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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis, preliminary cytotoxicity evaluation of new 3-formylchromone hydrazones and phosphorohydrazone derivatives of coumarin and chromone

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Abstract Reactions of chromone derivatives **1–4** with *N*-substituted hydrazines were described. Hydrazones (**6**, **7a**, **8a–c**, **9b–e**, **10e**, **11e**, **12**) were evaluated for cytotoxicity (MTT test) against HL-60 and NALM-6 leukemia cells. Phosphorohydrazone of 3-formylchromone **8a** and hydrazone of 2-amino-3-chromone derivative **11e** showed appreciable cytotoxicity. The cytotoxicity indices of **8a**, **11e**, and **12** were higher on drug-resistant HL-60 ADR cells in comparison to HL-60. Compounds **8a** and **11e** were tested for their ability to induce cytochrome *c* translocation from mitochondria to cytosol.

Keywords Chromone · Hydrazone · Cytotoxic · Cytochrome *c*

Introduction

In recent years, benzopyrone group-based compounds, such as chromone, coumarin and flavone, have come to the attention of many scientists due to their unique chemical and biological properties. Numerous studies of chromone compounds have proven their antitumor (Huang *et al.*, 2007; Huang *et al.*, 2009), antiviral (Ungwitayatorn *et al.*, 2004; Nunthanavanit *et al.*, 2008), antimicrobial (Hasan *et al.*, 2009; Amin *et al.*, 2010), insecticidal (Zhou and Yang, 2006; Zhao *et al.*, 2007), and antioxidant (Samee *et al.*, 2004; Samee *et al.*, 2008) activity. Moreover, compounds from this chemical group are present in many species of plants, fungi, lichens, and water plants, and possess varied biological activity (Witczak 1994; Lin *et al.*, 2001). The chemistry of 3-substituted chromones, mainly 3-formylchromones, has been developed thanks to the simple and convenient Vilsmeier–Haack method and proposed for the synthesis of 3-formylchromones from 2-hydroxyacetophenones, DMF, and $POCl_3$ (Nohara *et al.*, 1974).

3-Formylchromone and its derivatives have been extensively used for the synthesis of Schiff bases by reactions with a variety of nitrogen nucleophiles (Coutinho and Fernandes, 1992; Sosnovskikh *et al.*, 2008). The biological activity of these compounds is greatly varied. 3-Formylchromones and some of their condensation products with amines show antibacterial (El-Shaaer *et al.*, 1998), antimycobacterial (Lácová *et al.*, 1995), antimutagenic (Foltínová *et al.*, 2000), and cytotoxic (Baráth *et al.*, 2006) activity. On the other hand, antiproliferative (Roma *et al.*, 1998) and cytotoxic (Di Braccio *et al.*, 2003) properties of 2-aminochromone derivatives and the specific inhibitory activity of 6-chloro-/fluorochromones against topoisomerase (Ishar *et al.*, 2006) as well as that of Schiff bases of 3-formylchromone against thymidine phosphorylase (Khan *et al.*, 2009) have been described in the literature. Aminochromones have also been tested as agents potentially preventing thrombosis and delaying restenosis after percutaneous transluminal coronary angioplasty (Bonin *et al.*, 1993).

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In our earlier works, we discussed the synthesis of a series of phosphorohydrazone derivatives of coumarin and chromone, and reported the results of our research on their in vivo antitumour activity (Nawrot-Modranka *et al.*, 2004; Nawrot-Modranka *et al.*, 2006) and influence on the polymerization and viscosity of fibrin (Michalska *et al.*, 2008), which proved that these compounds have many interesting properties.

Continuing our previous studies on benzopyrones, we tested 12 compounds (**6**, **7a**, **8a–c**, **9b–e**, **10e**, **11e**, **12**) for their cytotoxic activity on HL-60 and NALM-6 leukemia cell lines. The most promising compounds **8a** and **11e** were examined for cytosolic cytochrome *c* levels. Some of these compounds (phosphorohydrazone derivatives **6**, **7a**, **8a**) were previously described by us (Nawrot-Modranka *et al.*, 2006), while the others (**8b–c**, **9b–e**, **10e**, **11e**, **12**) were first synthesized in the present study. Hydrazones (**8b–c**, **9b–e**, **10e**, **11e**, **12**) were prepared by treating the corresponding 3-formylchromone (**1**) and its derivatives (**2–4**) with suitable hydrazines (Scheme 1).

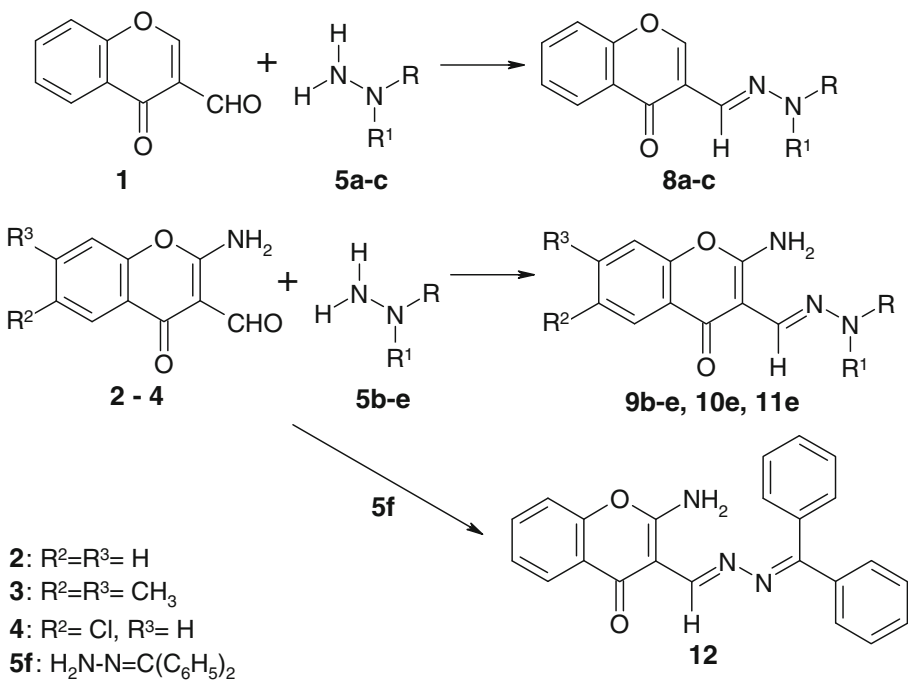
Results and discussion

Synthesis

3-Formylchromone is a very reactive compound and its reactions with nitrogen nucleophiles give a variety of products. The primary amino group can react at three electrophilic sites (C-2, C-4, CHO) of 3-formylchromones. The reaction of equimolar quantities of 3-formylchromone and an aromatic primary amine leads to a mixture of anil and a 1,4-adduct (Fitton *et al.*, 1975, 1979).

3-Formylchromones with aromatic amino carboxylic acids yield enamines of 2-hydroxy(alkoxy)-chroman-4-ones or 3-(aryliminomethyl)-chromones, depending on the reaction conditions (Stankovičová *et al.*, 2001). On the other hand, the results of recently published works (El-Shaaer *et al.*, 1998; Foltínová *et al.*, 2000; Nawrot-Modranka *et al.*, 2006; Wang *et al.*, 2006; Qi *et al.*, 2009) show that no products of 3-formylchromone with hydrazine derivatives have been obtained by rearrangement of the benzopyrone ring.

Scheme 1 Synthesis of hydrazones of 3-formylchromones



2: $R^2=R^3=H$
3: $R^2=R^3=CH_3$
4: $R^2=Cl$, $R^3=H$
5f: $H_2N\text{-}N=C(C_6H_5)_2$

a: $R=CH_3$, $R^1=P(S)(OCH_3)_2$
b: R= H, R^1= 2,5-dichlorophenyl
c: R= H, $R^1=COOCH_3$
d: R= H, $R^1=C_6H_5COOH$
e: R= H, $R^1=(CH_2)_2OH$

These observations confirm our results concerning the chemistry of 3-formylchromone (**1**) and 2-amino-3-formylchromones (**2**–**4**) with hydrazines (**5b**–**f**), showed in this article (Scheme 1).

The preparation of hydrazones **8b**–**c**, **9b**–**e**, **10e**, **11e**, **12** was carried out by stirring and heating the mixture of 3-formylchromone (**1**) or 2-amino-3-formylchromones (**2**–**4**) with substituted aromatic and aliphatic hydrazines in dry ethanol or toluene. The progress of the reaction was monitored by TLC. The optimal conditions for the synthesis of these compounds to obtain the highest possible yield were found. The functional groups in the aromatic ring of the studied hydrazines do not possess a significant influence on their reactivity; however, aromatic hydrazines are less reactive than aliphatic hydrazines. In the case of chromones, the $-NH_2$ group in position 2 causes decreased chemical reactivity.

The structures of the products (**8b**–**c**, **9b**–**e**, **10e**, **11e**, **12**) were determined by spectroscopic analysis (IR, ^{1}H NMR). All IR and ^{1}H NMR spectra indicate that the products of the presented reactions contain a chromone ring. The IR spectra of hydrazones **8b**–**c**, **9b**–**e**, **10e**, **11e**, and **12** revealed a strong band resulting from the stretching frequency of the carbonyl group of the pyrone at 1642–1665 cm^{-1}. ^{1}H NMR spectra of hydrazones **8b** and **8c** showed a singlet signal of H-2 of the chromone ring at δ 8.46 and 8.17 ppm, and a signal of the CH=N group at $\delta = 8.99$ and 8.71, respectively. The characteristic feature of 2-amino-3-formylchromone hydrazones (**9b**–**e**, **10e**, **11e**, and **12**) is that their ^{1}H NMR spectrum is a broad singlet at δ 8.57–9.30 (NH_2) and a singlet at δ 8.12 – 9.07 (CH=N).

The synthesized hydrazones are very stable in the open air and are insensitive to the influence of water.

Cytotoxicity

The cytotoxicity of the studied compounds was assayed against human lymphoblastic leukemia NALM-6 cells, promyelocytic leukemia HL-60 cells, and adriamycin-resistant HL-60 ADR cells. Warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin) and adriamycin were used as reference compounds. Cells were exposed to a broad range of drug concentrations (10^{-8} to 10^{-3} M) for 48 h, and cell viability was analyzed by MTT assay. Cytotoxic activity was expressed as the concentration (μM) of a tested compound required to reduce the cell survival fraction to 50% of the control (IC_{50} value). Results are given in Table 1. Two groups of compounds, namely **6**, **7a**, **8a** and **9e**, **10e**, **11e**, exhibited relatively high cytotoxicity against the studied cell lines. Compounds from the second group, all having an aromatic hydrazine R^1 substituent, seem to be the most promising as potential anticancer agents. Compound **11e**, with the IC_{50} value of 6.6 ± 0.7 μM for HL-60 cells, can be considered highly potent according to Kupchan's classification ($IC_{50} \leq 15$ μM) (Kupchan *et al.*, 1971). Apart from two other chromone derivatives with moderate cytotoxic effectiveness (**8b** and **12**), there exists yet another group of compounds with only negligible antiproliferative activity (**9b**–**d** and **8c**).

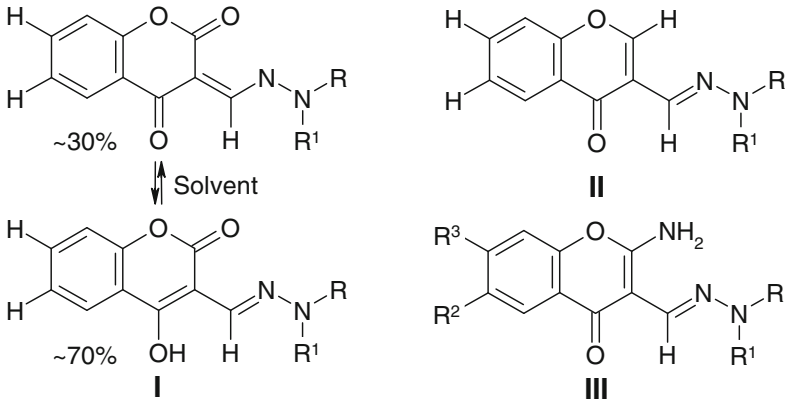
The effect of the three selected chromone derivatives on the viability of multidrug-resistant cells was also studied. For this purpose, cytotoxicities of **8a**, **11e**, **12** and adriamycin against maternal HL-60 cells and the multidrug-resistant HL-60 ADR subline (adriamycin-resistant, MRP1-overexpressing) were tested and compared. Multidrug resistance-associated protein 1 (MRP1) belongs to the family of cell membrane transporters with an ATP-binding cassette. This efflux pump is one of the factors of resistance to chemotherapeutic agents. Its overexpression in malignant cells plays an important role in decreasing the intracellular concentration of anticancer drugs, a phenomenon known as multidrug resistance (MDR) (Linton, 2007). As shown in Table 1, drug-resistant HL-60 ADR cells were over 100 times less sensitive to adriamycin in comparison to maternal HL-60 cells. But all three chromone derivatives were at least equally effective against both cell lines. Although the antitumor activity of **8a**, **11e**, and **12** was much lower than that of adriamycin, this was only the case against the drug susceptible HL-60 cells. In contrast, **11e** was only a little less effective than adriamycin against drug-resistant HL-60 ADR cells. It seems that chromone derivatives are poor substrates for transport by the MRP1 efflux pump, which means that they may be useful for treating drug-resistant tumors.

Cytosolic cytochrome *c* level

Compounds **8a** and **11e** were tested for their ability to induce cytochrome *c* translocation from mitochondria to cytosol. Such an assay is used to detect the early steps in initiating apoptosis in the cells. Upon apoptotic stimuli, the loss of normal mitochondrial physiology releases cytochrome *c* into cytosol, where, in the presence of deoxy-ATP, it mediates the activation of the adaptor molecule, that is, apoptosis protease activating factor 1 (Apaf-1). This complex known as apoptosome can recruit procaspase-9, which finally allows for the catalytic maturation of caspase-3 and other caspases. The proapoptotic signal is amplified by a caspase cascade leading to the cleavage of vital cellular proteins and is followed by cellular disintegration into apoptotic bodies and the disappearance of any traces of the cells (Garrido *et al.*, 2006).

In this study, HL-60 leukemia cells were treated with **8a** and **11e** in three concentrations, 0.2 × IC_{50}, 1 × IC_{50}, and 5 × IC_{50} for 0.5, 2, and 4 h. The time course of cytochrome *c* translocation from mitochondria to cytosol is shown in Fig. 1. Both chromone derivatives were able to

Table 1 Structure and IC_{50} values of the compounds tested for the cytotoxicity on NALM-6, HL-60, and HL-60-ADR leukemia cells



Symbol	Chemical structure					Cytotoxicity, IC_{50} (μM)*		
	Structure	R	R^1	R^2	R^3	NALM-6	HL-60	HL-60 ADR
6	I	CH_3	$P(S)(OC_2H_5)_2$	–	–	66.5 ± 5.7	86.3 ± 5.5	
7a	I	CH_3	$P(S)(OCH_3)_2$	–	–	56.5 ± 6.8	61.8 ± 3.8	
8a	II	CH_3	$P(S)(OCH_3)_2$	–	–	28.8 ± 7.0	34.4 ± 4.3	30.6 ± 2.2
8b	II	H	2,5-$C_6H_3Cl_2$	–	–	85.3 ± 8.9	264.6 ± 2 9.1	
8c	II	H	$COOCH_3$	–	–	309.2 ± 26.1	370.6 ± 40.6	
9b	III	H	2,5-$C_6H_3Cl_2$	H	H	499.9 ± 35.9	>1000.0	
9c	III	H	$COOCH_3$	H	H	407.1 ± 27.8	463.4 ± 58.3	
9d	III	H	4-C_6H_4COOH	H	H	627.7 ± 32.1	940.0 ± 57.3	
9e	III	H	CH_2CH_2OH	H	H	42.1 ± 3.4	48.5 ± 9.7	
10e	III	H	CH_2CH_2OH	CH_3	CH_3	65.3 ± 4.7	23.5 ± 6.7	
11e	III	H	CH_2CH_2OH	Cl	H	28.0 ± 5.9	6.6 ± 0.7	7.9 ± 0.6
12	III	–	$=C(C_6H_5)_2$	H	H	101.9 ± 16.1	56.2 ± 7.8	54.8 ± 4.2
Adriamycin							0.04 ± 0.01	4.3 ± 0.6
Warfarin						74.8 ± 8.3	486.0 ± 50.2	

* IC_{50} concentration of a test compound required to reduce the fraction of survival cells to 50% of that observed in control, non-treated cells. Each data represents the mean from concentration-survival curves of at least three experiments ±SD, each performed at five repeats. Statistically significant difference between the HL-60 and HL-60 ADR cells as calculated by *t* test, $p < 0.001$

initiate apoptosis after a short incubation time. However, the increase in the level of cytosolic cytochrome *c* after 0.5 h exposure to **11e** was much more pronounced as compared with **8a**. It should be noted that both of the tested chromone derivatives were able to induce the apoptotic process in a time/concentration dependent manner.

Experimental

Chemistry

Melting points were determined using a Boëthius apparatus and they are not corrected. ^{1}H NMR spectra were recorded with a Varian Mercury 300 MHz spectrometer using DMSO-d_6 and D_2O as solvents. IR spectra were recorded using an ATI Mattson Infinity Series FTIR in KBr. Elemental analysis data were obtained with a Perkin Elmer 2400 analyzer.

Compound **1** used for the synthesis was obtained according to the described procedure (Nohara *et al.*, 1974). Compounds **2–4** and all hydrazine derivatives were purchased from Aldrich and Alfa Aesar as "synthesis grade" and used without further purification.

Synthesis of compounds **6**, **7a**, **8a**

Compounds **6**, **7a**, and **8a** were synthesized according to the method previously described by the authors (Nawrot-Modranka *et al.*, 2006), from 3-formylchromone or methyl chromone-3-carboxylate with phosphorus hydrazides.

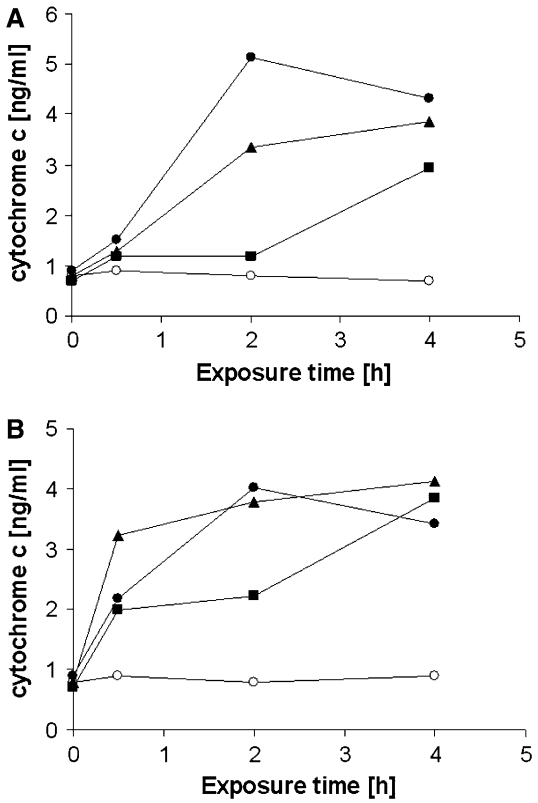


Fig. 1 Time course of cytosolic cytochrome *c* level in HL-60 leukemia cells exposed to **8a** (**a**) and **11e** (**b**). Cells were treated with tested compounds at the concentration of 0.2× IC_{50} (*filled square*), 1× IC_{50} (*filled triangle*), and 5× IC_{50} (*filled circle*). Control, non-treated cells (*open circle*). After 0.5, 2, and 4 h of incubation, the cells were lysed and quantified for cytochrome *c* level. Points on the curves are means of three determinations. SD values were excluded for clarity and did not exceed 15% of the mean value for each point

General procedure for the synthesis of 3-hydrazonomethylchromen-4-one derivatives **8b–c**

3-Formylchromone (0.6 mmol) was added at room temperature to a solution of 0.6 mmol hydrazine **5b** or **5c** in anhydrous ethanol (25 ml) and refluxed for 1 h. After cooling, the mixture was filtered, dried, and recrystallized from absolute ethanol.

3-[(2,5-Dichlorophenyl)hydrazonomethyl]chromen-4-one (**8b**)

Recrystallisation from ethanol; yellow solid. Yield: 90%, m.p. 214–215°C. ^{1}H NMR (DMSO-d_6) δ 6.82 (dd, $J = 2.6$ and 8.3 Hz, 1H, H-4, phenyl), 7.34 (d, $J = 8.3$ Hz, 1H, H-3, phenyl), 7.53 (t, $J = 7.9$ Hz, 1H, H-6, chromone ring), 7.61 (d, $J = 2.4$ Hz, 1H, H-6, phenyl), 7.72 (d, J = 8.5 Hz, 1H, H-8, chromone ring), 7.81–7.87 (m, 1H, H-7, chromone ring), 8.13 (dd, $J = 1.2$ and 7.9 Hz, 1H, H-5, chromone ring), 8.46 (s, 1H, H-2, chromone ring), 8.99 (s, 1H, CH), 10.29 (s, 1H, NH). IR (cm^{-1}, KBr): 3286 (NH), 1646 (C=O), 1581 (CH=N). Anal. calcd. for $C_{16}H_{10}N_2O_2Cl_2$ (333.18): C, 57.68; H, 3.03; N, 8.40. Found: C, 57.71; H, 2.56; N, 8.50%.

N′-[1-(4-oxo-chromen-4-yl)meth-(E)-ylidene]hydrazine carboxylic acid methyl ester (**8c**)

Recrystallisation from ethanol; yellow solid. Yield: 80%, m.p. 189–190°C. ^{1}H NMR (DMSO-d_6) δ: 3.68 (s, 3H, CH_3), 7.51–7.56 (m, 1H, H-6), 7.71 (d, $J = 8.5$ Hz, 1H, H-8), 7.82–7.87 (m, 1H, H-7), 8.11 (dd, $J = 1.2$ and 7.9 Hz, 1H, H-8), 8.17 (s, 1H, H-2), 8.71 (s, 1H, CH=N), 11.21 (bs, 1H, NH, disap. in D_2O). IR (cm^{-1}, KBr): 3229 (NH), 1632 (C=O), 1225(C–O). Anal. calcd. for $C_{12}H_{10}N_2O_4$ (246.22): C, 58.54; H, 4.09; N, 11.38. Found: C, 58.59; H, 3.75; N, 11.67%.

General procedure for the synthesis of 2-amino-3-hydrazonomethyl-chromen-4-one derivatives (**9b–d**)

An equimolar mixture of 2-amino-3-formylchromone (**2**) (1 mmol) and hydrazine **5b–d** was refluxed in 25 ml of anhydrous ethanol for 24 h. After cooling the solid was filtered and washed in cold alcohol to give TLC pure compounds.

2-Amino-3-[(2,5-dichlorophenyl)hydrazonomethyl]-chromen-4-one (**9b**)

Recrystallisation from ethanol; pale orange solid. Yield 80%, m.p. 284–285°C. ^{1}H NMR (DMSO-d_6) δ: 6.77 (dd, $J = 2.6$ and 8.3 Hz, 1H, H-4, phenyl), 7.14 (d, $J = 2.6$ Hz, 1H, H-6, phenyl), 7.34 (d, $J = 8.3$ Hz, 1H, H-3, phenyl), 7.39–7.46 (m, 2H, H-6, H-8, chromone ring), 7.66–7.71 (m, 1H, H-7, chromone ring), 8.02 (dd, $J = 1.6$ and 8.1 Hz, 1H, H-5, chromone ring), 8.87 (s, 1H, CH=N), 9.00 (bs, 2H, NH_2, disap. in D_2O), 9.92 (s, 1H, NH). IR (cm^{-1}, KBr): 3883 (NH_2), 1609 (CH=N), 1265 (C–O). Anal. calcd. for $C_{16}H_{11}N_3O_2Cl_2$ (348.19): C, 55.19; H, 3.18; N, 12.07. Found: C, 55.40; H, 2.73; N, 12.05%.

N′-[1-(2-Amino-4-oxo-chromen-3-yl)meth-(E)-ylidene]hydrazine carboxylic acid methyl ester (**9c**)

Recrystallisation from ethanol; pale orange solid. Yield 60%, m.p. 295°C dec. ^{1}H NMR (DMSO-d_6) δ: 3.67 (s, 3H, CH_3), 7.38–7.45 (m, 2H, H-6, H-8), 7.65–7.71 (m, 1H, H-7), 7.98 (dd, $J = 1.4$ and 8.1 Hz, 1H, H-5), 8.51 (s, 1H, CH), 9.13 (bs, 1H, NH_2, disap. in D_2O), 9.36 (bs, 1H, NH_2, disap. in D_2O), 11.05 (bs, 1H, NH, disap. in D_2O). IR (cm^{-1}, KBr): 3288, 1723 (CO, ester), 1608 (CH=N),

1221(C–O). Anal. calcd for $C_{12}H_{11}N_3O_4$ (261.24): C, 55.17; H, 4.24; N, 16.50. Found: C, 55.33; H, 3.78; N, 16.50%.

4-{*N*-[1-(2-Amino-4-oxo-chromen-3-yl)meth-(E)-ylidene]hydrazino}benzoic acid (**9d**)

Recrystallisation from ethanol; light yellow solid. Yield: 75%, m.p. 342–343°C. ^{1}H NMR (DMSO-d_6) δ: 6.90 (d, $J = 8.7$ Hz, 2H, H-2, H-3, phenyl), 7.39–7.46 (m, 2H, H-6, H-8, chromone ring), 7.67–7.72 (m, 1H, H-7, chromone ring), 7.79 (d, $J = 8.7$ Hz, 2H, H-5, H-6, phenyl), 8.00–8.03 (dd, $J = 1.7$ and 7.7 Hz, 1H, H-5, chromone ring), 8.53 (s, 1H, CH), 8.89–9.04 (bd, 2H, NH_2, disap. in D_2O), 10.62 (s, 1H, NH, disap. in D_2O), 12.22 (bs, 1H, COOH, disap. in D_2O). IR (cm^{-1}, KBr): 3254 (OH), 3140 (NH_2), 1665 (C=O), 1598 (CH=N), 1274 (C–O). Anal. calcd. for $C_{17}H_{13}N_3O_4$ (323.31): C, 63.16; H, 4.05; N, 13.00. Found: C, 63.03; H, 4.18; N, 12.66%.

2-Amino-3-[(2-hydroxyethyl)hydrazonomethyl]-chromen-4-one (**9e**)

2-Amino-3-formylchromone (**2**) (1.06 mmol) was added at room temperature to a solution of 3.51 mmol 2-hydroxyethylhydrazine (**5e**) in 30 ml of anhydrous EtOH and was refluxed for 2 h. The solution was evaporated, and the residue was treated with 20 ml of H_2O. The solid product was filtered off, washed in H_2O, dried, and crystallized from chloroform.

Light orange solid. Yield 75%, m.p. 166–168°C. ^{1}H NMR (DMSO-d_6) δ: 3.11 (q, $J = 6.0$ and 5.5 Hz, 2H, CH_2), 3.53 (q, $J = 5.5$ and 6.0 Hz, 2H, CH_2), 4.60 (t, $J = 5.5$ Hz, 1H, OH, disap. in D_2O), 6.68 (t, $J = 5.4$ Hz, 1H, NH, disap. in D_2O), 7.35–7.41 (m, 2H, H-6, H-8), 7.61–7.67 (m, 1H, H-7), 7.98 (dd, $J = 1.4$ and 7.7 Hz, 1H, H-5), 8.15 (s, 1H, CH), 8.69 (bs, 1H, NH_2, disap. in D_2O), 9.22 (bs, 1H, NH_2, disap. in D_2O). IR (cm^{-1}, KBr): 3271 (OH), 3115 (NH_2), 1642 (C=O), 1605 (CH=N), 1266 (C–O). Anal. calcd. for $C_{12}H_{13}N_3O_3$ (247.26): C, 58.29; H, 5.30; N, 16.99. Found: C, 58.30; H, 5.15; N, 16.75%.

2-Amino-6,7-dimethylo-3-[(2-hydroxyethyl)-hydrazonomethyl]chromen-4-one (**10e**)

2-Amino-6,7-dimethylchromone (**3**) (0.46 mmol) was added at room temperature to a solution of 0.46 mmol 2-hydroxyethylhydrazine (**5e**) in anhydrous toluene, and the reaction mixture was heated in an oil bath at 117°C for 24 h. Then toluene was removed in vacuum, and the residue was crystallized from acetone.

Dark orange solid. Yield of 35%, m.p. 209–210°C. ^{1}H NMR (DMSO-d_6) δ: 2.28 (s, 3H, CH_3), 2.32 (s, 3H, CH_3), 3.11(q, $J = 5.7$ and 5.5 Hz, 2H, CH_2), 3.53 (q, $J = 5.7$ and 6.0 Hz, 2H, CH_2), 4.59 (t, $J = 5.5$ and 5.4 Hz, OH, disap. in D_2O), 6.61 (t, $J = 5.4$ Hz, 1H, NH, disap. in D_2O), 7.18 (s, 1H, H-8), 7.71 (s, 1H, H-5), 8.15 (s, 1H, CH), 8.57 (bs, 1H, NH_2, disap. in D_2O), 9.14 (bs, 1H, NH_2, disap. in D_2O). IR (cm^{-1}, KBr): 3267 (NH), 3201 (OH), 3123 (NH_2), 1643 (C=O), 1607 (CH=N). Anal. calcd for $C_{14}H_{17}N_3O_3$ (275.29): C, 61.08; H, 6.22; N, 15.26. Found: C, 60.95; H, 6.04; N, 15.10%.

2-Amino-6-chloro-3-[(2-hydroxyethyl)-hydrazonomethyl]chromen-4-one (**11e**)

An equimolar mixture of 2-amino-6-chloro-3-formylchromone (**4**) (0.45 mmol) and 2-hydroxyethylhydrazine (**5e**) in 25 ml of toluene was refluxed at 181°C for 36 h. The mixture was cooled at −15°C, and the obtained solid was filtered off, washed in cold toluene, and crystallized from chloroform.

Yield 45%, m.p. 210–211°C. ^{1}H NMR (DMSO-d_6) δ: 3.12 (q, $J = 5.5$ and 5.3 Hz, 2H, CH_2), 3.54 (q, $J = 5.5$ and 5.7 Hz, 2H, CH_2), 4.60 (t, $J = 5.3$ Hz, 1H, OH, disap. in D_2O), 6.75 (bs, 1H, NH, disap. in D_2O), 7.46 (d, $J = 8.9$ Hz, 1H, H-8), 7.67 (dd, $J = 2.6$ Hz, 1H, H-7), 7.90 (d, $J = 2.6$ Hz, 1H, H-5), 8.12 (s, 1H, CH), 8.83 (bs, 1H, NH_2, disap. in D_2O), 9.30 (bs, 1H, NH_2, disap. in D_2O). IR (cm^{-1}, KBr): 3248(OH), 3071 (NH), 1647 (C=O), 1611 (CH=N), 1253 (C–O). Anal. calcd. for $C_{12}H_{12}N_3O_3Cl$ (281.70): C, 51.17; H, 4.29; N, 14.92. Found: C, 51.32; H, 4.11; N, 14.61%.

2-Amino-3-(benzylhydrylidenehydrazonomethyl)-chromen-4-one (**12**)

An equimolar mixture of 2-amino-3-formylchromone (**2**) (1 mmol) and hydrazine **5f** was refluxed in 30 ml of anhydrous ethanol for 18 h. The solid was filtered off and washed in cold acetone to give TLC pure compounds. Recrystallisation from acetone; small pale yellow solid.

Yield 55%, m.p. 264–265°C. ^{1}H NMR (DMSO-d_6) δ: 7.28–7.31 (m, 2H, phenyl), 7.36–7.54 (m, 6H, phenyl, 2H, chromone), 7.64–7.71 (m, 2H, phenyl and 1H, chromone ring), 7.99–8.02 (dd, $J = 1.4$ and 7.7 Hz, 1H, H-5, chromone ring), 9.07 (s, 1H, CH=N), 9.09 (bs, 1H, NH_2, disap. in D_2O), 9.13 (bs, 1H, NH_2, disap. in D_2O). IR (cm^{-1}, KBr): 3415 (NH_2), 1656 (C=O), 1611 (C=N), 1242 (C–O). Anal. calcd. for $C_{23}H_{17}N_3O_2$ (367.41): C, 75.19; H, 4.66; N, 11.44. Found: C, 75.20; H, 4.22; N, 11.59%.

Biological assays

Cell lines

Human acute lymphoblastic leukemia NALM-6 cells, acute promyelocytic leukemia HL-60 cells, and drug-resistant HL-60 ADR (adriamycin-resistant, MRP1-overexpressing) cells were used. The NALM-6 cell line was purchased from the German Collection of Microorganisms and Cell Cultures. The HL-60 cell line was obtained from the Institute of Immunology and Experimental Therapy (Wrocław, Poland), and the adriamycin-resistant subline HL-60 ADR was kindly provided by Prof. G. Bartosz, University of Łódź, (Poland). The cells were cultured in RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and antibiotic (25 μg/ml of gentamycin) at 37°C in 5% CO_2/95% air atmosphere.

Cytotoxicity assay by MTT

The cytotoxicity of the synthesized hydrazones, warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin), and adriamycin was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide, Sigma, St. Louis, USA] assay as described in (Hansen *et al.*, 1989). Briefly, after 46 h incubation with drugs (in concentrations from 10^{-8} to 10^{-3} M), the cells were treated with the MTT reagent, and incubation was continued for 2 h. MTT-formazan crystals were dissolved in 20% SDS and 50% DMF at pH 4.7, and absorbance was read at 562 nm on an ELISA-plate reader (ELX 800, Bio-Tek, USA). The values of IC_{50} (the concentration of the tested compound required to reduce the cell survival fraction to 50% of the control) were calculated from log-transformed concentration-survival curves by linear regression of the slopes and used as a measure of cellular sensitivity to a given treatment. As a control, cultured cells were grown in the absence of drugs. Data points represent means of 3–4 experiments, each performed at five repeats ±SD.

Determining cytosolic cytochrome c levels

HL-60 cells treated with **8a** or **11e** (in three concentrations: $0.2 \times IC_{50}$, $1 \times IC_{50}$ and $5 \times IC_{50}$) were used for the determination of cytosolic cytochrome *c* levels by enzyme-linked immunosorbent assay (Diaclone, Besancon, France). This is a tool for detecting the first early steps in initiating apoptosis in the cells through measurement of cytochrome *c* translocation from the mitochondria to cytosol. After 0.5, 2, and 4 h of incubation with the tested compound, the cells were spun down and washed twice with washing buffer. The detection of cytochrome *c* release from the mitochondria to cytosol was achieved by selective lysis of cell membrane, and the assay was performed according to the manufacturer's protocol. Briefly, 100-fold diluted cellular lysates were added to the microwells coated with anti-cytochrome *c* monoclonal antibody. Then, a biotin-conjugated monoclonal anti-cytochrome *c* antibody was added and bound to cytochrome *c* captured by the first antibody. In the detection step, streptavidin-HRP and a substrate reactive with HRP were used. Absorbance was read at $\lambda = 450$ nm on an ELISA-plate reader (ELX 800, Bio-Tek, USA). Simultaneously, a standard curve was prepared to evaluate cytochrome *c* sample concentration.

Conclusion

The nine new hydrazones of chromones (**8b**–**12**) were obtained as a result of condensation reactions of chromones with the selected hydrazines. All described compounds were studied for in vitro anticancer activity on NALM-6, HL-60 cell lines and few of them (**8a**, **11e**, **12**) was also studied on HL-60 adriamycin-resistant cells (ADR). Two of new synthesized derivatives **8a** and **11e** exhibit relatively high antileukemic in vitro activity. Tested compounds are able to induce apoptosis process in exposed cells in time/concentration-dependent manner. They are also poor substrates for MRP1 efflux membrane pump what suggests that they might be useful for treating drug-resistant tumors.

From our results, we can conclude that better biological activity is displayed by the compounds with aliphatic substituents in the hydrazone moiety. Additionally, the presence of Cl in chromone system at position 6, considerably improve the cytotoxic activity of the studied aliphatic compound **11e**. Our results encourage us to continue the research of biological activity of the proper hydrazone derivatives. That gives us the possibility to investigate structure–activity dependences by QSAR method.

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