, 10 μg/mL
		

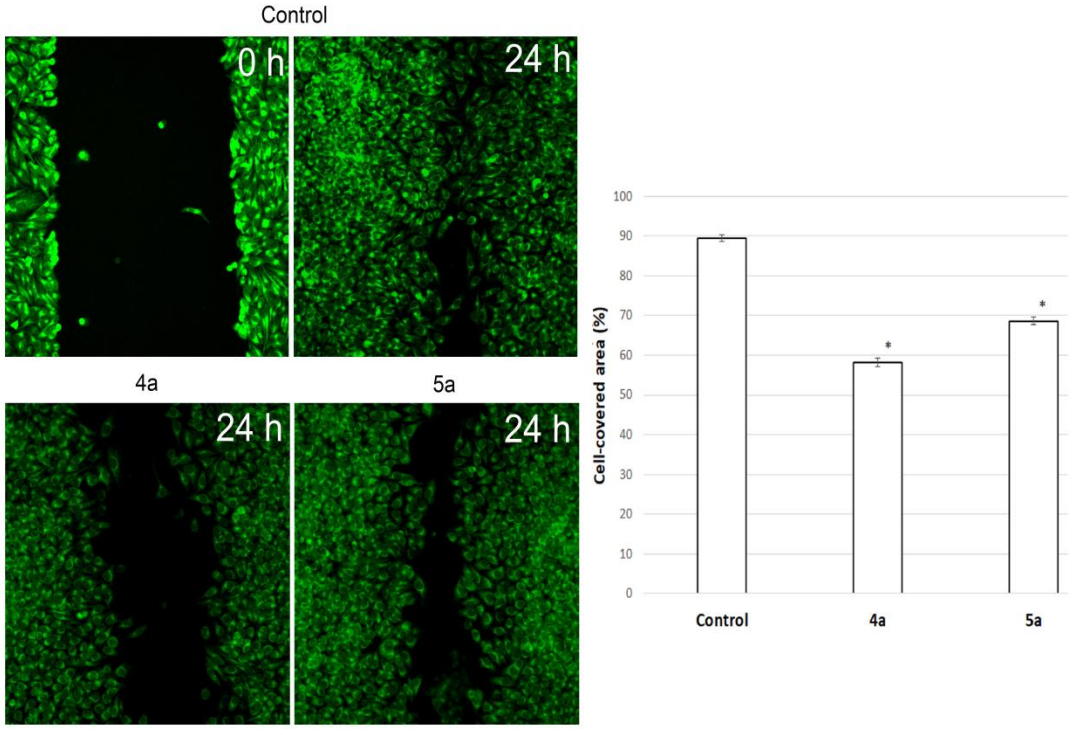
	Control	4a (5 μg/mL)	4a (10 μg/mL)	5a (5 μg/mL)	5a (10 μg/mL)
SubG1 (%)	3.62±0.23	11.32±0.95	14.36±1.22	9.82±0.70	10.31±1.21
G0/G1 (%)	67.51±1.12	53.04±1.01	50.62±1.92	58.91±1.72	57.78±2.04
S (%)	8.93±0.74	17.91±1.07	15.60±0.98	12.31±1.13	15.35±2.99
G2/M (%)	19.94±1.12	17.73±0.95	19.15±2.25	18.96±1.52	16.56±2.16

Inhibition of cell motility evaluated by scratch-test

Cell motility is a fundamental and ancient cellular behavior that contributes to metastasis. A major challenge in understanding metastatic tumour spread in patients is that the process cannot be observed or manipulated directly. Scratch-test is a simple model to assess the impact of different effects on cell motility and potential metastasis.

To assess the potential ability of compounds to inhibit metastasis associated with cell motility, a Scratch-test was performed on human cervical carcinoma (HeLa) cell line. We have found that nontreated HeLa cells filled the scratchad strip at 89.4 ± 0.8% while under treatment with compounds **4a** (10 μg/mL) and **5a** (20 μg/ml) cells fill 58.2 ± 1% and 68.6 ± 0.9% of scratched strip, respectively. Hela cells lose their ability to move under treatment and do not fill the scratched strip. Although compound **4a** shows greater cytotoxicity, **5a** appears to have a stronger inhibitory effect on cell motility. The presented results indicate that the tested compounds can effectively block the cellular movement of tumor cells.

Inhibition of cell motility evaluated by scratch-test



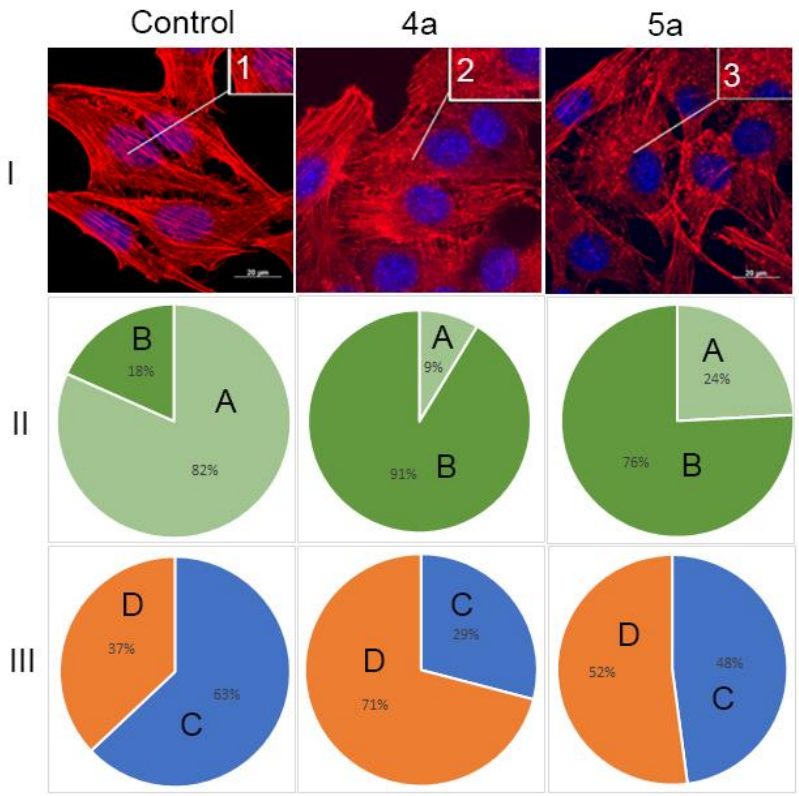
Microscopic images of the HeLa cells wound area in the scratch assay and wound area (%) in the scratch assay after 24h incubation post spiro-fused cycloadducts **4a** and **5a** treatment at concentration 10 μg/mL.

Actine cytoskeleton changes

The structure of the actin cytoskeleton of HeLa cells was assessed by the availability of stress fibers and the presence of filopodia-like protrusions after the impact of compounds **4a** and **5a**.

It was found using confocal microscopy that treatment with cycloadducts **4a** and **5a** led to significant changes of the actin cytoskeleton structure of tumor cells leading to the disappearance of stress fibers and changes in the number of filopodia-like deformations. Such treatment with compounds **4a** and **5a** led to decrease in the number of cells with stress fibers from 82% to 9% and 24% respectively. Similarly the number of cells with filopodia-like structures decreased from 63% in control sample to 29% and 48% in cells treated with either **4a** or **5a** respectively.

Actine cytoskeleton changes



State of actin cytoskeleton of HeLa cells after treatment with spiro-fused tryptanthrines and pyrrolo-[3,4-*a*]pyrrolizines **4a** and **5a**.
I: Images demonstrate the different stages of cell actin cytoskeleton.
II: Pie charts demonstrate percentage of cells with normal stress fibers (A) and disassembled stress fibers (B).
III: Pie charts demonstrate percentage of cells with filopodia-like deformations (C), and without filopodia-like deformations (D).
Inserts: 1 – stress fibers;
2, 3 - disassembled stress fibers.

Conclusions

In this study, we have established the ability of heterocyclic compounds containing spiro-fused pyrrolo[3,4-*a*]pyrrolizine and tryptanthrin framework to reduce the viability of both human erythroleukemia (K562) and human cervical carcinoma (HeLa) cell lines.

We have found that spiroadducts with all *cis* bridge-protons of pyrrolo[3,4-*a*]pyrrolizine moiety were more active in all cases. Replacement of hydrogen atom of pyrrole moiety by either alkyl or aryl group leads to significant decrease in activity of both formed cycloadducts.

Among target compounds only unsubstituted at pyrrole ring cycloadduct **4a** demonstrates significant activity with IC_{50} 1.9±0.2 μg/mL (K562, 72 h), while its diastereomer, **5a**, demonstrates nearly 7 fold lower activity with IC_{50} 14.9±0.5 μg/mL (K562, 72 h).

Third most potent ethyl substituted at pyrrole ring cycloadduct **4b** demonstrates intermediate activity with IC_{50} 7.8 ± 0.4 μg/mL (K562, 72 h) and its diastereomer, **5b**, was nearly 6 times weaker with IC_{50} 47.0 ± 0.7 μg/mL (K562, 72 h).

Both cycloadducts **4a** and **5a** were shown to downregulate growth of Hela cells, as well as arrest the cell cycle in the G2/M phase, lead to a decrease in the number of cells with normal stress fibers and filopodia-like deformations, induce apoptosis and decrease cell motility.

All obtained data make it possible to assume that spiro-fused pyrrolo[3,4-*a*]pyrrolizine and tryptanthrin framework may be considered as a new promising pharmacophore unit for further screenings.

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