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Original article

# Synthesis and *in vitro* biological evaluation of new polyamine conjugates as potential anticancer drugs

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ABSTRACT

The synthesis of new polyamine derivatives containing dimeric quinoline (**3a**–**c**), cinnoline (**4a**–**c**) and phthalimide (**7a**–**c** and **8a**–**c**) moieties is described. Three different polyamines: (1,4-bis(3-aminopropyl) piperazine (**a**), 4,9-dioxa-1,12-dodecanediamine (**b**), 3,3′-diamino-*N*-methyldipropylamine (**c**) were used as linkers. The new compounds were obtained according to known procedures. Their biological activity was assessed *in vitro* in a highly aggressive melanoma cell line A375. Polyamine diimides containing phthalimide moieties demonstrated no inhibitory activities against melanoma cells. Quinoline diamides were more efficient than cinnoline ones. Mainly cytostatic activity exerted as altered cell cycle profiles was observed at the concentrations causing about 50% reduction of adherent cell proliferation. Based on their structure as well as their biological activity, we assume that some of the newly synthesized compounds may act as DNA bisintercalators. This study might be useful for further designing and developing anticancer drugs with potent activities.



## 1. Introduction

Treatment of cancer is a major challenge for the contemporary medicine. A large percentage of antineoplastic drugs are small molecules that interact with DNA [1]. The non-covalent interactions of molecules with DNA can be classified into three modes of binding: intercalation, minor groove binding and major groove binding [2]. The term "intercalation" was first introduced by Lerman in 1961 [3]. It is used to describe the process of inserting planar, polyaromatic molecules between adjacent base pairs of the double helix. Stacking and charge transfer interactions are main driving forces for intercalation but hydrogen bonding and electrostatic forces also contribute to stabilization of the DNA–intercalator complex [4,5]. Intercalation leads to topological changes in double helix (unwinding and lengthening at the intercalation site) [1,6,7] and disrupts transcription, replication and DNA repair processes [4,7,8]. It is well accepted that antitumor activity of intercalators may be closely related to the ability of these compounds to stabilize the DNA–intercalator–topoisomerase II ternary complex [5].

Some of the monointercalators are valuable anticancer drugs, currently used in the treatment of acute leukemias, ovarian and breast cancers, but their clinical effectiveness is limited by their undesirable side effects and development of multidrug resistance [9]. In order to overcome these limitations bisintercalators have been designed [5].

Bisintercalators constitute a group of compounds that interact reversibly with the DNA double helix. These agents share common structural features such as the presence of two extended electron deficient, planar, polycyclic aromatic systems separated by a spacer chain which must be long enough to enable double intercalation according to the neighbour exclusion principle [4,5]. Incorporating two intercalating systems into DNA double helix results in higher DNA affinity and sequence selectivity in comparison with monointercalating agents. Moreover, groove or phosphate interaction of basic chain connecting two intercalating moieties may provide additional increase in the binding capacity to DNA [6].

It was discovered that many tumor cells (neuroblastoma, melanoma, human lymphocytic leukemia, colonic, lung tumor cell lines, murine L1210 cells) contain elevated polyamine levels and an active polyamine transporter (the PAT) for importing exogenous

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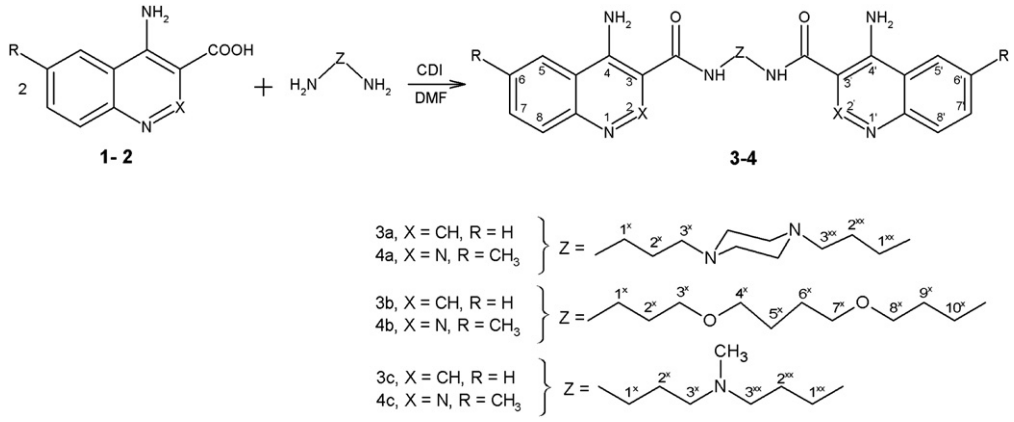



**Scheme 1.** Synthesis of quinoline and cinnoline derivatives.

polyamines. Due to the broad structural tolerance of the PAT, different chains similar to endogenous polyamines may be potential vectors carrying different moieties into rapidly dividing cells [10–12]. Designing bisintercalators with such polyamine linkers has given an opportunity for selective drug delivery, improving their efficacy and safety [6].

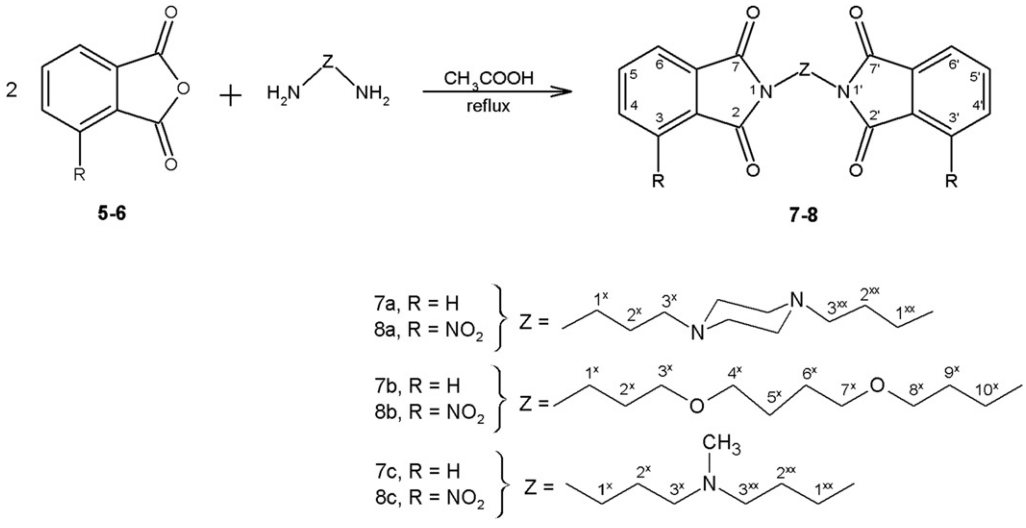
Many research groups have been interested in designing various groups of dimeric agents of diverse chemical structure and biological properties, such as echinomycin antibiotics [13–15], 7*H*-pyridocarbazole derivatives [16–19], bisanthracyclines [9,20–22], bisnaphthalimides [23–26], bisacridines [27–30] and bisimidazoacridones [31–33]. Many of them turned out to be potent anticancer drugs e.g. Elinafide (LU79553), bisnaphthalimide that progressed to clinical trials against solid tumors [34].

In our search for new therapeutics, we have synthesized some dimeric quinoline, cinnoline and phthalimide derivatives and performed a first evaluation of their biological activity. The chemical structure of synthesized derivatives may suggest their activity as potential bisintercalators. They contain two planar polyaromatic systems able to form hydrogen bonds with DNA bases, connected with polyamine linkers. Structural modifications introduced into terminal aromatic moieties as well as using as linkers different polyamines: 1,4-bis(3-aminopropyl)piperazine (**a**), 4,9-dioxa-1,12-dodecanediamine (**b**), 3,3′-diamino-*N*-methyldipropylamine (**c**), also playing potentially an important role in the activity of such derivatives [33,35], were assessed for their cytotoxic and cytostatic activity on melanoma cell line A375.

## 2. Chemistry

The starting materials 4-aminoquinoline-3-carboxylic acid **1** and 4-aminocinnoline-3-carboxylic acid **2** were obtained by the intramolecular Friedel–Crafts cyclocondensation of the corresponding (arylaminomethylene)(cyano)acetamide, or (arylhydrazono)(cyano)acetamide and hydrolysis of obtained carboxamides, according to the procedures described earlier [36–39]. The final products were formed by condensation of the acids **1** and **2** with 0.5 equiv. of appropriate polyamine in the presence of 1,1′-carbonyldiimidazole (CDI) in DMF [40]. The synthetic pathway leading to target bis(4-aminoquinoline-3-carboxamides) **3a**–**c** and bis(4-aminocinnoline-3-carboxamides) **4a**–**c** is given in Scheme 1.

Compounds bearing two phthalimide **7a**–**c** or 3-nitrophthalimide **8a**–**c** moieties were obtained by the condensation of phthalic **5** or 3-nitrophthalic anhydride **6** with corresponding polyamines in refluxing acetic acid, according to methods described previously [41]. A general procedure for the synthesis of compounds **7a**–**c** and **8a**–**c** is illustrated in Scheme 2.




**Scheme 2.** Synthesis of phthalimide derivatives.

Compounds **3a**, **3b**, **4a** were converted into hydrochlorides to increase their solubility in the culture medium. Molecular formulas and molecular weights of all derivatives were established on the basis of an elemental analysis.

## 3. Biological evaluation

Preliminary biological activity of newly synthesized polyamine derivatives was investigated *in vitro* on highly aggressive melanoma cell line A375. The antiproliferative activity was determined by the MTT-based assay (Fig. 1). Briefly, the melanoma cell line was allowed to adhere for 6 h. The tested derivatives were then added, and cells were incubated for 44 h. The results were expressed as relative number of viable adherent cells. The compounds showing at least 50% growth inhibition ($IC_{50}$) at 30 μM were considered in further evaluation. Changes in cell cycle distribution were assessed by flow cytometry after 24 h of treatment at the concentrations 20 μM and 50 μM (Fig. 2). Cell death was visualized and quantified by fluorescence microscopy after 44 h of treatment at the same concentrations (Fig. 3).

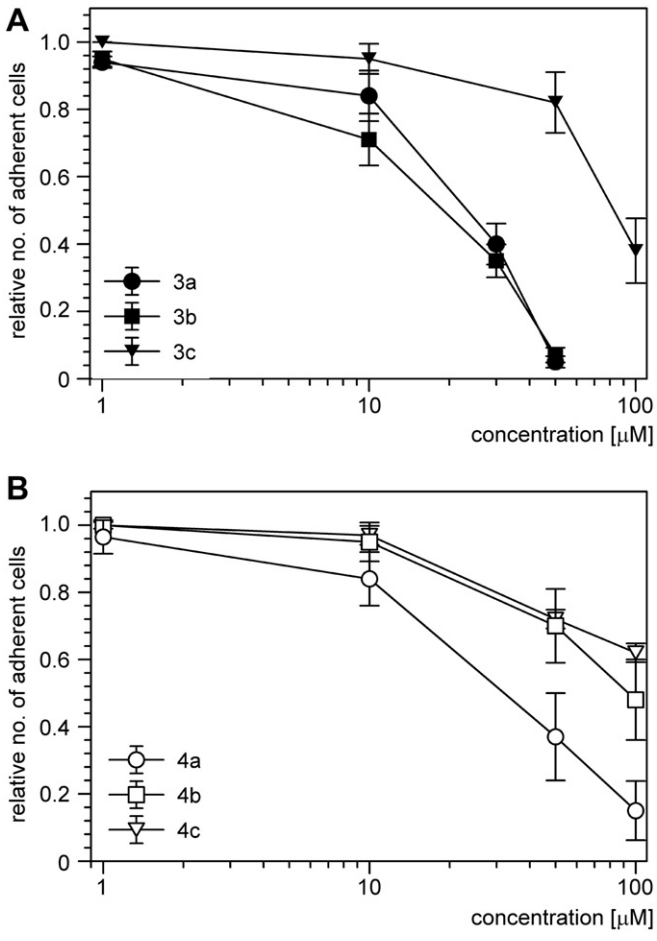



**Fig. 1.** The inhibitory effects of new quinoline (A) and cinnoline (B) derivatives on melanoma cell proliferation. Viable, adherent A375 melanoma cells were quantified by MTT assay after 44 h of culturing in the presence of different concentrations of newly synthesized derivatives as described in Experimental protocols. The mean of the absolute absorbance values given by drug-treated cells was divided by the mean of the absolute absorbance of vehicle-treated control sample and expressed as relative number of viable adherent cells. The data are mean ± SD of three independent experiments ($P < 0.05$, except for 1 μM for all compounds and 10 μM for **3c**, **4b**, **4c**).

## 4. Results and discussion

### 4.1. Some of newly synthesized compounds reduce proliferation of melanoma cell line A375

Tetrazolium derivative reduction (MTT) assay was used to assess the influence of the new dimeric quinoline, cinnoline, phthalimide and nitrophthalimide derivatives on the metabolic activity of adherent melanoma cells (A375) in relation to untreated control cells. Concentration–response course analysis was performed to determine drug concentrations required to inhibit the growth of cancer cells by 50% ($IC_{50}$) after incubation for 44 h. Newly synthesized compounds were tested in a wide range of concentrations, from 1 μM to 100 μM. Treatment of melanoma cells with new quinoline (**3a**–**c**) and cinnoline (**4a**–**c**) derivatives resulted in concentration-dependent inhibition of adherent cell proliferation (Fig. 1). Quinoline derivatives were found to have a higher antiproliferative activity than cinnoline ones. The lowest $IC_{50}$ values, below 20 μM, were obtained for quinoline derivatives **3a** and **3b** (Table 1). Much higher value of $IC_{50}$ was obtained for **3c** derivative, about 88 μM. Among cinnoline derivatives the most active was **4a** containing 1,4-bis(3-aminopropyl)piperazine as the linker. Much lower effects on cellular proliferation were observed after treatment with other cinnoline derivatives **4b**, **4c**. $IC_{50}$ values about 100 μM as for **4b** and higher as in the case of **4c** were obtained. The exposure of A375 cells to phthalimide (**7a**–**c**) and nitrophthalimide (**8a**–**c**) derivatives at the indicated range of concentrations did not inhibit proliferation of melanoma cells. For comparison, in the same experimental conditions 5 μM cisplatin inhibited the growth of A375 cells to 49% ± 6% of control (not shown).

Only derivatives with the highest antiproliferative potential **3a**, **3b** and **4a** were chosen for the next experiments.

### 4.2. Effects of quinoline and cinnoline derivatives on cell cycle progression in melanoma cells

Many anticancer drugs interact with cells leading to cell growth arrest or cell death. To gain insight into the mechanisms through which tested compounds reduced the number of adherent cells, flow cytometric analysis was performed after incubation of A375 melanoma cells with newly synthesized compounds at selected concentrations for 24 h. Separation of cells in $G_0/G_1$, S and $G_2/M$ phase was based on fluorescence intensity after staining with propidium iodide. Representative profiles are shown in Fig. 2. In untreated cells, a predominant number of A375 melanoma cells were accumulated in $G_0/G_1$ phase. Quinoline derivatives induced a marked reduction in the number of cells in the $G_0/G_1$ phase and an accumulation of cells in the S phase of the cell cycle. This effect was clearly visible in cell populations treated with **3a** already at 20 μM, whereas in the case of cells treated with **3b** at the concentration of 50 μM. Cells treated with **3a** at the concentration of 50 μM started to accumulate in hypodiploid $subG_1$ (not shown). In turn, new cinnoline derivative **4a** at the concentration of 20 μM and 50 μM caused a cell accumulation mainly in $G_2/M$ phase.

Results showing changes in cell cycle profiles of melanoma cells suggested that newly synthesized derivatives are rather cytostatic than cytotoxic agents. To confirm these observations cell death analysis was performed.

### 4.3. Newly synthesized derivatives are not cytotoxic agents in A375 cells

Cell death was assessed in melanoma cells treated with **3a**, **3b** and **4a** at indicated concentrations for 44 h. Morphological characteristics of apoptotic and necrotic cells in fluorescence microscopy

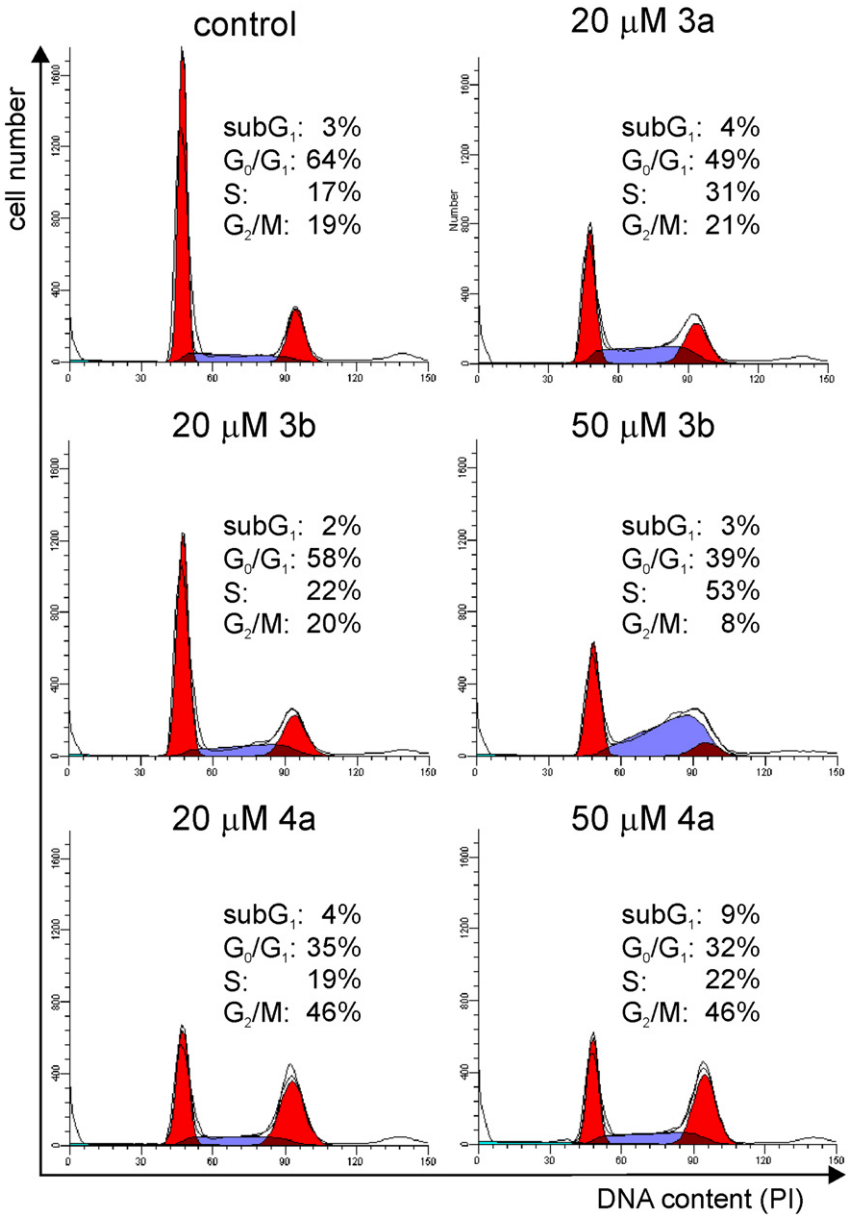



**Fig. 2.** Diverse effects of quinoline and cinnoline derivatives on cell cycle progression in A375 cell cultures. Distribution of the A375 cells through the cell cycle phases was quantified by flow cytometric analysis of propidium iodide-stained cells after 24 h culturing with new derivatives or vehicle. Representative histograms and data from three independent experiments are shown. To calculate percentages of the cells in each fraction ModFit LT 3.0 software was used.

after staining with acridine orange and ethidium bromide (AO/EB) is shown in Fig. 3A. More than 200 cells were then analyzed and percentages of apoptotic or necrotic cells were calculated (Fig. 3B). The exposure of A375 cells to quinoline and cinnoline derivatives at the concentrations in the range of $IC_{50}$ did not induce apoptosis or necrosis in A375 cells. The highest increase in the amount of apoptotic cells was observed after treatment with **3a**. At the concentration of 50 μM about 30% cells with morphological features of apoptosis after AO/EB staining were observed (Fig. 3B). This compound, which is one of the most efficient in reducing proliferation of adherent melanoma cells, accumulated about 14% of cells in $subG_1$ when assessed at 50 μM by PI staining and flow cytometry analysis (not shown). Other compounds were much less efficient in inducing cell death even when used at 50 μM. Barely 8% and 14% apoptotic cells were noted with **3b** and **4a** derivatives, respectively (Fig. 3B). This is in agreement with the results of cell cycle analysis obtained after 24 h of treatment, where **4a** was more efficient than **3b** in inducing the appearance of hypodiploid $subG_1$ cells (Fig. 2).

In summary, we could assume that in the applied conditions apoptotic cell death was not the major cause of reduced proliferation of melanoma cells.

## 5. Conclusions

The present paper has reported the synthesis of new polyamine derivatives containing dimeric quinoline, cinnoline and phthalimide moieties. Three different linkers have been employed for this study. The first biological studies have revealed that some of the newly synthesized polyamine diamides reduced proliferation of melanoma cells. Quinoline derivatives **3a** and **3b** containing 1,4-bis(3-aminopropyl)piperazine or 4,9-dioxa-1,12-

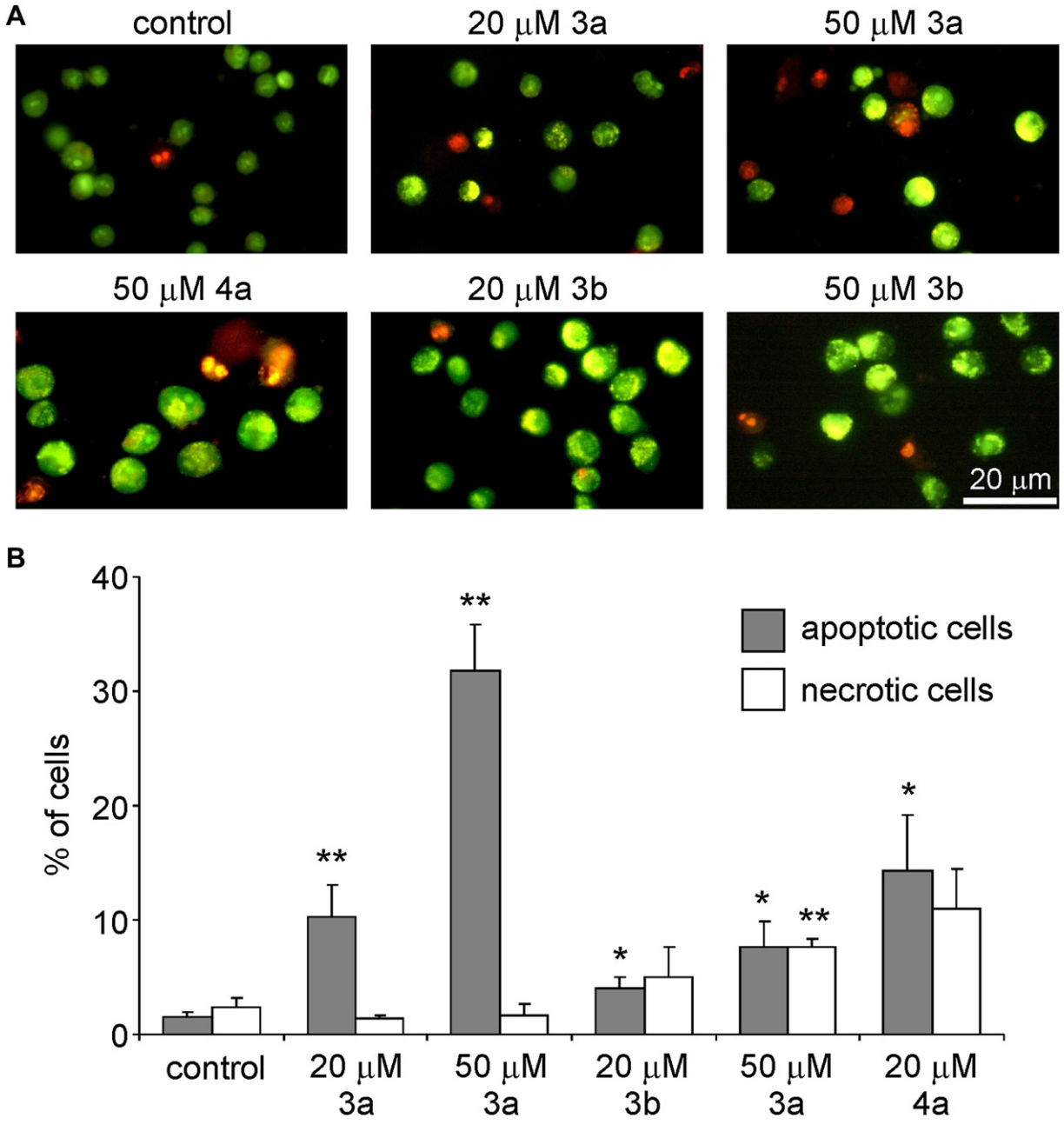



**Fig. 3.** Cell death analysis of A375 melanoma cell line by acridine orange and ethidium bromide (AO/EB) double staining. The apoptotic and necrotic cells were analyzed after 44 h of culturing in the presence of quinoline and cinnoline derivatives at indicated concentrations. (A) Cells were stained with nucleic acid selective fluorochromes: membrane-permeable acridine orange and impermeable ethidium bromide. Representative microscopic fields are shown. Viable cells had bright green chromatin with organized structure. In early apoptotic cells, the chromatin was condensed or fragmented but still stained green. In late apoptotic cells, it was condensed or fragmented and stained orange, and necrotic cells had bright orange chromatin with organized structure. (B) Ten different fields were randomly selected for counting 200 cells and the percentages of apoptotic and necrotic cells were calculated. Bars presenting percentages of apoptotic and necrotic cells are shown. The data are mean ± SD of three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$).

dodecanediamine respectively, were more efficient than cinnoline compounds **4a, 4b** having corresponding linkers. None of the tested phthalimide derivatives **7** or nitrophthalimide derivatives **8**, as well as quinoline and cinnoline ones with 3,3′-diamino-*N*-methyldipropylamine linker **3c** and **4c** showed any significant anticancer activity *in vitro*. Our results demonstrate that new quinoline **3a** and **3b** and cinnoline **4a** derivatives do not induce cell death, whereas efficiently inhibit adherent cell proliferation and induce cell cycle disturbance. Interestingly, quinoline derivatives **3a** and **3b** induce an apparent increase in the portion of S phase cells, whereas cinnoline derivative **4a** increase the percentages of cells in $G_2$/M phase. Cell cycle arrest at $G_1$/S and $G_2$/M transitions has been observed in mammalian cancer cells in response to several drugs. S phase arrest often revealed as the consequence of drug treatment is associated with inhibition of DNA synthesis. Most recently, we observed S phase arrest of A375 melanoma cells used in the current study after treatment with cisplatin which was concomitant to cytostatic but not cytotoxic effect on cancer cells [42]. It would be of interest to evaluate the molecular basis of discrepancies between quinoline and cinnoline derivatives in their influence on cancer cell cycle. More information about the mechanism of anticancer activity and further

**Table 1**
*In vitro* growth inhibition of melanoma cells A375 by new polyamine derivatives containing dimeric quinoline (**3a**–**c**) and cinnoline (**4a**–**c**) moieties.

| Compound | $IC_{50}$ values (μM) |
|---|---|
| **3a** | 16.8 ± 2.3 |
| **3b** | 15.5 ± 4.2 |
| **3c** | 87.9 ± 16.8 |
| **4a** | 31.6 ± 13.2 |
| **4b** | 100.5 ± 0.7 |
| **4c** | 115.9 ± 9.5 |

After 44 h incubation of melanoma cells A375 with tested compounds, colorimetric MTT assay was used to assess inhibition of cell growth. $IC_{50}$ (concentration of tested compounds causing 50% inhibition of cell growth compared to an untreated control) was then calculated.
Values represent the mean ± standard deviation of at least three separate experiments.

structural modifications might improve the potency of newly synthesized compounds.

## 6. Experimental protocols

### 6.1. Chemistry

#### 6.1.1. General experimental information

Reagents and solvents were purchased from common commercial suppliers. The melting points were measured on the electrothermal apparatus in open capilares and are uncorrected. Elemental analyses were carried out with a Perkin Elmer series II. CHNS/O Analyzer 2400 and were within ±0.4% of the theoretical values. $^1$H NMR and $^{13}$C NMR spectra were recorded on a Varian Mercury (300 MHz) spectrophotometer in $CDCl_3$/DMSO-$d_6$ solutions with TMS as an internal standard. The spectra data of new compounds refer to their free bases. Chemical shifts were expressed in $\delta$ (ppm) and the coupling constants *J* in hertz (Hz). The following abbreviations are used to describe peak patterns when appropriate: s (single), d (doublet), t (triplet), m (multiplet), bs (broad singlet).

#### 6.1.2. General procedure for the synthesis of ***3a*–*c***

Compound **1** (10 mmol) and CDI (10 mmol) in DMF (100 ml) were stirred for 1 h in room temperature. Then the appropriate polyamine (6 mmol) was added and stirring was continued for additional 2 h. At the end of the reaction the mixture was heated at about 100 °C and filtered. The solvent was removed under reduced pressure; 20 ml of $H_2O$ was added to the residue and left for 24 h at 5 °C. Then the solid was filtered off, washed with $H_2O$ and crystallized from DMF/$H_2O$.

*6.1.2.1. N,N′-(Piperazine-1,4-diyldipropane-3,1-diyl)bis(4-aminoquinoline-3-carboxamide) (**3a**).* Yield: 69.4%, m.p. 266.7–267.8 °C; $^1$H NMR (DMSO) $\delta_H$: 1.63–1.78 (m, 4H, C-2$^x$H$_2$, C-2$^{xx}$H$_2$), 2.37 (t, *J* = 7.0 Hz, 4H, C-3$^x$H$_2$, C-3$^{xx}$H$_2$), 3.21–3.37 (cluster, 8H piperazine CH$_2$, 4H, C-1$^x$H$_2$, C-1$^{xx}$H$_2$), 7.45 (t, *J* = 7.5 Hz, 2H, C-7H, C-7′H), 7.65 (t, *J* = 7.6 Hz, 2H, C-7H, C-7′H), 7.78 (d, *J* = 8.3 Hz, 2H, C-5H, C-5′H), 8.29 (d, *J* = 8.2 Hz, 2H, C-8H, C-8′H), 8.34 (bs, 4H, 2 NH$_2$), 8.56 (t, *J* = 5.3 Hz, 2H, 2 CONH), 8.80 (s, 2H, C-2H, C-2′H) ppm. $^{13}$C NMR (DMSO) $\delta_C$: 25.91, 37.48, 52.24, 55.44, 103.52, 118.34, 122.84, 124.49, 128.58, 130.32, 148.04, 148.96, 152.54, 167.96 ppm. Anal. Calcd for $C_{30}H_{36}N_8O_2 \cdot 4HCl \cdot 4H_2O$: C, 47.50; H, 6.38; N, 14.77; Found: C, 47.61; H, 6.49; N, 14.59.

*6.1.2.2. N,N′-[Butane-1,4-diylbis(oxypropane-3,1-diyl)]bis(4-aminoquinoline-3-carboxamide) (**3b**).* Yield: 69%, m.p. 196.3–197.8 °C; $^1$H NMR (DMSO) $\delta_H$: 1.45–1.63 (m, 4H, C-5$^x$H$_2$, C-6$^x$H$_2$), 1.68–1.87 (m, 4H, C-2$^x$H$_2$, C-9$^x$H$_2$), 3.25–3.49 (cluster, 12H, C-1$^x$H$_2$, C-10$^x$H$_2$, C-3$^x$H$_2$, C-4$^x$H$_2$, C-7$^x$H$_2$, C-8$^x$H$_2$), 7.46 (t, *J* = 7.5 Hz, 2H, C-7H, C-7′H), 7.67 (*J* = 7.5 Hz, 2H, C-6H, C-6′H), 7.78 (d, *J* = 8.2 Hz, 2H, C-5H, C-5′H), 8.37 (d, *J* = 8.2 Hz, 2H, C-8H, C-8′H), 8.30 (bs, 4H, 2 NH$_2$), 8.49 (t, *J* = 5.3, 2H, 2 CONH), 8.80 (s, 2H, C-2H, C-2′H) ppm. $^{13}$C NMR (DMSO) $\delta_C$: 26.12, 29.05, 36.61, 67.67, 69.79, 103.34, 116.94, 119.81, 124.40, 126.91, 134.28, 137.13, 141.93, 157.35, 165.42 ppm. Anal. Calcd for $C_{30}H_{36}N_6O_4 \cdot 2HCl \cdot H_2O$: C, 56.69; H, 6.34; N, 13.22; Found: C, 56.33; H, 6.62; N, 12.92.

*6.1.2.3. N,N′-[(Methylimino)dipropane-3,1-diyl]bis(4-aminoquinoline-3-carboxamide) (**3c**).* Yield: 55%, m.p. 224.5–225.3 °C; $^1$H NMR (DMSO) $\delta_H$: 1.63–1.82 (m, 4H, C-2$^x$H$_2$, C-2$^{xx}$H$_2$), 2.19 (s, 3H, CH$_3$), 2.40 (t, *J* = 6.9 Hz, 4H, C-3$^x$H$_2$, C-3$^{xx}$H$_2$), 3.25–3.37 (m, 4H, 4H, C-1$^x$H$_2$, C-1$^{xx}$H$_2$), 7.47 (t, *J* = 7.6 Hz, 2H, C-7H, C-7′H), 7.68 (t, *J* = 7.6 Hz, 2H, C-6H, C-6′H), 7.79 (d, *J* = 8.3 Hz, 2H, C-5H, C-5′H), 8.35 (d, *J* = 8.3 Hz, 2H, C-8H, C-8′H), 8.30 (bs, 4H, 2 NH$_2$), 8.56 (t, *J* = 5.3 Hz, 2H, 2 CONH), 8.80 (s, 2H, C-2H, C-2′H) ppm. $^{13}$C NMR (DMSO) $\delta_C$: 26.80, 37.56, 41.75, 55.12, 103.60, 118.37, 122.80, 124.45, 130.24, 148.21, 149.03, 152.45, 167.96 ppm. Anal. Calcd for $C_{27}H_{31}N_7O_2 \cdot 4H_2O$: C, 58.15; H, 7.04; N, 17.58; Found: C, 58.63; H, 6.72; N, 17.68.

#### 6.1.3. General procedure for the synthesis of ***4a*–*c***

Compound **2** (10 mmol) and CDI (10 mmol) in DMF (100 ml) were stirred for 1 h in room temperature. Then the appropriate amine (6 mmol) was added and stirring was continued for additional 2 h. At the end of the reaction the mixture was heated at about 100 °C and filtered. The solvent was removed under reduced pressure. The residue was dissolved in methanol, acidified with 10% HCl, heated with charcoal and filtered. The filtrate was neutralized with 10% $NH_4OH$ to obtain the free bases. The precipitate was filtered off and crystallized from $CH_3OH/H_2O$.

*6.1.3.1. N,N′-(Piperazine-1,4-diyldipropane-3,1-diyl)bis(4-amino-6-methylcinnoline-3-carboxamide) (**4a**).* Yield: 78%, m.p. 246.8–248.4 °C; $^1$H NMR (DMSO) $\delta_H$: 1.66–1.81 (m, 4H, C-2$^x$H$_2$, C-2$^{xx}$H$_2$), 2.49 (t, *J* = 7.0 Hz, 4H, C-3$^x$H$_2$, C-3$^{xx}$H$_2$), 2.52 (s, 6H, 2CH$_3$), 3.31–3.46 (cluster, 8H piperazine CH$_2$, 4H, C-1$^x$H$_2$, C-1$^{xx}$H$_2$), 7.65 (d, *J* = 8.6 Hz, 2H, C-8H, C-8′H), 7.96 (bs, 2H, C-4NH$_2$, C-4′NH$_2$), 8.08 (d, *J* = 8.6 Hz, 2H, C-7H, C-7′H), 8.19 (s, 2H, C-5H, C-5′H), 9.05 (bs, 2H, C-4NH$_2$, C-4′NH$_2$), 9.37 (t, *J* = 5.9 Hz, 2H, 2 CONH) ppm. $^{13}$C NMR (DMSO) $\delta_C$: 21.82, 25.96, 37.45, 52.27, 55.42, 115.90, 120.83, 127.42, 128.36, 133.28, 138.32, 143.08, 147.29, 167.11 ppm. Anal. Calcd for $C_{30}H_{38}N_{10}O_2 \cdot 4HCl \cdot 6H_2O$: C, 43.69; H, 6.60; N, 16.98; Found: C, 43.70; H, 6.54; N, 16.93.

*6.1.3.2. N,N′-[Butane-1,4-diylbis(oxypropane-3,1-diyl)]bis(4-amino-6-methylcinnoline-3-carboxamide) (**4b**).* Yield: 56.7%, m.p. 211.5–212.1 °C; $^1$H NMR (DMSO) $\delta_H$: 1.52–1.61 (m, 4H, C-5$^x$H$_2$, C-6$^x$H$_2$), 1.75–1.87 87 (m, 4H, C-2$^x$H$_2$, C-9$^x$H$_2$), 2.52 (s, 6H, 2 CH$_3$), 3.32–3.49 (cluster, 12H, C-1$^x$H$_2$, C-10$^x$H$_2$, C-3$^x$H$_2$, C-4$^x$H$_2$, C-7$^x$H$_2$, C-8$^x$H$_2$), 7.70 (dd, *J* = 8.6, 1.5 Hz, 2H, C-8H, C-8′H), 7.98 (bs, 2H, C-4NH$_2$, C-4′NH$_2$), 8.11 (d, *J* = 8.6 Hz, 2H, C-7H, C-7′H), 8.20 (s, 2H, C-5H, C-5′H), 9.01 (bs, 2H, C-4NH$_2$, C-4′NH$_2$), 9.15 (t, *J* = 5.9 Hz, 2H, 2 CONH) ppm. $^{13}$C NMR (DMSO) $\delta_C$: 21.50, 26.08, 29.42, 36.34, 68.23, 69.88, 115.89, 120.86, 127.42, 128.28, 133.29, 138.36, 143.05, 147.25, 167.13 ppm. Anal. Calcd for $C_{30}H_{38}N_8O_4$: C, 62.70; H, 6.66; N, 19.49; Found: C, 62.36; H, 6.85; N, 19.20.

*6.1.3.3. N,N′-[(Methylimino)dipropane-3,1-diyl]bis(4-amino-6-methylcinnoline-3-carboxamide) (**4c**).* Yield: 55%, m.p. 225.6–227.1 °C; $^1$H NMR (DMSO) $\delta_H$: 1.68–1.83 (m, 4H, C-2$^x$H$_2$, C-2$^{xx}$H$_2$), 2.18 (s, 3H, CH$_3$), 2.41 (t, *J* = 6.8 Hz, 4H, C-3$^x$H$_2$, C-3$^{xx}$H$_2$), 2.52 (s, 6H, 2 CH$_3$),

3.38–3.47 (m, 4H, C-1$^{x}H_2$, C-1$^{xx}H_2$), 7.69 (dd, $J$ = 8.6, 1.5 Hz, 2H, C-8H, C-8′H), 7.97 (bs, 2H, C-4$NH_2$, C-4′$NH_2$), 8.14 (d, $J$ = 8.6 Hz, 2H, C-7H, C-7′H), 8.19 (s, 2H, C-5H, C-5′H), 9.07 (bs, 2H, C-4$NH_2$, C-4′$NH_2$), 9.28 (t, $J$ = 5.9 Hz, 2H, 2 CONH) ppm. $^{13}$C NMR (DMSO) $\delta_C$: 21.52, 26.89, 37.29, 41.64, 55.43, 115.91, 120.85, 127.46, 128.34, 133.27, 138.34, 143.06, 147.27, 167.10 ppm. Anal. Calcd for $C_{27}H_{33}N_8O_4 \cdot H_2O$: C, 60.77; H, 6.61; N, 23.62; Found: C, 60.92; H, 6.40; N, 23.65.

*6.1.4. General procedure for the synthesis of **7a–c** and **8a–c***

To a solution of phthalic anhydride (10 mmol) or 3-nitrophthalic anhydride (10 mmol) in acetic acid (15 ml) appropriate polyamines (5 mmol) were added. The reaction was refluxed for 7 h, poured into ice bathing water. The precipitate was filtered off and crystallized from appropriate solvent.

*6.1.4.1. N,N′-Diphthaloyl-1,4-bis(3-aminopropyl)piperazine (**7a**).* Yield: 86.9%, m.p. 178.3–179.9 °C (EtOH); $^1$H NMR ($CDCl_3$) $\delta_H$: 1.74–1.86 (m, 4H, C-2$^{x}H_2$, C-2$^{xx}H_2$), 2.03–2.64 (cluster, 8H piperazine $CH_2$, 4H, C-3$^{x}H_2$, C-3$^{xx}H_2$), 3.72 (t, $J$ = 7.0 Hz, 4H, C-1$^{x}H_2$, C-1$^{xx}H_2$), 7.64–7.74 (m, 4H, C-4H, C-4′H, C-5H, C-5′H), 7.76–7.90 (m, 4H, C-3H, C-3′H, C-6H, C-6′H) ppm. $^{13}$C NMR ($CDCl_3$) $\delta_C$: 25.41, 36.89, 53.15, 56.21, 123.20, 132.38, 133.84, 168.44 ppm. Anal. Calcd for $C_{26}H_{28}N_4O_4$: C, 67.75; H, 6.08; N, 12.17; Found: C, 67.45; H, 6.18; N, 12.08.

*6.1.4.2. N,N′-Diphthaloyl-4,9-dioxa-1,12-dodecanediamine (**7b**).* Yield: 94.8%, m.p. 117.0–118.8 °C (EtOH); $^1$H NMR ($CDCl_3$) $\delta_H$: 1.46–1.64 (m, 4H, C-5$^{x}H_2$, C-6$^{x}H_2$), 1.89–1.98 (m, 4H, C-2$^{x}H_2$, C-9$^{x}H_2$), 3.32 (t, $J$ = 6.0 Hz, 4H, C-1$^{x}H_2$, C-10$^{x}H_2$), 3.45 (t, $J$ = 6.1 Hz, 4H, C-3$^{x}H_2$, C-8$^{x}H_2$), 3.79 (t, $J$ = 6.7 Hz, 4H, C-4$^{x}H_2$, C-7$^{x}H_2$), 7.68–7.73 (m, 4H, C-4H, C-4′H, C-5H, C-5′H), 7.81–7.87 (m, 4H, C-3H, C-3′H, C-6H, C-6′H) ppm. $^{13}$C NMR ($CDCl_3$) $\delta_C$: 26.55, 28.94, 35.97, 68.55, 70.92, 123.22, 132.30, 133.89, 168.42 ppm. Anal. Calcd for $C_{26}H_{28}N_2O_6$: C, 67.17; H, 6.03; N, 6.03; Found: C, 67.18; H, 5.96; N, 6.09.

*6.1.4.3. N,N′-Diphthaloyl-3,3′-diamino-N-methyldipropylamine (**7c**).* Yield: 50.1%, m.p. 105.8–106.8 °C (EtOH); $^1$H NMR ($CDCl_3$) $\delta_H$: 1.73–1.88 (m, 4H, C-2$^{x}H_2$, C-2$^{xx}H_2$), 2.17 (s, 3H, $CH_3$), 2.39 (t, $J$ = 7.0 Hz, 4H, C-3$^{x}H_2$, C-3$^{xx}H_2$), 3.69–3.76 (m, 4H, C-1$^{x}H_2$, C-1$^{xx}H_2$), 7.65–7.74 (m, 4H, C-4H, C-4′H, C-5H, C-5′H), 7.77–7.87 (m, 4H, C-3H, C-3′H, C-6H, C-6′H) ppm. $^{13}$C NMR ($CDCl_3$) $\delta_C$: 26.54, 36.59, 41.79, 55.43, 123.22, 132.27, 133.87, 168.39 ppm. Anal. Calcd for $C_{23}H_{23}N_3O_4$: C, 68.41; H, 5.70; N, 10.40; Found: C, 68.11; H, 5.32; N, 10.39.

*6.1.4.4. N,N′-Di(3-nitrophthaloyl)-1,4-bis(3-aminopropyl)piperazine (**8a**).* Yield: 86.6%, m.p. 203.0–204.6 °C (DMF/$H_2O$); $^1$H NMR (DMSO) $\delta_H$: 1.60–1.71 (m, 4H, C-2$^{x}H_2$, C-2$^{xx}H_2$), 1.91–2.18 (cluster, 8H piperazine $CH_2$, 4H, C-3$^{x}H_2$, C-3$^{xx}H_2$), 3.60 (t, $J$ = 6.6 Hz, 4H, C-1$^{x}H_2$, C-1$^{xx}H_2$), 7.98–8.08 (m, 2H, C-4H, C-4′H), 8.15 (dd, $J$ = 7.5, 0.9 Hz, 2H, C-3H, C-3′H), 8.25 (dd, $J$ = 8.0, 0.9 Hz, 2H, C-5H, C-5′H) ppm. $^{13}$C NMR (DMSO) $\delta_C$: 25.73, 36.72, 52.54, 55.83, 122.89, 126.54, 127.87, 133.56, 135.82, 144.21, 163.12, 165.81 ppm. Anal. Calcd for $C_{26}H_{26}N_6O_8$: C, 56.69; H, 4.72; N, 15.25; Found: C, 56.58; H, 4.51; N, 15.35.

*6.1.4.5. N,N′-Di(3-nitrophthaloyl)-4,9-dioxa-1,12-dodecanediamine (**8b**).* Yield: 75.17%, m.p. 104.6–106.0 °C (EtOH); $^1$H NMR (DMSO) $\delta_H$: 1.20–1.35 (m, 4H, C-5$^{x}H_2$, C-6$^{x}H_2$), 1.66–1.90 (m, 4H, C-2$^{x}H_2$, C-9$^{x}H_2$), 3.20 (t, $J$ = 6.7 Hz, 4H, C-1$^{x}H_2$, C-10$^{x}H_2$), 3.36 (t, $J$ = 6.8 Hz, 4H, C-3$^{x}H_2$, C-8$^{x}H_2$), 3.64 (t, $J$ = 6.8 Hz, 4H, C-4$^{x}H_2$, C-7$^{x}H_2$), 8.0–8.08 (m, 2H, C-4H, C-4′H), 8.15 (dd, $J$ = 7.5, 0.9 Hz, 2H, C-3H, C-3′H), 8.26 (dd, $J$ = 8.0, 0.9 Hz, 2H, C-5H, C-5′H) ppm. $^{13}$C NMR (DMSO) $\delta_C$: 25.88, 27.72, 35.96, 67.83, 69.78, 122.91, 126.50, 127.92, 133.50, 135.89, 144.03, 163.16, 165.78 ppm. Anal. Calcd for $C_{26}H_{26}N_4O_{10}$: C, 56.31; H, 4.69; N, 10.10; Found: C, 56.28; H, 4.39; N, 10.18.

*6.1.4.6. N,N′-Di(3-nitrophthaloyl)-3,3′-diamino-N-methyldipropylamine (**8c**).* Yield: 52%, m.p. 148.4–150.1 °C (EtOH); $^1$H NMR (DMSO) $\delta_H$: 1.76–1.89 (m, 4H, C-2$^{x}H_2$, C-2$^{xx}H_2$), 2.16 (s, 3H, $CH_3$), 2.41 (t, $J$ = 7.0 Hz, 4H, C-3$^{x}H_2$, C-3$^{xx}H_2$), 3.66–3.73 (m, 4H, C-1$^{x}H_2$, C-1$^{xx}H_2$), 8.0–8.1 (m, 2H, C-4H, C-4′H), 8.13 (dd, $J$ = 7.5, 0.9 Hz, 2H, C-3H, C-3′H), 8.22 (dd, $J$ = 8.0, 0.9 Hz, 2H, C-5H, C-5′H) ppm. $^{13}$C NMR (DMSO) $\delta_C$: 22.68, 35.41, 52.65, 123.02, 126.62, 128.02, 134.57, 135.98, 144.07, 163.26, 165.90 ppm. Anal. Calcd for $C_{23}H_{21}N_5O_8$: C, 55.75; H, 4.27; N, 14.13; Found: C, 55.58; H, 4.32; N, 13.98.

## 6.2. Bioassays

*6.2.1. Cell line and cell culture conditions*

A375, a human melanoma cell line with high metastatic potential was maintained in RPMI 1640 medium (Lonza, Switzerland), supplemented with 10% fetal bovine serum (FBS), penicillin (10 U/ml) and streptomycin (50 μg/ml) in standard conditions. For experiments, culture medium was substituted with fresh medium containing 0.5% FBS. In all experiments, cells in logarithmic phase of growth were used.

*6.2.2. Cell proliferation analysis*

To determined $IC_{50}$ value (concentration of tested compounds causing 50% inhibition of cell growth) colorimetric MTT assay was used. A375 cells were seeded in 24-well plates and 6 h later adherent cells were exposed to tested compounds at different concentrations. After 44 h, the MTT reagent (thiazolyl blue tetrazolium bromide; Sigma–Aldrich; 0.84 mg/ml in PBS) was added to each well for additional 3 h. After removal of the medium, dimethyl sulfoxide was added to each well to dissolve the blue formazan crystals and the absorbance was determined at 540 nm. Data show the mean of at least three independent experiments ± SD. $IC_{50}$ values were calculated by concentration–response curve fitting using a Microsoft Excel-based analytic method.

*6.2.3. Cell cycle analysis*

Flow cytometric analysis measuring cellular DNA content was performed to evaluate the distribution of the cells through the cell cycle phases. Melanoma cells A375 were incubated with tested compounds at indicated concentrations for 24 h. Untreated and treated cells were harvested, washed with PBS, fixed in ice-cold 70% ethanol and stained with propidium iodide/RNAse solution (BD Pharmingen, San Diego, CA, USA). The cell cycle profiles were obtained by flow cytometry (Becton–Dickinson FACSCalibur, San Diego, CA, USA). Up to 10,000 cells per sample were analyzed. ModFit LT 3.0 software was used to calculate the percentages of cells in each cell cycle phase.

*6.2.4. Cell death analysis by acridine orange/ethidium bromide double staining*

The apoptotic and necrotic cells were monitored by double staining with acridine orange and ethidium bromide using a fluorescence microscope (Olympus BX 41, Japan). A375 cells were cultured for 44 h with or without tested compounds at indicated concentrations. To the combined cell populations (adherent and floating) in 100 μl PBS, 20 μl of staining solution (1:1) mixture of

100 μg/ml EB and 100 μg/ml AO (Sigma Chemical Co.) was added. In each experiment, more than 200 cells were analyzed and then percentages of apoptotic or necrotic cells were calculated.

#### 6.2.5. Statistical analysis

Data represent mean ± SD from at least three separate experiments. The significance of an apparent difference in mean values for any tested parameter was validated by a Student's paired *t* test. The difference was considered significant if $P < 0.05$.

## Authors' contributions

MSz participated in the design of the study, preparing the manuscript and carried out the synthesis of the compounds. AS-M took the lead on performing biological study, carried out cell culture, cell death assay, MTT assay and helped to draft the manuscript. KK and MS contributed to cell cycle analysis including data interpretation. WL was involved in interpretation of spectral and analytical data of obtained compounds. AS and MC acquired funding for the project, worked on the design of the study, discussion and wrote the manuscript. All authors read and approved the final manuscript.

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