Send Orders for Reprints to reprints@benthamscience.ae

Medicinal Chemistry, **2018**, *14*, **1-9**

RESEARCH ARTICLE

Synthesis and Biological Evaluation of Acridine/Acridone Analogs as Potential Anticancer Agents

Monika Gensicka-Kowalewska[a], Mirosława Cichorek[b], Anna Ronowska[c], Milena Deptuła[b], Ilona Klejbor[d] and Krystyna Dzierzbicka[*a]

[a]*Department of Organic Chemistry, Gdansk University of Technology, Narutowicza St 11/12, PL 80-233 Gdansk, Poland;* [b]*Department of Embryology, Medical University of Gdansk, Debinki St. 1, 80-210 Gdansk, Poland;* [c]*Department of Lab Med, Medical University of Gdansk, Debinki 7 Bldg 27, PL-80211 Gdansk, Poland;* [d]*Department of Anatomy & Neurobiology, Medical University of Gdansk, Debinki St. 1, 80-210 Gdansk, Poland*

Abstract: ***Background*:** The lack of efficacious therapy for advanced melanoma and neuroblastoma makes new approaches necessary. Therefore, many scientists seek new, more effective, more selective and less toxic anticancer drugs.

***Objective*:** We propose the synthesis of the new functionalized analogs of 1-nitroacridine/4-nitroacridone connected to tuftsin/retro-tuftsin derivatives as potential anticancer agents.

***Method*:** Acridine and acridone analogues were prepared by Ullmann condensation and then cyclization reaction. As a result of nucleophilic substitution reaction 1-nitro-9-phenoxyacridine or 1-chloro-4-nitro-9(10*H*)-acridone with the corresponding peptides, the planned acridine derivatives (**10a-c**, **12**, **17-a-d**, **19**) have been obtained. The cytotoxic activity of the newly obtained analogs was evaluated against melanotic (Ma) and amelanotic (Ab) melanomacell lines and neuroblastoma SH-SY5Y by using the XTT method. Apoptosis and cell cycle were analyzed by flow cytometry.

***Results*:** Among the investigated analogs compound 12 exhibited the highest potency comparable to dacarbazine action for amelanotic Ab melanoma cells. FLICA test (flurochrome-labeled inhibitors of caspases) showed that this analog significantly increased the content of cells with activated caspases (C+) among both neuroblastoma lines and only Ab melanoma line. Using phosphatidylserine (PS) externalization assay, 12 induced changes in the Ab melanoma plasma membrane structure as the externalization of phosphatidylserine (An+ cells). These changes in neuroblastoma cells were less pronounced.

***Conclusion*:** Analog 12 could be proposed as the new potential chemotherapeutic against amelanotic melanoma form especially.

ARTICLEHISTORY

Received: April 24, 2018
Revised: August 18, 2018
Accepted: September 24, 2018

DOI:
10.2174/1573406414666181015145120

Keywords: Acridine, Acridone, Tuftsin, Retro-tuftsin, Melanoma, Neuroblastoma.

1. INTRODUCTION

Cancer, which is mostly caused by a mutation of a normal cell, is one of the most common malignant diseases obsessing mankind [1, 2]. Tumor cells resistance to many anticancer drugs is a huge concern for effective cancer chemotherapy [3]. Hence, there is a high demand to develop new drugs with improved pharmacological properties, enhanced antitumor activity and lower toxicity. Acridines have attracted considerable interest because of their broad range of biological activity. Initially, acridine derivatives were applied as pigments and dyes [4]. The first clinical application of acridines was targeted against bacterial [5] and parasitic [6] infections. Their anticancer properties were discovered in the 70s [7, 8]. Actually, the anticancer activity of acridine/acridone analogs has attracted incoming interest. To date, many derivatives of acridine were synthesized and tested for antitumor activity [9, 10].

One of the ways to effective delivery of drugs to the target site in the body is using carrier-drug conjugates, in which the active ingredient is bound by a covalent bond with a carrier, *e.g.* peptides [11]. One of such peptides is tuftsin (TKPR), natural tetrapeptide, present in the peripheral blood of humans and other mammals, where it stimulates certain white blood cells [12]. The activity of tuftsin is generally directed towards the activation of non-specific elements of the immune system.

*Address correspondence to this author at Department of Organic Chemistry, Chemical Faculty, Gdansk University of Technology, G. Narutowicza 11/12, 80-233 Gdansk, Poland; Tel.: +48-58-347-20-54;
E-mail: krydzier@pg.edu.pl.

1573-4064/18 $58.00+.00

Although a lot of acridines/acridones and tuftsin analogs were prepared, attempts for new derivatives are still made. More effective anticancer drugs with lower side effects still leaves something to be desired. From this reason and taking into account our experience in the synthesis of compounds containing molecules of various mechanisms of action as acridine/acridone derivatives and current literature overview, we propose the synthesis of the new functionalized analogs of 1-nitro-acridine/4-nitroacridone connected to tuftsin/retro-tuftsin derivatives.

In this work, we synthesized a series of tuftsin/retro-tuftsin analogs with acridine and acridone derivatives containing isopeptide bond between ε-amino group of lysine and carboxyl group of aliphatic amino acids such as Gly, β-Ala, and Val. The newly synthesized tuftsin/retro-tuftsin compounds (**10a-c**, **12**, **17a-d**, **19**) were evaluated for their anticancer activities against Ab and Ma melanoma cells and neuroblastoma SHSY5Y. In the past decades, a steady increase in the incidence of malignant melanoma has been observed. The patients diagnosed with early-stage malignant melanoma are cured by primary surgical treatment but for individuals with metastatic melanoma, there is no satisfactory systemic chemotherapy. A similar situation occurs with neuroblastoma. Melanoma lines differ *e.g.* the level of melanogenesis that changes many biological features. Cells with hypomelanization have a higher rate of growth and are easy to culture *in vitro* in comparison to melanotic form [13]. Thus in our model, we used pairs of biological forms of the melanoma: amelanotic Ab, melanotic Ma and the neuroblastoma SHSY5Y: cholinergic DC, dopaminergic NC, to compare the chemosensitivity of biological variants of the examined tumors. The lack of efficacious therapy for advanced melanoma and neuroblastoma makes new approaches necessary**.**

2. EXPERIMENTAL

2.1. Chemistry

All chemicals and solvents were of reagent grade and were used without further purification. The reactions were monitored by TLC on Sigma-Aldrich F254 silica gel precoated plates. The following solvent systems (by vol.) were used for TLC development: $CHCl_3$-MeOH (3:1, v/v) (A), $CHCl_3$-MeOH (9:1, v/v) (B), $CHCl_3$-MeOH-NH_3 (7:1:0.01, v/v/v) (C), $CHCl_3$-MeOH-NH_3 (5:1:0.01, v/v/v) (D). MS spectra were recorded on matrix-assisted laser desorption/ ionization-time on flight mass spectrometry (MALDI-TOF MS, Biflex III Bruker). The detection was carried out using UV and ninhydrin. ^{1}H and ^{13}C NMR spectra were measured in dimethyl sulfoxide (DMSO) or CD_3OD solutions with a Varian Unity 500 Plus spectrometer, using TMS as an internal standard. Chemical shifts (δ) are given in ppm. Multiplicity is indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Conditions of chromatographic HPLC separation and detection of the examined compounds (**10a-c**, **12**, **17a-d**, **19**): column - Poroshell EC-C18 (3.0x150 mm, 2.7 µm, Agilent Technologies; column temperature - 40 °C; injection volume - 2 µL; flow rate - 0.4 mL/min; eluents: (A) 0.1 % HCOOH in water, (B) 0.1 % HCOOH in ACN/MeOH (1:1, v/v); gradient program: 0 min. 90% (A) 10% (B), 20 min. 0% (A) 100% (B), 30 min. 0% (A) 100% (B); post time - 10 min; UV-Vis detection; wavelengths UV: 254 nm; Vis: 580 nm; peak width > 0.1 min (2s); ESI MS detection.

Tuftsin and retro-tuftsin derivatives **1**, **2a-c** and **3** were prepared according to the method described previously [14-17]. The general preparation of 1-nitro-9-phenoxy acridine (**8**) and 1-chloro-4-nitro-9(10*H*)-acridone (**15**) was described in the literature [18-20].

2.1.1. General Procedure for the Synthesis of Acridine with Tuftsin/Retro-Tuftsin Derivatives 10a-c and 12

A mixture of 1-nitro-9-phenoxyacridine (**8**) (0.12 mmol) and tuftsin or retro-tuftsin derivatives (**1**, **2a-b**, **3**) (0.24 mmol) in phenol was stirred at 50°C for 24 h under argon atmosphere. After completion, a solution of ethyl acetate (5 mL) was added and the mixture was extracted with 5% KOH (5 mL x 4). The organic phases was dried with anhydrous $MgSO_4$, and filtered. After evaporation of the solvent, the reaction mixture was purified using preparative thin layer chromatography (TLC) in solvent B to obtain compounds **9a-c**, **11**.

To a mixture of derivatives **9a-c**, **11** in *N*,*N*-dimethylformamide (DMF) (1 mL) diethylamine (DEA) (0.4 mL) was added. The mixture was left for 1h at room temperature. After completion, evaporation of the solvent followed by purification with preparative TLC in solvent C or D was done to obtain derivatives without the 9-fluorenylmethoxycarbonyl (Fmoc) group. Then, these derivatives were suspended in MeOH saturated with hydrochloric acid in diethyl ether (Et_2O). Evaporation of the solvent followed by purification with preparative TLC in solvent C or D, provided the corresponding product **10a-c**, **12**.

2.1.2. General Procedure for the Synthesis of Acridone with Tuftsin/Retro-Tuftsin Derivatives 17a-d and 19

To a mixture of 1-chloro-4-nitro-9(10*H*)acridone (**17**) (0.12 mmol) and tuftsin or retro-tuftsin derivatives (**1**, **2a-c**, **3**) (0.24 mmol) in anhydrous DMF (3 mL), anhydrous triethylamine (TEA) (0.48 mmol) was added. The mixture was stirred at room temperature for 24 h under argon atmosphere. After completion, evaporation of the solvent followed by purification with preparative TLC in solvent B was done to obtain derivatives **16a-d**, **18**.

To a mixture of derivatives (**16a-d**), (**18**) in DMF (1 mL) DEA (0.4 mL) was added. The mixture was left for 1h at room temperature. Evaporation of the solvent followed by purification with preparative TLC in solvent C or D, provided the corresponding derivatives without the Fmoc group. Then, the mixture of derivatives was suspended in MeOH saturated with hydrochloric acid in diethyl ether (Et_2O). Evaporation of the solvent followed by purification with preparative TLC in solvent C or D, provided the corresponding product **17a-d**, **19**.

2.2. Biological Studies

2.2.1. Transplantable Melanomas

The original transplantable melanotic melanoma (Ma) was derived from a spontaneous melanoma of the skin that had appeared in a bred of a golden hamster in 1959 [21]. The

amelanotic melanoma line (Ab) originated from the Ma form by a spontaneous alteration. The loss of pigment was accompanied by changes in many biological features of Ab line - faster tumor growth rate, shorter animal survival and changes in ultrastructure of the cells. Once established, these melanomas possessed a considerable degree of phenotypic stability over decades of passaging [21]. Since their discovery, each melanoma line is maintained *in vivo* by consecutive, subcutaneous transplantations of tumor material every 21 (Ma) or 11 (Ab) days. This melanoma model is known as Bomirski hamster's melanomas. The experiments' procedures were approved by the Animal Ethics Committee at Medical University of Gdansk and conducted in accordance with National Health and Medical Research Council's guide for the care and use of laboratory.

Melanoma cells were isolated for each experiment from solid tumors by a non-enzymatic method [13]. The suspension consisted of 95 - 98% viable cells (estimated by trypan blue test). Melanoma cells were cultured in an RPMI medium (Sigma-Aldrich) supplemented with 10% of FBS (fetal bovine serum; Sigma-Aldrich) and antibiotics (0.05 mg streptomycin and 50 U penicillin per 1 mL; Sigma-Aldrich). Each independent experiment was performed on the cells isolated from a single tumor.

2.2.2. Neuroblastoma

Neuroblastoma SH-SY5Y is a cloned subline of SK-N-SH of human neuroblastoma originally established from the bone marrow biopsy of a neuroblastoma patient in the early 1970s. Human SH-SY5Y neuroblastoma cells, between 10th and 25th passage, were used for the experiments. Cells were seeded at a density of 4 x 10^3 cells/cm^2 on 25 cm^2 tissue culture flask in DMEM medium (Sigma-Aldrich) with 10% FBS (Sigma-Aldrich) and antibiotics (0.05 mg streptomycin and 50 U penicillin per 1 mL; Sigma-Aldrich). Cholinergic differentiation of DC variant was obtained by combined addition of 1 mM/L dibutyryl cAMP (cAMP; Sigma-Aldrich) and 0.001 mM/L all-trans-retinoic acid (RA; Sigma-Aldrich) for 48 hrs [22]. At this time the medium has been replaced for the medium without differentiating agents and tested analogs were added and culture was continued for the next 72 hrs. Cell line was purchased from the American Type Culture Collection (ATCC).

2.2.3. Analogs Treatment

New synthesized analogs at concentrations of 0.1-150 µM were used. All the solutions used in the experiments were prepared with a suitable cultivation medium for the cell lines and the main stock was dissolved in water containing 5% of dimethyl sulfoxide (DMSO) (Sigma-Aldrich). For induction of death, thecells were incubated in the media without (spontaneous death) or with analog for 48 and/or 72 hrs at 37°C. After that time, the cells were harvested and used for analysis.

2.2.4. Cells Viability Assay (XTT)

The cell viability was determined by XTT assay (Roche Diagnostic, USA), which measures the cells ability to reduce tetrazolium salt XTT (2,3-bis- (2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) to water-soluble formazan product. Cells were seeded at a density of $5x10^3$ neuroblastoma, Ab melanoma and $50x10^3$ Ma melanoma cells into 96-well plates with suitable cultivation media. After 24 hrs, the media were exchanged and the cells were stimulated with the appropriate concentrations (0.1; 1; 10; 20; 40; 50; 100; 150 µM) of the tested analogs for 48 and 72 hrs. Orange-colored formazan product was quantified at 450 nm in a microplate reader (Multiscan FC, Thermoscientific USA). Cells proliferation was normalized with respect to the non-treated control (100%) and the half maximal inhibitory concentration IC_{50} was estimated. For analogs where IC_{50} was not possible to be determined, the percentage of living cells after treatment with 150 µM concentration was calculated.

2.2.5. Apoptosis Determination by Flow Cytometry

Melanoma cells were seeded in 6-well plates at concentration of 200 x 10^3/well and 500 x 10^3/well for Ab and Ma lines respectively. Neuroblastoma cells were seeded at density of 10^5 in 25 cm^3 culture flask. Cells were cultured without (control, spontaneous death) and with 100-µM analog **12**. The acridine **7** as a basic acridine for analog **12** synthesis has also been tested at IC_{50} (15 µM) for Ab melanoma and 100 µM for Ma melanoma lines. Cells were cultured for 48 and/or 72 hrs. For the tumor cells death analysis two tests for the apoptosis estimation have been chosen: test for the detection of cells with activated caspases (FLICA test) and Annexin-V_FITC binding to the phosphatidylserine (PS) presented in the outer phospholipids layer of the plasma membrane (PS externalization), propidium iodide (PI) DNA staining as a marker of apoptotic bodies. Cells were analyzed using a flow cytometry method. After incubation, the cells were analyzed using a C6 flow cytometer (Becton Dickinson Immunocytometry Systems, USA). After gating out small-sized (*e.g.* noncellular debris) objects, 10 000 events were collected from each sample. Results were analyzed off-line using Cyflogic v.1.2.1 software.

2.2.7. Activated Caspases

We used FLICA test (flurochrome-labeled inhibitors of caspases) to estimate the cells containing activated caspases [23]. The idea of this method is based on the fact that fluorochrome-labeled inhibitors of caspases covalently react with reactive enzymatic center of activated caspase. We used FITC labeled pan-inhibitor of caspases VAD-FMK which detects the most active caspases in the cell. After 48 and 72 hrs, the cells were collected and $1x10^6$ cells from each experimental point were incubated with 5 µM FITC-VAD-FMK (CaspACETMFITC-VAD-FMK, Promega, USA) for 30 min at room temperature in the dark, washed and resuspended in PBS.

2.2.8. Phosphatidylserine (PS) Externalization Assay

The early apoptotic change of the plasma membrane structure as PS externalization - was determined by Annexin V-FITC (BD Pharmingen, USA) according to the manufacturer's instructions. The staining allows determining populations of viable (An-/PI-) and with PS externalization (An+; sum of early An+PI- and late apoptotic An+PI+) cells.

TFAxThr-Lys(Fmoc)-Pro-Arg(NO_2)-OCH_3

1

TFA-Thr-Lys(FmocX)-Pro-Arg(NO_2)-OCH_3

2a-c

2a: X=Gly
2b: X=b-Ala
2c: X=Val

TFAxArg(NO_2)-Pro-Lys(Fmoc)-Thr-OCH_3

3

Fig. (1). Structure of tuftsin (**1**, **2a-c**) and retro-tuftsin (**3**) derivatives.

2.2.9. Sub G1 Phase – Apoptotic Bodies

Damaged cells as cells with a reduced DNA content (loss of DNA from permeabilized cells due to apoptotic DNA fragmentation; apoptotic bodies) were evaluated by the cell cycle analysis method [24]. Using the nucleic acid dye propidium iodide (PI) apoptotic cells were represented by a sub G1 region of cell cycle histogram. Ethanol-fixed $1x10^6$ cells were resuspended in 1 mL of staining solution (RNaseA 200 µg/mL and PI (5 µg/mL in PBS), incubated for 30 min in 37°C, in the dark.

2.2.10. Statistical Analysis

The statistical analysis was performed using the data analysis software system STATISTICA version 12, StatSoft Inc. (2016). Nonparametric Mann-Whitney U test with the Bonferroni correction; p-value lower than 0.05 (for one pair)

And 0.025 (for two pairs) were considered statistically significant. Data are expressed as mean ± SD.

3. RESULTS AND DISCUSSION

3.1. Chemistry

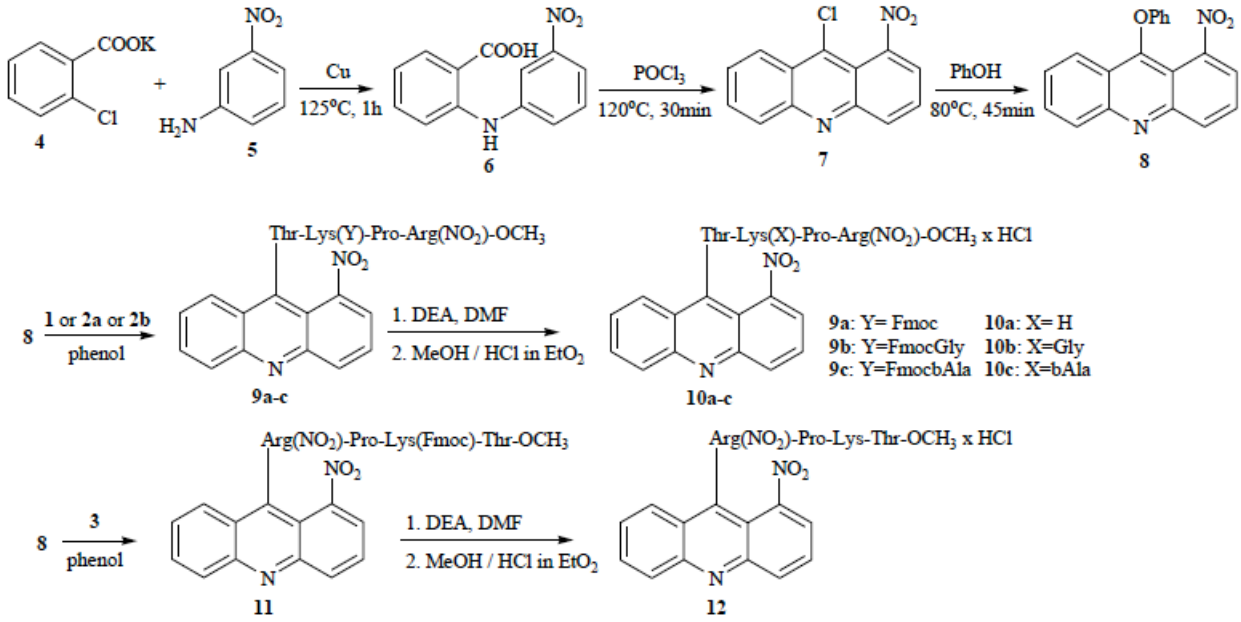
The synthesis of adequate protected tuftsin and retro-tuftsin derivatives (**1**, **2a-c**, **3**) Fig. **(1)** was performed according to the method described in the literature [17] by using mixed anhydride method with isobutyl chloroformate and *N*-methylmorpholine (NMM) in anhydrous DMF.

The synthesis of acridine linked to tuftsin/retro-tuftsin derivatives (**10a-c**, **12**) was carried out according to the reactions presented in Scheme **1**. It was started from the Ullmann condensation reaction of the potassium salt of *o*-chlorobenzoic acid **4** and *m*-nitroaniline **5** in the presence of copper at 125°C to give *N*-(3'-nitrophenyl)anthranilic acid **6**, which was refluxed in $POCl_3$ to afford the 9-chloroacridine derivative **7**. Then, acridine **7** was converted by the reaction with phenol to stable 1-nitro-9-phenoxyacridine **8** [18]. As a result of nucleophilic substitution reaction, 1-nitro-9-phenoxyacridine **8** with the corresponding peptides (**1**, **2a**, **2b** or **3**) in phenol gave analogs (**9a-c**, **11**). Next, compounds **9a-c** and **11** were selectively deprotected with DEA and were dissolved in MeOH and performed to the corresponding hydrochloride (**10a-c**, **12**) by using HCl in anhydrous Et2O.

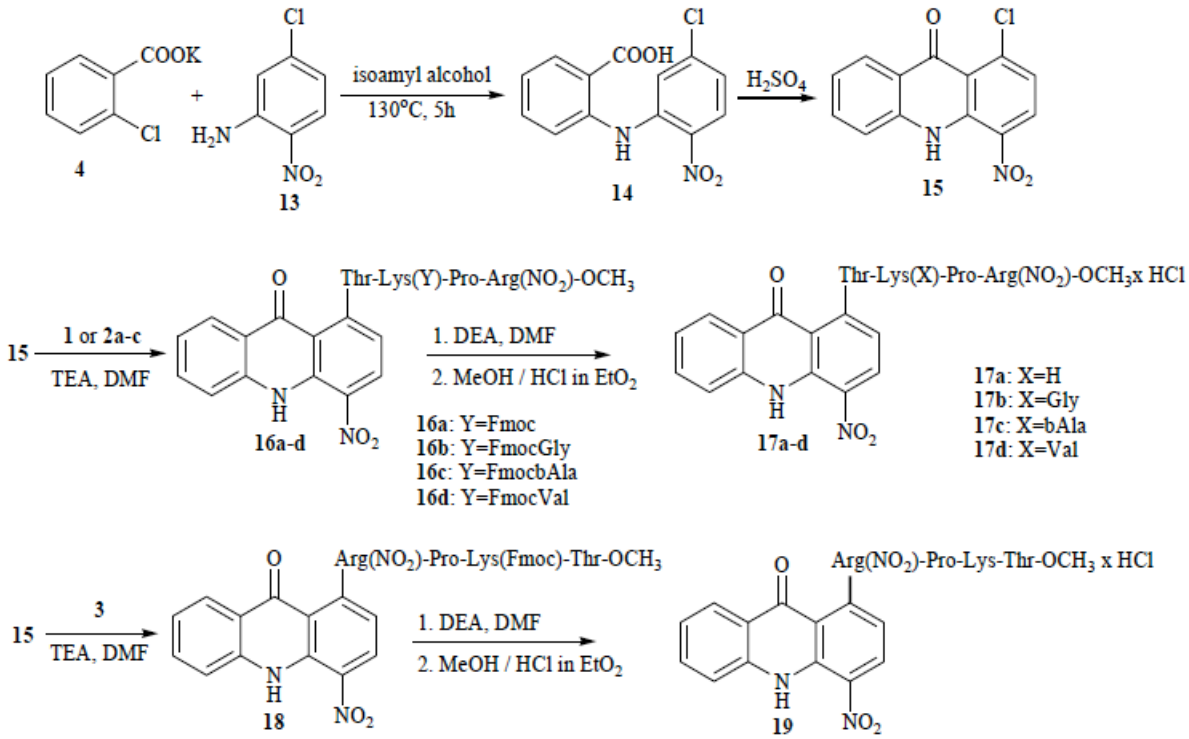
The synthesis of acridone with tuftsin/retro-tuftsin derivatives (**17a-d**, **19**) was carried out according to the reactions presented in Scheme **2**. 1-Chloro-4-nitro-9 (10 *H*)acridone **15** was obtained by cyclization of the anthranilic acid **14**, which was prepared from the Ullmann condensation of the potassium salt of *o*-chlorobenzoic acid **4** with 5-chloro-2-nitroaniline **13** in isoamyl alcohol, in the presence of a catalytic amount of copper dust. Acridone analogs (**16a-d**, **18**) were obtained on the basis of nucleophilic substitution reaction of 1-chloro-4-nitro-9 (10*H*) acridone **15** with tuftsin/retro-tuftsin derivatives (**1**, **2a-c**, **3**) in anhydrous DMF, in the presence of triethylamine. The protecting groups were removed by DEA and MeOH/HCl to obtain compounds (**17a-d**, **19**).

3.2. Biological Assays

As targets for the new analogs action estimations, we chose neuroblastoma and melanoma cells, tumors with not satisfactory therapies results. Melanoma and neuroblastoma



Scheme 1. Synthesis of acridine derivatives (**10a-c**, **12**).



Scheme 2. Synthesis of acridone derivatives (**17a-d**, **19**).

are not homogenous types of tumors, they have different biological variants. Thus the effectiveness of a potential chemotherapeutic estimated at one form not always causes the same effects on others. Nowadays, oncology is looking for individual therapies against the biological types of a tumor. Melanoma and neuroblastoma develop from normal cells (melanocytes, neurons) of the same embryonic origin – neuroectoderm. SH-SY5Y is a neuroblast-like cell line of the dopaminergic phenotype (NC variant; SH-SY5Y-NC; mainly uses the neurotransmitter dopamine) but can be differentiated to cholinergic phenotype *in vitro* (DC variant SH-SY5Y-DC; mainly uses the neurotransmitter acetylcholine). The Bomirski hamster melanomas model has two basic forms: melanotic Ma (produces melanin) line and amelanotic Ab (lack of melanin production). The cytotoxicity of the synthesized analogs was estimated by XTT test and the apoptotic death features as the caspases activation and plasma membrane changes (phosphatidylserine, PS, externalization) were analysed.

3.2.1. Cells Viability Assay (XXT)

As shown in Table **1** only analog **12** decreased cells viability in concentrations that allowed to estimate the IC_{50} doses as 61 μM, 96 μM, 48 μM, for Ab melanoma, NC and DC forms of SH-SY5Y neuroblastoma respectively. Among Ma melanoma cells incubated with the highest (150 μM) dose of analog **12** about 60% were still alive (mitochondrial activity was not inhibited). Analog **10b** seemed to influence Ab melanoma line but only at the highest dose - 50% of cells have inhibited mitochondrial activity. Analog **17b** at 150 μM dose influence mitochondria in 40% and 50% of Ab melanoma and SH-SY5Y-DC cells respectively. Thus, for further examination of tumor cells death, analog **12** has been chosen. The 9-chloro-1-nitroacridine **7**, precursor for the tested analogs, was especially active against Ab melanoma line (IC_{50} 15 μM). Its IC_{50} doses against SH-SY5Y neuroblastoma were 51 μM, 36 μM for NC and DC forms respectively.

3.2.2. The Dying Cells Plasma Membrane Structure Changes

Compound **12** induces changes in the Ab melanoma plasma membrane structure as the externalization of phosphatidylserine (An+ cells). After 48 hrs 54% of Ab melanoma cells were An+, significantly more than in control cells incubated without **12** but this value did not increase significantly after additional 24 hrs (data not shown in Table. **2**, Fig. **2A**). Though Ma melanoma after additional 24 hrs has only 34% An+ cells (data not shown) almost the same as after 48 hrs (Table. **2**). After 72 hrs incubation with **12** about 14% of both neuroblastoma have changes in the plasma membrane structure, two folds more than in cells incubated without **12** (Table. **2**, Fig. **2B**).

3.2.3. Caspases Activation

Analog **12** significantly increased the content of cells with activated caspases (C+) especially in Ab melanoma line and DC cholinergic form of SH-SY5Y. After 48 hrs about 32% of Ab melanoma cells were C+ (Table **2**, Fig. **2A**). In the same culture condition after 72 hrs among Ma melanoma was only about 10% of cells with activated caspases, similar to the control without **12** (Table **2**) and it did not change after additional 24 hrs (data not shown). After 72 hrs incubation with **12**, there was about 27% and 16% C+ cells among DC cholinergic and NC dopaminergic cells respectively (Table **2**, Fig. **2B**).

Table 1. **Analogs influence on melanoma and neuroblastoma cells viability by XTT assay. Melanotic (Ma) and amelanotic (Ab) melanoma cells and dopaminergic (NC) and cholinergic (DC) neuroblastoma SHSY5Y cells were used as targets. Values are means ± SD from at least 3 experiments. IC_{50} - half maximal inhibitory concentration; ne analog not estimated.**

Name of Analog	Melanoma						Neuroblastoma SHSY5Y			
	Melanotic (Ma Line)		Amelanotic (Ab Line)				Dopaminergic (NC Line)		Cholinergic (DC) Line	
	% of Viability with 150 µM		IC 50 [µM]		% of Viability with 150 µM		IC 50 [µM]	% of Viability with 150 µM	IC 50 [µM]	% of Viability with 150 µM
	48h	72h	48h	72h	48h	72h	72h	72h	72h	72h
10a	102.1±10.6	93.6 ± 14.5	-	-	129±40	117.5 ± 35.9	-	69.9±8.6	-	63.6±5.1
10c	161.5±36.1	137.4±12.6	-	-	110.9±25.4	91.3±14.2	-	97.6±8.2	-	76.8±11.8
10b	111.4±1.7	100.4±10.1	97.5±34.8	-		50.2±20.9	-	95.6±0.6	-	88.3±8.3
17a	113.7±32.7	91.3±24.2	-	-	74.6±28.9	88 ± 15.9	-	110±8.6	-	111.3±2.8
17c	80.3±4	76.2±12.2	-	-	58.8±10.5	78.4±11.7	-	ne	-	ne
17b	83.4±4.2	77.2±4.4	-	-	76.5±22.4	60.4±25.4	-	71.6±0.6	-	48.9±4.8
17d	79±1.4	85±10.3	-	-	99.2±27.6	92.1±14.3	-	ne	-	ne
12	59.9±23	58.5±32.2	80.1±31.6	61±41.1	-	-	95.5±5.8	-	48.3±6.8	-
19	117.7±23.2	73.4±8.4			110.3±26	97.1±4.7		ne		ne
7	65.4±20.7	48.5±14.2	14.3±13.3	3.4±3.2	-	-	51±8.9	-	36.2±4.5	-
Dacarbazine	71.8±17.4	69.4±10	-	68.5±20.9	47.5±9.7	-	ne	ne	ne	ne
Cisplatine	**ne**	**ne**	**ne**	**ne**	**ne**	**ne**	**2.5±0.6**	**-**	**1.8±0.9***	-

* According to observed cells morphology changes test has been finished after 48 hrs.

Table 2. **Percentage of the melanotic Ma and amelanotic Ab melanoma cells; dopaminergic NC and cholinergic DC variant of SH-SY5Y neuroblastoma cells with apoptotic death features as activated caspases and phosphatidylserine (PS) externalization after incubation without (control) and with analog 12 (+12; 100 µM). The basic compound analog 7 has also been tested against melanoma lines at concentrations: 100 µM for Ma line and IC_{50} for Ab line. Values are means ± SD. Statistical analysis by U Mann-Whitney test; * Statistically significant increase in comparison to values without analog 12 (control). * Statistically significant lower sensitivity of Ma line in comparison to Ab line. 3-5 for Ma melanoma, neuroblastoma and 6-8 experiments for Ab melanoma have been done. ne - not estimated.**

Cells	Incubation Time in hrs	Percentage of Cells with/in								
		Activated Caspases			PS Externalization			Sub G1		
		Control	+ 12	+7	Control	+ 12	+ 7	Control	+12	+7
Melanotic Ma melanoma	48	9.8.±8.8	9.8±2.4**	4.2±2.8**	28.8±8.8	32.7±8.9**	19.6±6.7	16.0±7.7	14.9±13.3	17.5±5.5
Amelanotic Ab melanoma	48	9.9±5.6	**31.5±7.1***	15.3±4.9*	24.7±11.6	**54.2±9.6***	33.4±11.4	16.5±7.1	**41.3±8.8***	17.5±5.2
Dopaminergic SH-SY5Y-NC	72	7.1±2.6	**16.2±8.4***	ne	7.8±4.6	**13.3±3.8**	**ne**	0.8±0.1	1.6±0.5	**ne**
Cholinergic SH-SY5Y-DC	72	8.8±2.1	**27.2±2.7***	ne	7.4±2.3	**14.5±5.4***	**ne**	1.2.±0.5	1.4±0.5	**ne**

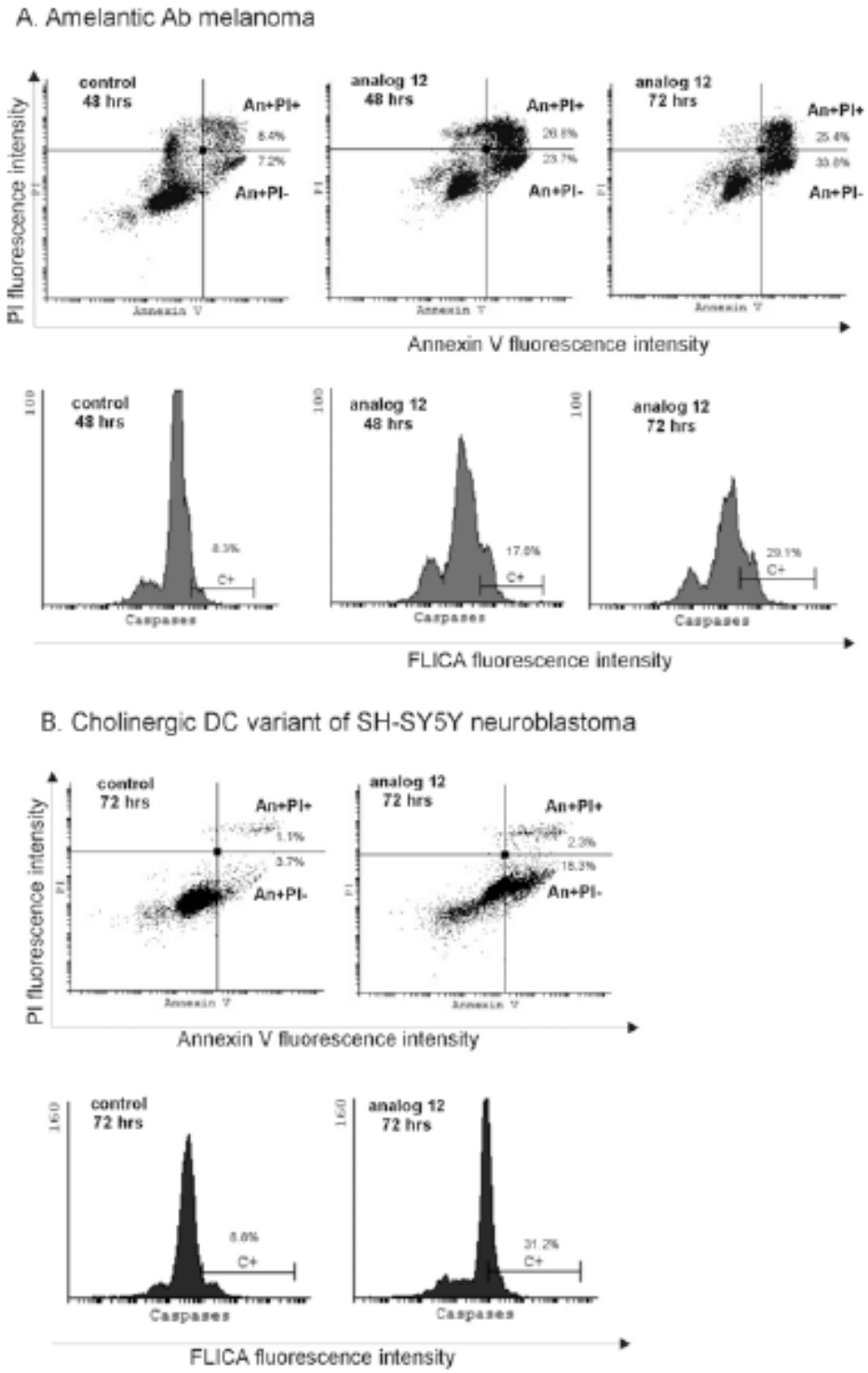


Fig. (2). Flow cytometric analysis of amelanotic Ab melanoma (A) and cholinergic SH-SY5Y-DC (B) cells with externalized phosphatidylserine (Annexin V test) and with active caspases (FLICA test). Density plots and histograms of a representative experiment. The plots are divided into quadrants containing: lower right An+PI- early apoptotic, upper right An+PI+ late apoptotic. The marker C+ in histograms pointed cells with activated caspases.

3.2.4. Damaged Cells in Sub G1 Phase

The content of cells in sub-G1 illustrates the effectiveness of analog **12** on the examined cells (Table **2**). More than 40% of Ab melanoma and only 15% of Ma melanoma cells treated for 48 hrs with **12** located in sub G1phase as apoptotic bodies. After an additional 24 hrs these values were 54% and 33% respectively (data not shown). Analog **12** does not increase the content of neuroblastoma cells in sub G1 phase significantly although other features of apoptosis (externalization of phosphatidylserine, activation of caspases) have been observed.

CONCLUSION

In conclusion, we first report the evaluated series of new tuftsin/retro-tuftsin analogs combined with acridine/acridone derivatives for their anticancer activities against amelanotic Ab and melanotic Ma melanoma cells and two forms of SHSY5Y neuroblastoma: DC cholinergic and NC dopaminergic. Analog **12** induces apoptotic changes as caspases activation and externalization of phosphatidylserine in the plasma membrane in the examined cells. Among the tested lines, amelanotic melanoma cells were especially sensitive to analog (acridine linked retro-tuftsin) **12** action. We suspected that the tuftsin recognized by cell-surface receptors would increase the new drugs action against tested tumor lines. Adding retro-tuftsin increased the entering of **12** into the cells that we checked for Ab cells under the fluorescence microscope. But except for the increased incorporation of **12** to Ab, the cells we observed decreased the activity of it in comparison to **7**. Tuftsin recognizes its receptor neutropilin (NRP) and activates some signaling ways and associates with a variety of coreceptors thus it could influence cell biology through the different signaling pathways. NRP presence has been documented in melanoma as in neuroblastoma cells. The expression of NRP seemed to be connected with the melanoma progression and aggressiveness [25,26]. Except for the influence of tuftsin through the receptor, there are also other observations of its capacity to interact directly with DNA thus it reduced the acridine binding to DNA. High NRP1 expression inhibited the action of oncogene-targeted (BRAF) therapies in melanoma. To sum up, it is very difficult to explain why adding retro-tuftsin to acridine **7** decreased its cytotoxicity, it needs further examination.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This work was financially supported by the Polish National Science Center, grant no. 2014/13/B/NZ7/02234.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

REFERENCES

[1] Barot, K.P.; Nikolova, S.; Ivanov, I.; Ghate, M.D. Novel anticancer agents and targets: recent advances and future perspectives. *Mini Rev. Med. Chem.*, **2013**, *13*, 1239-1255.

[2] Kaplan-Ozen, C.; Tekiner-Gulbas, B.; Foto, E.; Yildiz, I.; Diril, N.; Aki, E.; Yalcin, I. Benzothiazole derivatives as human DNA topoisomerase II alpha inhibitors. *Med. Chem. Res.*, **2013**, *22*, 5798-5808.

[3] Prasad, V.V.S.R.; Reddy, G.D.; Kathmann, I.; Amareswararao, M. Peters, G.J. Nitric oxide releasing acridone carboxamide derivatives as reverters of doxorubicin resistance in MCF7/Dx cancer cells. *Bioorg. Chem.*, **2016**, *64*, 51-58.

[4] Geddes, C.D. Optical thin film polymeric sensors for the determination of aqueous chloride, bromide and iodide ions at high pH, based on the quenching of fluorescence of two acridinium dyes. *Dyes Pigm.*, **2000**, *45*, 243-251.

[5] Dana, S.; Prusty, D.; Dhayal, D.; Gupta, M.K.; Dar, A.; Sen, S.; Mukhopadhyay, P.; Adak, T.; Dhar, S.K. Potent antimalarial activity of acriflavine *in vitro* and *in vivo*. *ACS Chem. Biol.*, **2014**, *9*, 2366-2373.

[6] Di Giorgio, C.; De Meo, M.; Chiron, J.; Delmas, F.; Nikoyan, A.; Jean, S.; Dumenil, G.; Timon-David, P.; Galy, J.P. Synthesis and antileishmanial activities of 4,5-di-substituted acridines as compared to their 4-mono-substituted homologues. *Bioorg. Med. Chem.*, **2005**, *13*, 5560-5568.

[7] Gamega, S.A.; Spicer, J.A.; Atwell, G.J.; Finlay, G.J.;Baguley, B.C.; Denny, W.A. Structure-activity relationships for substituted bis(acridine-4-carboxamides): A new class of anticancer agents.*J. Med. Chem.*, **1999**, *42*, 2383-2393.

[8] Gensicka-Kowalewska, M.; Cholewiński, G.; Dzierzbicka, K. Recent developments in the synthesis and biological activity of acridine/acridone analogues.*RSC Advances*, **2017**, *7*, 15776-15804.

[9] Malachowska-Ugarte, M.; Cholewinski, G.; Dzierzbicka, K.; Trzonkowski, P. Synthesis and biological activity of novel mycophenolic acid conjugates containing nitro-acridine/acridone derivatives. *Eur. J. Med. Chem.*, **2012**, *54*, 197-201.

[10] Kukowska, M. Amino acid or peptide conjugates of acridine/ acridone and quinoline/quinolone-containing drugs. A critical examination of their clinical effectiveness within a twenty-year timeframe in antitumor chemotherapy and treatment of infectious diseases. *Eur. J. Pharm. Sci.*, **2017**, *109*, 587-615.

[11] Kukowska-Kaszuba, M.; Dzierzbicka, K. Synthesis and structure-activity studies of peptide-acridine/ acridone conjugates. *Curr. Med. Chem.*, **2007**, *14*, 3079-3104.

[12] Siebert, A.; Gensicka-Kowalewska, M.; Cholewiński, G.; Dzierzbicka, K. Tuftsin – properties and analogs. *Curr. Med. Chem.*, **2017**, *24*, 3711-3727.

[13] Cichorek, M.; Kozłowska, K.; Bryl, E. The activity of caspases in spontaneous and camptothecin-induced death of melanotic and amelanotic melanoma cells. *Cancer Biol. Ther.*, **2007**, *6*, 346-353.

[14] Kofoed, T.; Hansen, H.F.; Ørum, H.; Koch, T. PNA synthesis using a novel Boc/acyl protecting group strategy. *J. Pept. Sci.*, **2001**, *7*, 402-412.

[15] Dzierzbicka, K.; Trzonkowski, P.; Sewerynek, P.; Kolodziejczyk, A.M.; Myśliwski, A. Synthesis and biological activity of tuftsin, its analogue and conjugates containing muramyl dipeptides or nor-muramyl dipeptides. *J. Pept. Sci.*, **2005**, *11*, 123-135.

[16] Dzierzbicka, K.; Wardowska, A.; Rogalska, M.; Trzonkowski, P. New conjugates of muramyl dipeptide and nor-muramyl dipeptide linked to tuftsin and retro-tuftsin derivatives significantly influence their biological activity. *Pharmacol. Rep.*, **2012**, *64*, 217-223.

[17] Januchta, W.; Serocki, M.; Dzierzbicka, K.; Cholewinski, G.; Skladanowski, A. Synthesis of functionalized new conjugates of batracylin with tuftsin/retro-tuftsin derivatives and their biological evaluation. *Eur. J. Med. Chem.*, **2015**, *106*, 85-94.

[18] Ledochowski, A. Ledacrin - anticancerous medicine 1-nitro-9(3-dimethyloamino-propylamino)-acridine-2HCl-H_2O. *Mater. Med. Pol.*, **1976**, *8*, 237-251.

[19] Capps, D.B. Substituted 1-amino-4-nitroacridinones, pharmaceutical compositions comprising the same and processes for their production. European Patent E.P. 0145226, October 31, 1984.

[20] Dzierzbicka, K.; Kołodziejczyk, A.M.; Wysocka-Skrzela, B.; Myśliwski, A.; Sosnowska, D. Synthesis and antitumor activity of conjugates of muramyldipeptide, normuramyl dipeptide, and des-muramylpeptides with acridine/acridone derivatives. *J. Med. Chem.*, **2001**, *44*, 3606-3615.

[21] Bomirski, A.; Slominski, A.; Bigda, J. The natural history of a family of transplantable melanomas in hamsters. *Cancer Metastasis Rev.*, **1988**, *7*, 95-118.

[22] Bielarczyk, H.; Jankowska, A.; Madziar, B.; Matecki, A.; Michno, A.; Szutowicz, A. Differential toxicity of nitric oxide, aluminum, and amyloid b-peptide in SN56 cholinergic cells from mouse septum.*Neurochem. Int.*, **2003**, *42*, 323-331.

[23] Smolewski, P.; Grabarek, J.; Halicka, H.D.; Darzynkiewicz, Z. Assay of caspase activation *in situ* combined with probing plasma membrane integrity to detect three distinc stages of apoptosis. *J. Immunol. Methods*, **2002**, *265*, 111-121.

[24] Darżynkiewicz, Z.; Williamson, B.; Carswell, E.; Old, L. Cell cycle-specific effects of tumor necrosis factor. *Cancer Res.*, **1984**, *44*, 83-90.

[25] Guo, H.F.; Kooi, C.W.V.. Neuropilin functions as an essential cell surface receptor. *J. Biol. Chem*., **2015**, *290*, 29120-26.

[26] Graziani, G.; Lacal, P.M.. Neuropilin-1 as therapeutic target for malignant melanoma. *Front Oncol.*, 2015, *5*, 125-129.