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Synthesis and Biological Evaluation of New Bischromone Derivatives with Antiproliferative Activity

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The synthesis of new bischromone derivatives (**4a–c** and **5a–c**) as potential anticancer drugs is described. The difference in the reactivity between 4-oxo-4*H*-chromene-3-carboxylic acid **2** (or its methyl ester **3**) and 4-oxo-4*H*-chromene-3-carbonyl chloride **1** with three different polyamines: 3,3′-diamino-*N*-methyldipropylamine (**a**), 1,4-*bis*(3-aminopropyl)piperazine (**b**), 4,9-dioxa-1,12-dodecanediamine (**c**) resulted in the formation of two different groups of products, compounds **4a–c** and **5a–c**, designed in agreement with the bisintercalators' structural requirements. The transformation of 4-oxo-4*H*-chromene-3-carboxylic acid into 2*H*-chromene-2,4(3*H*)-diones (**5**) was confirmed by the NMR and XRD experiments. Compounds **4a** and **5a** were evaluated *in vitro* in the highly aggressive melanoma cell line A375. An enhanced induction of apoptosis and cell cycle arrest clearly revealed that compound **5a** was more potent than **4a**. Compound **5a** was also more active in diminishing the adhesive potential of melanoma cells. Current studies support the notion that small changes in the three-dimensional structure of molecules might have a substantial impact on their biological activity.

Keywords: Antiproliferative activity / A375 melanoma cells / Bischromone derivatives

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Supporting Information available online

Introduction

4-Oxo-4*H*-1-benzopyran derivatives were found to have a wide range of biological properties including the anti-inflammatory [1], antimicrobial [2], anticancer [3], and anti-HIV [4] activity. Bischromones constitute a group of compounds which are also pharmaceutically important. Their activity depends on the position of attachment, the nature of the linker and possibly the coplanarity of the two chromone (1,4-benzopyrone) rings [5].

The structure of the chromone ring meets the requirements for the terminal moiety of bisintercalators. The connection of such two almost planar unsaturated systems with the polyamine linker may lead to the formation of molecules which exhibit specific biological properties, e.g., antitumor activity and are capable of binding to DNA *via* a bisintercalative binding mode (simultaneous insertion of two planar systems between the adjacent base-pairs according to a neighbor exclusion principle) [6, 7]. Bisintercalators constitute a versatile and very promising group of compounds extensively studied by many research groups worldwide. Lots of them turned out to be potent anticancer drugs, e.g., elinafide

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(LU79553), bisnaphthalimide which progressed to clinical trials against solid tumors [8].

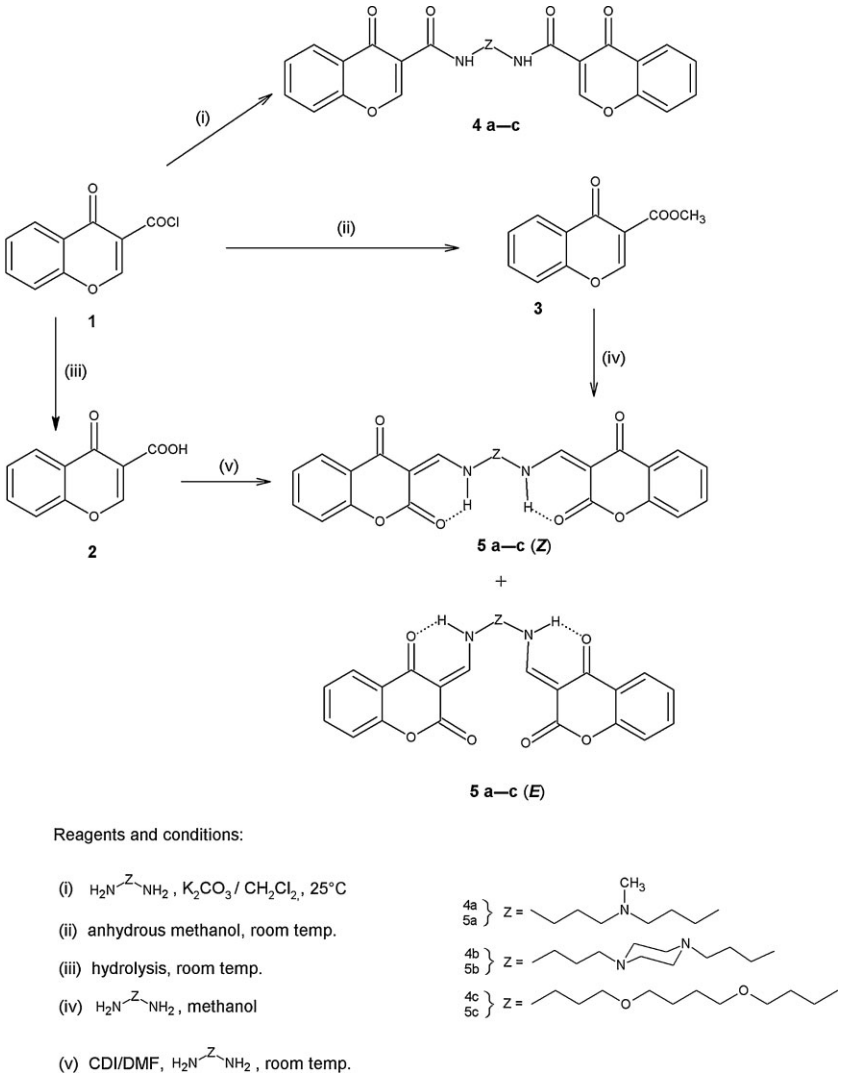
Taking into consideration our earlier studies on potential anticancer drugs [9], we decided to design the bischromone derivatives structurally related to known bisintercalators, containing two chromone rings tethered at the 3,3′ position by three different polyamines: 3,3′-diamino-*N*-methyldipropylamine (**a**), 1,4-*bis*(3-aminopropyl)piperazine (**b**), and 4,9-dioxa-1,12-dodecanediamine (**c**). To get information on the biological activity of the synthesized compounds we planned to evaluate their action on melanoma cell line A375 *in vitro*.

Results and discussion

Chemistry

The compounds were prepared by the procedure depicted in Scheme 1. The starting material, 4-oxo-4*H*-chromene-3-carbonyl chloride **1** was synthesized from 4-oxo-4*H*-chromene-3-carbaldehyde obtained by *Wilsmeier–Haack* reaction, according to published procedures [10–12]. 4-Oxo-4*H*-1-benzopyran-3-carboxylic acid **2** was prepared by hydrolysis of 4-oxo-4*H*-chromene-3-carbonyl chloride **1**, whereas condensation of **1** with methanol at room temperature gave methyl 4-oxo-4*H*-1-benzopyran-3-carboxylate **3**.

As a consequence of the diverse reactivity of 4-oxo-4*H*-chromene-3-carbonyl chloride **1** and 4-oxo-4*H*-chromene-3-carboxylic acid **2** (as well as its methyl ester **3**) with primary polyamines (**a–c**), two different groups of compounds **4a–c** and **5a–c** were synthesized. The reaction of **1** with polyamines (**a–c**) led to *bis*(4-oxo-4*H*-chromene-3-carboxamides) **4a–c** [13]. Instead, the products obtained from the reaction of **2** with the polyamines (**a–c**) in the presence of 1,1′-carbonyldiimidazole (CDI) were identified as the stereoisomeric mixture ($E/Z \sim 2{:}1$) of 2*H*-chromene-2,4(3*H*)-dione derivatives **5a–c**.



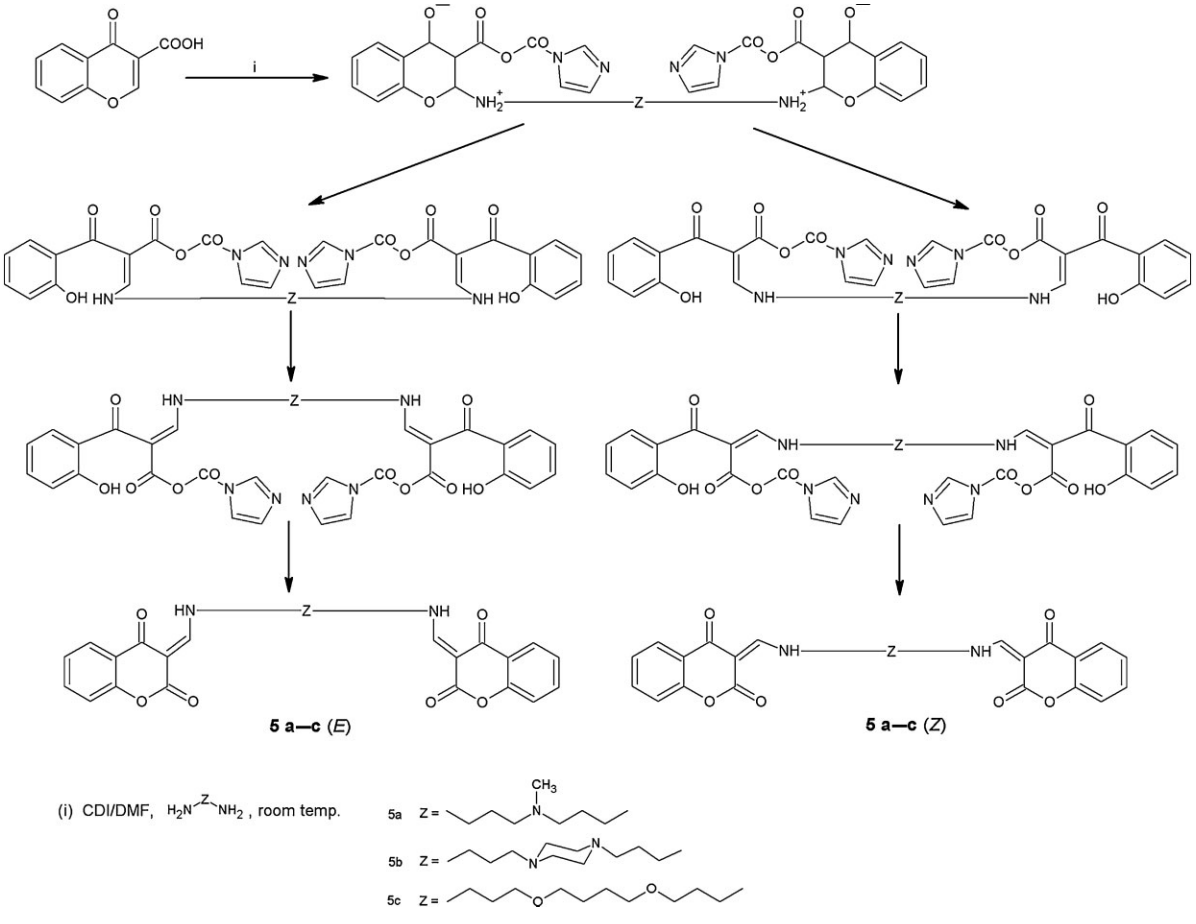
Scheme 1. The synthetic pathway to the compounds **4a–c** and **5a–c** under study.

The two geometrical isomers of compounds **5a–c** were also formed in the transformation of methyl 4-oxo-4*H*-1-benzopyran-3-carboxylate **3** under the influence of polyamines (**a–c**), as described in Ref. [13].

The purity and structure of all synthesized compounds were fully confirmed by physical data, elemental analyses, IR, and $^{1}H/^{13}C$ NMR spectroscopy in solution. Especially the 2D correlation $^{1}H–^{1}H$ COSY and $^{1}H–^{13}C$ HETCOR experiments, supported with additional DFT-level GIAO [14] based calculations of NMR chemical shifts [15], allowed the complete identification and assignment of all carbon and hydrogen atoms in the *E*/*Z*-isomers of **5**. Indeed, the ^{1}H NMR spectra of isomeric products **5** in $CDCl_3$ solution, instead of a characteristic NH amide triplet at ~9.4 ppm, exhibited two very broad low-field signals at ~11.9 and ~10.5 ppm (disappearing on shaking with D_2O), which were univocally assigned [15] to the exchangeable NH protons involved in an intramolecular H-bond with the keto and lactone oxygen atoms, respectively (Scheme 1). Simultaneously, two doublets were observed at ~8.5 ppm ($J = 15.2$ Hz) and ~8.4 ppm ($J = 14.3$ Hz) due to the exocyclic =CH proton in the *Z* and *E* isomer of **5**, respectively. These two signals were exchanged to the singlets by shaking with D_2O. An isomeric *E*/*Z* ratio, determined in this way from the 300 MHz ^{1}H NMR spectra of both isomers of **5** in the mixture, was established as ~2:1. The ^{13}C NMR spectra revealed that each of the isomers (*E*)-**5** and (*Z*)-**5** is represented in solution by their two conformations, which also appear in the ratio of ~2:1.

Both isomers of **5** were obtained as a consequence of the two-way transformation of the γ-pyrone ring occurring under the influence of the nucleophilic attack of primary amines at C2 of the CDI-activated carboxylic group of 4-oxo-4*H*-chromene-3-carboxylic acid (**2**). This rearrangement was also described by Alderete et al. [16], where the reactivity of the acid was enhanced with dimethylsulfamoyl chloride. The proposed mechanism for the transformation of **5** in the presence of CDI is shown in Scheme 2. The conversion into 2*H*-chromene-2,4(3*H*)-diones was also observed for the methyl 4-oxo-4*H*-1-benzopyran-3-carboxylate derivative as described by Budzisz et al. [13].

The aforementioned change into the two geometric isomers was confirmed by the XRD experiments for **5a**



Scheme 2. A proposed mechanism for the reaction of compounds **5a–c** according to Ref. [16].

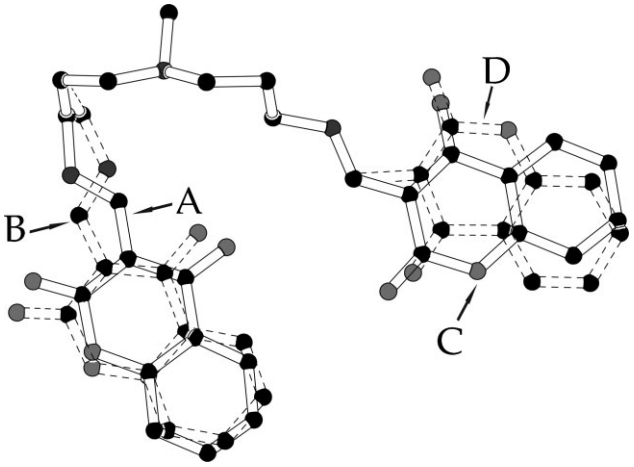


Figure 1. Disorder identified in the crystal structure of compound **5a**.

both at room and low temperatures. Its molecular structure is shown in Supporting Information Fig. S1, while the details of the structure determination are presented in Supporting Information Table S1. The structure reveals disorder in the crystal state caused by the presence of two different isomers (*E* and *Z*; Fig. 1). The populations of these stereoisomers, as determined for the low-temperature structure, are presented in Table 1 and are very similar to that found for the room-temperature structures. The total ratio of *E* to *Z* is 2:1. The intramolecular H-bond N_5A-H$\cdots O_8$A and N_5C-H$\cdots O_{16}$C $\{S_1^1(6)$ in the nomenclature of the graph set theory [17]$\}$ stabilizes the coplanarity of the cyclic systems with C_6–N_5–H fragments. The end groups of the molecule are almost parallel to each other ($\sim 5^\circ$), which allows for stacking interaction in the crystal (Supporting Information Fig. S2). Apart from an internal H-bond, the N_5 atom is a donor for intermolecular H-bonds N_5-H$\cdots O_8$ and N_5-H$\cdots O_{16}$ (Supporting Information Table S2), which forms two chains $C_1^1(14)$ on the first level and the ring $R_2^2(12)$ on the second level of a graph set.

Table 1. Populations of *E* and *Z* isomers of (3,3′)-3,3′-{(methylimino)*bis*[propane-3,1-diyliminomethylylidene]}*bis*(2*H*-chromene-2,4(3*H*)-dione) (**5a**) in the crystal state.

	I cyclic system		II cyclic system	
	A	B	C	D
Isomers	*Z*	*E*	*E*	*Z*
Occupancy (%)	52	48	86	14

A, B, C, and D denote disordered cyclic groups (Fig. 1).

Biological *in vitro* evaluation

Only **4a** (as hydrochloride) and **5a** both stable under experimental conditions were subjected to biological *in vitro* evaluation. Biological activity was investigated on the highly aggressive melanoma cell line A375. The antiproliferative activity was determined by the MTT-based assay and the acid phosphatase activity (APA) assay. Cell death was visualized by fluorescence microscopy and apoptosis was quantified by flow cytometric analysis.

To determine the drug concentration required to inhibit the growth of melanoma cells by 50% (IC_{50}), the tetrazolium derivative reduction (MTT) assay was used. Metabolic activity of adherent melanoma cells in relation to control cells was assessed. We found a significant difference in the activity of the tested compounds. Compound **5a** was found to have higher antiproliferative activity, with an IC_{50} value of 16 ± 2.2 μM (Fig. 2). A lower effect on cellular proliferation was observed after treatment with compound **4a** with an IC_{50} value of 46.8 ± 3.2 μM. To confirm the ability of these compounds to inhibit melanoma cell proliferation, the APA assay

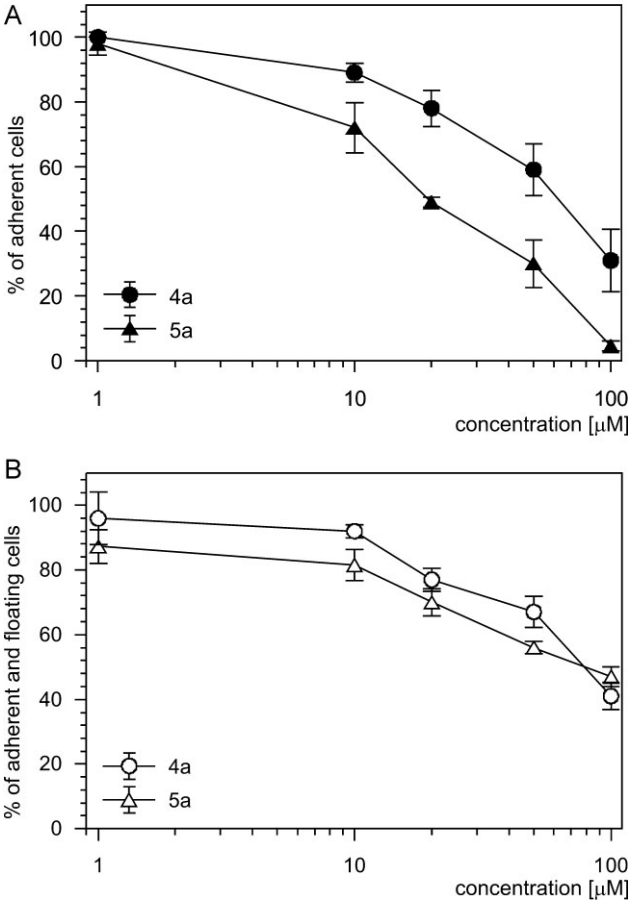


Figure 2. The influence of newly synthesized compounds **4a** and **5a** on A375 cell proliferation.

was used. In this method, both adherent and floating cells were analyzed. The same culture conditions, time of culturing and drug concentration range were retained. A similar effect on inhibition of A375 cell proliferation was observed after treatment with compound **4a**. For comparison, 100 μM **4a** inhibited the growth of A375 cells to 31 and 41% of control using the MTT and APA methods, respectively. Much bigger differences were observed after treatment with **5a**. For example, this compound at 100 μM inhibited the growth of melanoma cells to 4.6% of control when the MTT assay was used, and to 47% of control when the APA assay was applied. These results suggested that the newly synthesized compound **5a** was active at inhibition of both cell proliferation and adhesive potential of A375 cells.

To evaluate the ability of the new compounds to detach A375 cells from the surface, a more detailed analysis was performed. In the MTT assay, only adherent cells were analyzed since cells which lost contact with the surface (floating cells) were removed during the procedure. In the APA assay, both adherent and floating cells were analyzed. The percentages of floating viable cells were calculated by subtracting the percentages of adherent cells (MTT assay) from the percentages of all cells (APA assay). As shown in Fig. 3, compound **5a** reduced the adhesive potential of A375 melanoma cells in a concentration dependent manner. After incubation with **5a** at 50 and 100 μM, about 30% and more than 40% of A375 cells, respectively, lost contact with the surface, but remained viable (Fig. 3). In contrast to **5a**, compound **4a** did not influence the adhesion process and only about 10% of the A375 cells were detached from the surface when compound **4a** at 100 μM was used.

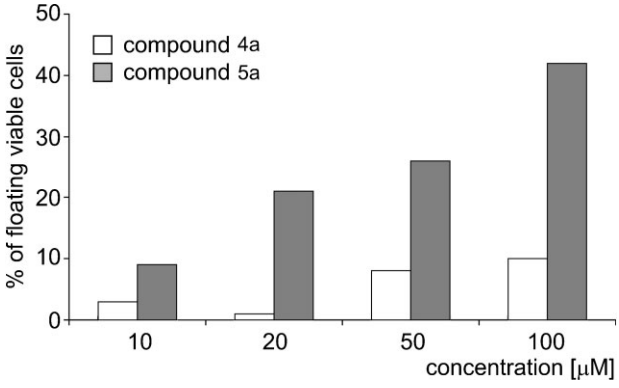


Figure 3. The influence of the new compounds **4a** and **5a** on the adhesive potential of A375 melanoma cells. The percentages of A375 cells which were detached from the surface (floating viable cells) after 44 h of incubation with the new compounds were calculated by subtracting the percentages of the adherent cells from the percentages of all cells (adherent and floating). The percentage of adherent viable cells was determined by MTT assay while the percentage of adherent and floating viable cells was assessed by APA assay, as described in the Experimental section.

The cell cycle analysis was performed in adherent A375 cells exposed to the newly synthesized compounds **4a** and **5a** (Fig. 4). Flow cytometric analysis of the DNA content was performed to analyze the accumulation of A375 cells in the cell cycle phases.

We found that the new compound **5a** induced a reduction in the percentages of cells in the G_0/G_1 and G_2/M phases, and an accumulation of cells in the S phase of the cell cycle, irrespective of the concentration. When the concentration of 50 μM was used, an accumulation of cells in hypodiploid $subG_1$ was additionally observed. Compound **4a** used at 20 and 50 μM did not introduce any changes in the cell cycle distribution when compared with control cells. Our results suggested that the newly synthesized compound **5a** was rather a cytostatic than a cytotoxic agent, whereas compound

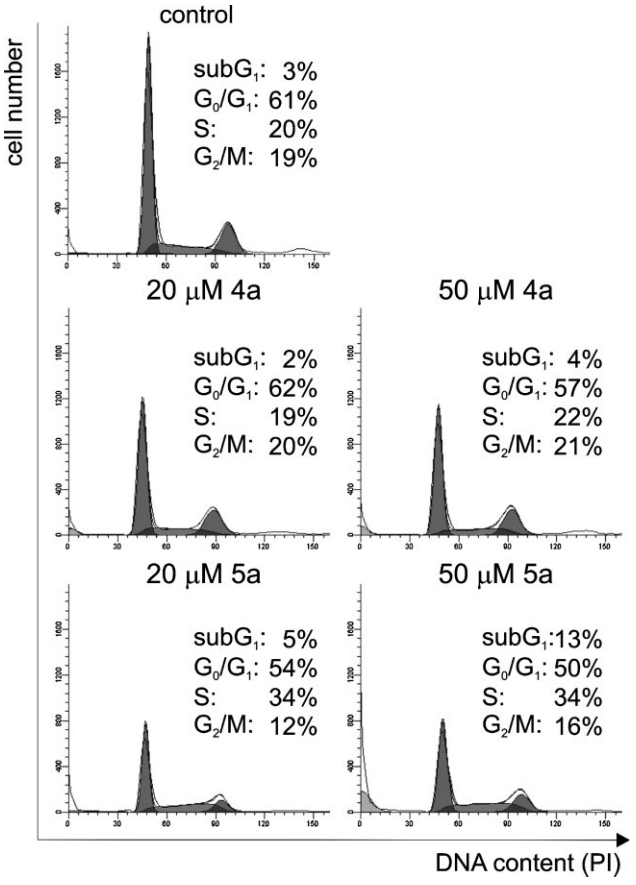


Figure 4. Effects of the new compounds **4a** and **5a** on cell cycle distribution. DNA content measured by flow cytometry at two different concentrations after 24 h of treatment with the new compounds. Percentages of the cells in each fraction were calculated using the ModFit 3.0 software. Representative histograms of one out of three independent experiments are shown.

4a was inactive at the same concentrations. To confirm these observations, cell death analysis was performed.

To examine whether the new compounds induced apoptosis in melanoma A375 cells, we employed a flow cytometric analysis of annexin V/PI-stained cells (Fig. 5A), and fluorescence microscopy for acridine orange (AO)/ethidium bromide (EB)-stained cells (Fig. 5B). The percentages of apoptotic (annexin-positive) and necrotic (exclusively PI-positive) cells were determined after 22 h of treatment with compounds **4a** and **5a** at two concentrations. The exposure of A375 cells to **4a** at a concentration close to the IC_{50} value and higher, did not induce significantly the percentage of apoptotic cells. About 11% of annexin V-positive cells were noted after treatment with 50 μM concentration. A higher concentration of **4a** slightly increased the apoptotic cells to 16% at 75 μM concentration. On the contrary, compound **5a** induced a higher increase in the amount of apoptotic cells. About 20 and 30% of A375 cells treated with **5a** at the concentration 50 and 75 μM, respectively, were annexin V positive. Those cells showed morphological features of apoptosis in AO/EB staining. Our result demonstrated that both compounds only slightly increased the percentages of necrotic cells. About 7 and 9% of necrotic cells were noted after treatment with 75 μM compounds **4a** and **5a**, respectively.

Conclusion

The present work describes the synthesis of six bischromone derivatives **4a–c** and **5a–c**, structurally related to bisintercalators. The condensation of 4-oxo-4*H*-chromene-3-carbonyl chloride **1** with corresponding polyamines (**a–c**) gave compounds **4a–c**, respectively, which was in agreement with previous studies. On the other hand, the reaction of 4-oxo-4*H*-chromene-3-carboxylic acid **2** with corresponding polyamines (**a–c**) in the presence of CDI resulted in the formation of stereoisomeric (*E*/*Z* ∼ 2:1) mixtures of compounds **5a–c**, respectively. Geometric isomers of **5a–c** were also formed in the transformation of methyl 4-oxo-4*H*-1-benzopyran-3-carboxylate **3** under the influence of the polyamines (**a–c**) used.

The structure of all synthesized compounds was fully confirmed by physical data, elemental analyses, IR, $^{1}H/^{13}C$ NMR

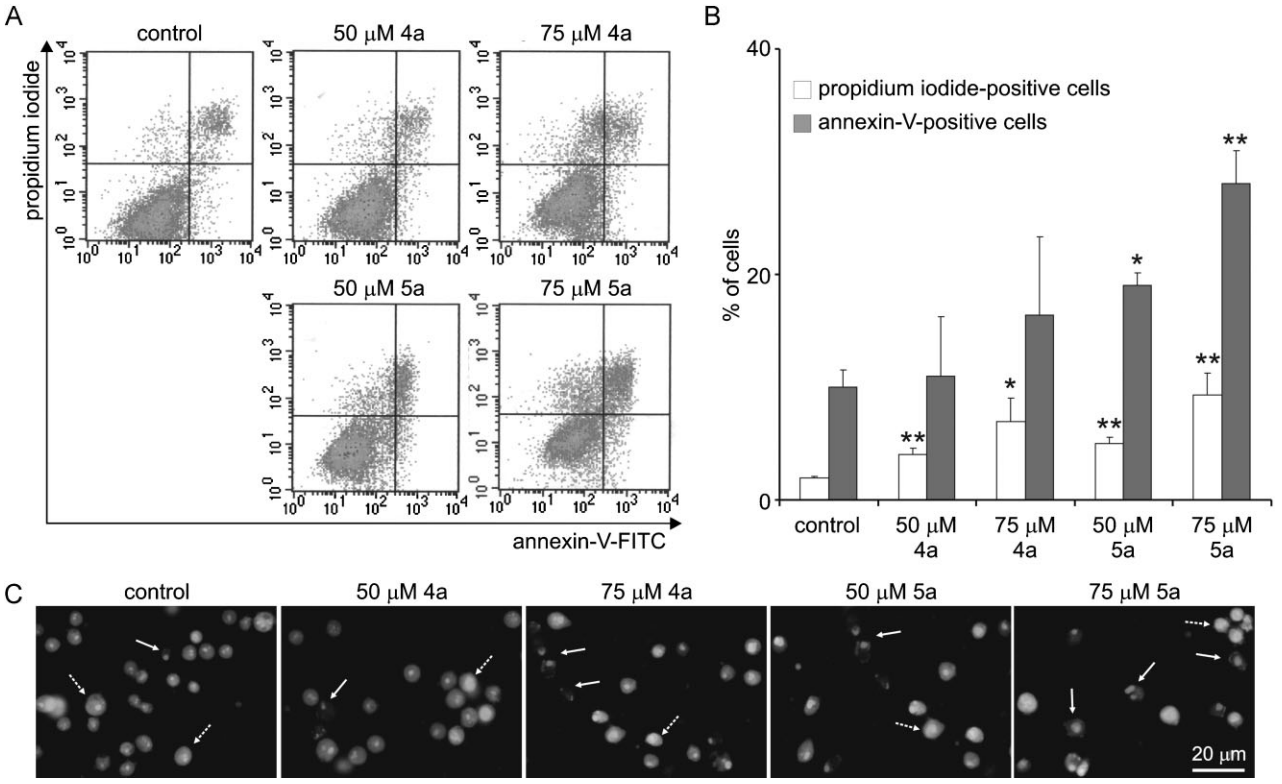


Figure 5. Cell death analysis of A375 melanoma cell line after treatment with new compounds **4a** and **5a**. (A) Externalization of phosphatidylserine was determined by annexin V/PI staining and analyzed by flow cytometry. Representative histograms from one typical experiment are shown. (B) Bars showing average percentages of annexin V- and PI-positive cells are presented. The data are means ± SD of three independent experiments ($^{*}p < 0.05$, $^{**}p < 0.01$). (C) Cells were stained with nucleic acid selective fluorochromes: membrane-permeable AO and impermeable EB. Representative microscopic fields presenting non-apoptotic, apoptotic and necrotic cells are shown.

spectroscopy in solution, and XRD experiments for the crystal of **5a**.

The outcome of our chemical investigations clearly confirmed a significant influence of the electron-withdrawing substituents at the 3-position of the chromone system on the reactivity of the γ-pyrone ring with respect to nitrogen nucleophiles. It also showed that in the case of 4-oxo-4*H*-1-benzopyran-3-carboxylic acid (**2**), the presence of CDI did not favor the amide formation but gave the stereoisomeric (*E*/*Z* ∼ 2:1) mixture of 2*H*-chromene-2,4(3*H*)-dione derivatives (**5a–c**). Methyl 4-oxo-4*H*-1-benzopyran-3-carboxylate (**3**) underwent similar transformations.

When biological activity is concerned, the observed differences in antiproliferative activity of the examined compounds **4a** and **5a** were significant, with **5a** being more active, especially when the induction of apoptosis and cell cycle arrest are considered. Compound **5a** was also more potent in diminishing the adhesive potential of melanoma cells. The current studies support the notion that small changes in the three-dimensional structure of the molecules might have a substantial impact on their biological activity.

Our results suggest that selected bischromone derivatives may be worthy of future research for designing new entities with an increased cytotoxic/cytostatic activity.

Experimental

General

Reagents and solvents were purchased from common commercial suppliers. The purity of the products was routinely confirmed by TLC on Merck plates (Kieselgel 60 F_{254}); the appropriate solvents were used, and spots were visualized under UV light. The melting points were measured on an Electrothermal apparatus in open capillaries 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. IR spectra were recorded in KBr using a Mattson Infinity Series FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were acquired on a Varian Mercury-300 spectrometer (at 300.06/75.45 MHz, for the ^{1}H/^{13}C nuclei) in $CDCl_3$ solution with TMS as internal standard. Chemical shifts are expressed in δ (ppm) and the coupling constants *J* in Hertz (Hz). The following abbreviations were used to describe the signal patterns when appropriate: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), vbr (very broad), and p (pseudo).

Chemistry

*General procedure for the synthesis of **4a–c***

A solution of 4-oxo-4*H*-chromene-3-carbonyl chloride **1** in 200 mL of methylene chloride was added for a period of 5 min to a stirred mixture of appropriate polyamines (**a–c**), powdered potassium carbonate and 100 mL of methylene chloride, keeping the temperature at 25°C with ice bath cooling. After 1 h, the solids were filtered off and the filtrate was concentrated. Additional material was recovered by dissolving the carbonate filter cake in water and filtering off the product. Recrystallization from DMF/H_2O gave pure amides.

*N,N′-[(Methylimino)dipropane-3,1-diyl]bis(4-oxo-4H-chromene-3-carboxamide) (**4a**)*

Yield: 55% as yellow crystals, mp 108.5–110.5°C; IR (KBr) *ν*: 1667, 1614 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 9.39 (t, *J* ∼ 5.5 Hz, 2H, 2 CONH), 8.96 (s, 2H, OCH=), 8.27 (dd, *J* = 8.1, 1.7 Hz, 2H, CH_{ar}), 7.69–7.76 (m, 2H, CH_{ar}), 7.43–7.55 (m, 4H, CH_{ar}), 3.53 (pq, *J* ∼ 6.5 Hz, 4H, NCH_2), 2.49 [t, *J* = 6.9 Hz, 4H, $CH_2N(CH_3)$], 2.26 (s, 3H, NCH_3), 1.77–1.88 (m, 4H, $CH_2CH_2CH_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 177.11 ($C{=}O_{unsaturated\ keto}$), 162.74 ($C{=}O_{amide}$), 162.21 (OCH=), 156.08 (C_q), 134.52, 126.30 (3 × CH_{ar}), 124.39 (C_q), 118.46 (CH_{ar}), 116.02 (C_q), 55.62 (CH_2), 42.22 (CH_3), 37.85, 27.39 (2 × CH_2) ppm. Anal. calcd. for ($C_{27}H_{27}N_3O_6 \times {}^1/_2H_2O$) C, 65.05, H, 5.66, N, 8.42. Found: C, 64.90, H, 5.46, N, 8.59.

*N,N′-(Piperazine-1,4-diyldipropane-3,1-diyl)bis(4-oxo-4H-chromene-3-carboxamide) (**4b**)*

Yield: 60% as pale yellow crystals, mp 201.3–202.3°C; IR (KBr) *ν*: 1664, 1613 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 9.38 (t, *J* ∼ 5.4 Hz, 2H, 2CONH), 8.99 (s, 2H, OCH=), 8.30 (dd, *J* = 7.9, 1.4 Hz, 2H, CH_{ar}), 7.75–7.79 (m, 2H, CH_{ar}), 7.50–7.53 (m, 4H, CH_{ar}), 3.53 (pq, *J* ∼ 6.8 Hz, 4H, NCH_2), 2.47–2.51 (cluster, 8H CH_2 piperazine + 4H CH_2N), 1.82–1.88 (m, 4H, $CH_2CH_2CH_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 177.17 ($C{=}O_{unsaturated\ keto}$), 162.77 ($C{=}O_{amide}$), 162.24 (OCH=), 156.14 (C_q), 134.53, 126.22 (3 × CH_{ar}), 124.37 (C_q), 118.42 (CH_{ar}), 115.99 (C_q), 56.09, 53.20, 37.74, 26.74 (5 × CH_2) ppm. Anal. calcd. for ($C_{30}H_{32}N_4O_6 \times 2{}^1/_2H_2O$) C, 61.10, H, 6.32, N, 9.50. Found: C, 60.95, H, 6.33, N, 9.67.

*N,N-[Butane-1,4-diylbis(oxypropane-3,1-diyl)]bis(4-oxo-4H-chromene-3-carboxamide) (**4c**)*

Yield: 73% as yellow crystals, mp 110.5–112.1°C; IR (KBr) *ν*: 1667, 1615 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 9.37 (t, *J* ∼ 5.5 Hz, 2H, 2 CONH), 8.95 (s, 2H, OCH=), 8.27 (dd, *J* = 7.9, 1.4 Hz, 2H, CH_{ar}), 7.70–7.78 (m, 2H, CH_{ar}), 7.51–7.57 (m, 2H, CH_{ar}), 7.48 (t, *J* = 7.5 Hz, 2H, CH_{ar}), 3.48–3.58 (cluster, 6H, NCH_2 + 6H CH_2O), 1.86–1.93 (m, 4H, $CH_2CH_2CH_2$), 1.67–1.72 (m, 4H, $CH_2CH_2CH_2CH_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 177.08 ($C{=}O_{unsaturated\ keto}$), 162.73 ($C{=}O_{amide}$), 162.16 (OCH=), 156.09 (C_q), 134.49, 126.18 (3 × CH_{ar}), 124.33 (C_q), 118.38 (CH_{ar}), 115.96 (C_q), 70.95, 68.58, 36.90, 29.61, 26.46 (5 × CH_2) ppm. Anal. calcd. for ($C_{30}H_{32}N_2O_8$) C, 65.68, H, 5.87, N, 5.11. Found: C, 65.40, H, 5.68, N, 5.24.

*General procedure for the synthesis of **5a–c***

*From 4-oxo-4H-chromene-3-carboxylic acid (**2**)*

10 mmol of **2** and 10 mmol of 1,1-carbonyl-diimidazole (CDI) in DMF (100 mL) were stirred for 1 h at room temperature. Then the appropriate polyamines (**a–c**) (6 mmol) were added and stirring was continued for an additional 2 h. The mixture was filtered, the solvent was removed under reduced pressure, water was added and the whole was left for 12 h at +5°C. The solids (precipitate) were filtered off, washed with H_2O, and crystallized from DMF/H_2O.

*From methyl 4-oxo-4H-1-benzopyran-3-carboxylate (**3**)*
The appropriate polyamines (**a**–**c**) (6 mmol) in MeOH (0.5 mL) were added at room temperature to a solution of **3** (10 mmol) in MeOH (5 mL). The solid crude products, which were precipitated after 24 h, were filtered off, dried, and crystallized from MeOH.

*(3,3′)-3,3′-{(Methylimino)bis[propane-3,1-diyliminomethylylidene]}bis(2H-chromene-2,4(3H)-dione) (**5a**)*

*From 4-oxo-4H-chromene-3-carboxylic acid (**2**)*
Yield: 96.5% as dark red crystals of an *E*/*Z* (∼2:1) isomeric mixture of **5a**, mp 167.8–168.5°C (from DMF/H_2O); IR (KBr) *ν*: 1700, 1638 (C=O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ_H: ∼11.95 (vbr ps, 1.33H, =CHN*H*, *E* forms), ∼10.55 (vbr asymmetric massif, 0.67H, =CHN*H*, *Z* forms), 8.56 (d, $J = 15.2$ Hz, 0.67H, =C*H*NH, *Z* forms), 8.42 (d, $J = 14.3$ Hz, 1.33H, =C*H*NH, *E* forms), 8.11–8.09 (m, 0.67H, CH_{ar}, *Z* forms), 8.08–8.06 (m, 1.33H, CH_{ar}, *E* forms), 7.58–7.50 (m, 2H, CH_{ar}), 7.26–7.18 (m, 4H, CH_{ar}), 3.79 (pq, $J \sim 6.3$ Hz, 1.33H, NCH_2, *Z* forms), 3.74 (pq, $J \sim 6.3$ Hz, 2.67H, NCH_2, *E* forms), 2.57–2.49 [m, 4H, C*H*$_2$N(CH_3)], 2.21 (s, 3H, NCH_3), 1.99–1.89 (m, 4H, $CH_2CH_2CH_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) (a) the *E* isomer (only lines of the major conformation are given) δ_C: 180.98 ($C{=}O_{keto}$), 164.05 ($C{=}O_{ester}$), 162.24 (=CHN), 154.76 (C_q), 134.16, 125.78, 123.93 ($3 \times CH_{ar}$), 120.69 (C_q), 117.25 (CH_{ar}), 96.87 (C_q), 55.85, 49.78 ($2 \times CH_2$), 40.91 (CH_3), 27.55 (CH_2) ppm; (b) the *Z* isomer (only lines of the major conformation are given) δ_C: 178.46 ($C{=}O_{keto}$), 164.94 ($C{=}O_{ester}$), 160.58 (=CHN), 154.64 (C_q), 134.21, 126.37, 124.09 ($3 \times CH_{ar}$), 120.86 (C_q), 117.25 (CH_{ar}), 96.69 (C_q), 56.53, 50.38 ($2 \times CH_2$), 40.77 (CH_3), 27.17 (CH_2) ppm. Anal. calcd. for $C_{27}H_{27}N_3O_6$: C, 66.24, H, 5.51, N, 8.58. Found: C, 65.90, H, 5.46, N, 8.59.

*From methyl 4-oxo-4H-1-benzopyran-3-carboxylate (**3**)*
Yield: 42% as yellow crystals mp 167.9–168.5°C (from MeOH); its analytical, IR, and $^1H/^{13}C$ NMR spectroscopic data were in full accordance with the results described above.

*(3)-3-[({3-[4-(3-{[(2,4-Dioxo-2H-chromen-3(4H)-ylidene)-methyl]amino}propyl)piperazin-1-yl]propyl}amino)-methylene]-2H-chromene-2,4(3H)-dione (**5b**)*

*From 4-oxo-4H-chromene-3-carboxylic acid (**2**)*
Yield: 73.5% as creamy crystals of an *E*/*Z* (∼2:1) isomeric mixture of **5b**, mp 233.6–234.2°C (from DMF/H_2O); IR (KBr) *ν*: 1693, 1640, 1605 (C=O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ_H: ∼11.90 (vbr s, 1.33H, =CHN*H*, *E* forms), ∼10.45 (vbr s, 0.67H, =CHN*H*, *Z* forms), 8.58 (d, $J = 15.1$ Hz, 0.67H, =C*H*NH, *Z* forms), 8.44 (d, $J = 14.2$ Hz, 1.33H, =C*H*NH, *E* forms), 8.12 (dd, $J = 7.8$, 1.6 Hz, 0.67H CH_{ar}, *Z* forms), 8.04 (dd, $J = 7.8$, 1.3 Hz, 1.33 H CH_{ar}, *E* forms), 7.60–7.55 (m, 2H, CH_{ar}), 7.29–7.24 (m, 4H, CH_{ar}), 3.70 (q, $J = 6.1$ Hz, 1.33H, NCH_2, *Z* forms), 3.65 (q, $J = 6.3$ Hz, 2.67H NCH_2, *E* forms), 2.65–2.43 (m, 12H, $CH_2N + CH_2$ piperazine), 1.93–1.85 (m, 4H, $CH_2CH_2CH_2$) ppm.^{13}C NMR (75 MHz, $CDCl_3$) (a) the *E* isomer (only lines of the major conformation are given) δ_C: 181.02 ($C{=}O_{keto}$), 164.07 ($C{=}O_{ester}$), 162.50 (=CHN), 154.90 (C_q), 134.15, 125.67, 123.90 ($3 \times CH_{ar}$), 120.75 (C_q), 117.28 (CH_{ar}), 96.81 (C_q), 55.84, 54.81 ($2 \times CH_2$), 52.90 ($2 \times CH_2$), 49.52, 26.48 ($2 \times CH_2$) ppm; (b) the *Z* isomer (only lines of the major conformation are given) δ_C: 178.60 ($C{=}O_{keto}$), 164.85 ($C{=}O_{ester}$), 160.79 (=CHN), 154.79 (C_q), 134.20, 126.39, 124.04 ($3 \times CH_{ar}$), 120.91 (C_q), 117.23 (CH_{ar}), 96.68 (C_q), 55.23, 54.82 ($2 \times CH_2$), 53.10 ($2 \times CH_2$), 49.88, 26.38 ($2 \times CH_2$) ppm. Anal. calcd. for $C_{30}H_{32}N_4O_6$: C, 66.16, H, 5.87, N, 10.28. Found: C, 65.92, H, 6.11, N, 10.67.

*From methyl 4-oxo-4H-1-benzopyran-3-carboxylate (**3**)*
Yield: 55% as white crystals, mp 233.6–234.1°C (from MeOH); its analytical, IR, and $^1H/^{13}C$ NMR spectroscopic data were in full accordance with the results described above.

*(3,3′)-3,3′-[(1,16)-6,11-Dioxa-2,15-diazahexadecane-1,16-diylidene]bis(2H-chromene-2,4(3H)-dione) (**5c**)*

*From 4-oxo-4H-chromene-3-carboxylic acid (**2**)*
Yield: 60% as white crystals of an *E*/*Z* (∼2:1) isomeric mixture of **5c**, mp 168.3–170.3°C (from DMF/H_2O); IR (KBr) *ν*: 1697, 1647, 1608 (C=O) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ_H: ∼11.89 (vbr s, 1.33H, =CHN*H*, *E* forms), ∼10.38 (vbr s, 0.67H, =CHN*H*, *Z* forms), 8.54 (dd, $J = 15.1$, 4.3 Hz, 0.67H, =C*H*NH, *Z* forms), 8.40 (dd, $J = 14.2$, 4.6 Hz, 1.33H, =C*H*NH, *E* forms), 8.11 (dd, $J = 7.8$, 1.4 Hz, 0.67H, CH_{ar}, *Z* forms), 8.03 (dd, $J = 7.8$, 1.5 Hz, 1.33H, CH_{ar}, *E* forms), 7.60–7.54 (m, 2H, CH_{ar}), 7.28–7.22 (m, 4H, CH_{ar}), 3.76–3.67 (m, 4H, CH_2O), 3.60–3.54 (m, 4H, NCH_2), 3.54–3.50 (m, 4H, OCH_2), 2.02–1.95 (m, 4H, $CH_2CH_2CH_2$), 1.79–1.70 (m, 4H, $CH_2CH_2CH_2CH_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) (a) the *E* isomer (only lines of the major conformation are given) δ_C: 181.15 ($C{=}O_{keto}$), 163.96 ($C{=}O_{ester}$), 162.43 (=CHN), 154.91 (C_q), 134.14, 125.66, 123.84 ($3 \times CH_{ar}$), 120.71 (C_q), 117.27 (CH_{ar}), 96.86 (C_q), 71.21, 67.76, 48.75, 29.96, 26.43 ($5 \times CH_2$) ppm; (b) the *Z* isomer (only lines of the major conformation are given) δ_C: 178.50 ($C{=}O_{keto}$), 164.98 ($C{=}O_{ester}$), 160.68 (=CHN), 154.77 (C_q), 134.17, 126.38, 124.02 ($3 \times CH_{ar}$), 120.90 (C_q), 117.19 (CH_{ar}), 96.73 (C_q), 71.31, 67.23, 49.16, 29.87, 26.36 ($5 \times CH_2$) ppm. Anal. calcd. for $C_{30}H_{32}N_2O_8$: C, 65.68, H, 5.83, N, 5.10. Found: C, 65.30, H, 5.74, N, 5.22.

*From methyl 4-oxo-4H-1-benzopyran-3-carboxylate (**3**)*
Yield: 50% as white crystals, mp 168.5–170.3°C (from MeOH); its analytical, IR, and $^1H/^{13}C$ NMR spectroscopic data were in full accordance with the results described in above.

X-ray crystallography

The crystals suitable for an X-ray experiment were obtained by the slow evaporation method from DMF solution. The data were collected with a Bruker CCD APEX II diffractometer using the Mo radiation. The experiment was carried out at −173°C (100 K). Data processing was performed by means of Bruker packages of programs [18]. The structure was solved by direct methods using SHELXTL [19] and refined by full-matrix least squares [20]. Non-hydrogen atoms were refined anisotropically except that belonging to the least populated cyclic system. All H-atoms were refined isotropically at calculated positions, by using the riding model. Supporting Information Tables S1 and S2 as well as Supporting Information Fig. S1 and Fig. 1 were prepared with Platon [21], while Supporting Information Fig. S2 was prepared with the Mercury software [22]. Final coordinates and details of the measurement, solution, and refinement were deposited with the Cambridge Crystallographic Data Centre No. CCDC 829311. All details of crystal data may be obtained free of

charge *via* www.ccdc.cam.ac.uk/conts/retrieving.html or from Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; or deposit@ccdc.cam.ac.uk.

Biological *in vitro* evaluation

Cell line and cell culture conditions

A375, a human melanoma cell line with a high metastatic potential, was grown in a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Lonza, Switzerland), supplemented with 10% fetal bovine serum (FBS), penicillin (10 U/mL), and streptomycin (50 μg/mL). For experiments, the culture medium was substituted with fresh medium containing 0.5% FBS. In experiments, cells in the logarithmic phase of growth were used.

Analysis of cell proliferation

Cell proliferation was measured by two methods: a colorimetric tetrazolium derivative reduction (MTT) and the APA assay. Cells were seeded in 24-well plates, and 6 h later adherent cells were exposed to tested compounds at indicated concentrations for 44 h. To determine IC_{50} values, the concentration of tested compounds causing 50% inhibition of cell growth, the MTT assay was used as described previously [9]. To confirm the influence of newly synthesized compounds on the proliferation potential of A375 melanoma cells, the APA assay was used. Briefly, cells on plates were centrifuged (2500 rpm, 10 min) and washed with PBS. To each well 0.1 M sodium acetate (pH 5.0), 0.1% Triton X-100 and 5 mM *p*-nitrophenyl phosphate were added. After 2 h of incubation at 37°C 1 M NaOH was added. The absorbance at 405 nm was recorded using a microplate reader (Infinite M200 PRO, TECAN, Austria). In the MTT assay, only adherent cells were assessed, whereas in the APA assay both adherent and floating cells were analyzed.

Cell cycle analysis

Cell cycle analysis was performed on propidium iodide-stained cells, as described previously [23]. A375 cells were treated with the newly synthesized compounds at indicated concentrations for 24 h and then analyzed for the DNA content. The percentages of a cell population in subG_1, G_0/G_1, S or G_2/M phases were calculated using the ModFit LT 3.0 software (Verify software, Topsham, MN, USA).

Flow cytometric analysis of cell death

A375 cells were treated with the new compounds at indicated concentrations. Adherent and floating cells were combined and analyzed together. Flow cytometry was used to assess apoptosis. The detection of apoptotic and necrotic cells was carried out by dual staining with FITC-conjugated annexin V and propidium iodide (PI) (Roche Diagnostics, Mannheim, Germany), as described previously [24]. After 22 h of drug treatment, cells were washed in cold PBS and incubated with 50 μL staining solution containing 1 μL annexin V-FITC. After 10 min of incubation, 50 μL staining solution containing 2 μL of 20 μg/mL PI was added. This method allowed for the discrimination of viable, non-apoptotic cells (unstained with either fluorochrome; lower left panel) from apoptotic cells (stained with annexin V; upper and lower right panels) and necrotic cells (stained only with PI; upper left panel).

Acridine orange/ethidium bromide staining

Cell death was monitored using fluorescent dyes: AO and EB. Briefly, A375 cells were cultured for 22 h with or without the tested compounds at indicated concentrations, then collected by centrifugation and resuspended in 30 μL of staining solution, a mixture (1:1) of EB (100 μg/mL) and AO (100 μg/mL). They were examined by ultraviolet fluorescence microscopy (Olympus BX 41).

Statistical analysis

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

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