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Antiproliferative and antioxidative effects of novel hydrazone derivatives bearing coumarin and chromene moiety

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Abstract A series of new hybrid molecules with a hydrazone fragment were synthesized and characterized by Fourier transform-infrared spectroscopy, nuclear magnetic resonance, mass spectrometry, and elemental analysis. The nuclear magnetic resonance spectra of the hydrazones 4a-c showed exchange of syn and antiperiplanar conformers around the amide bond, the more stable being the antiperiplanar one. The nuclear Overhauser effect spectroscopy (NOESY) spectra confirm E configuration around C=N bond. The tested compounds exhibited concentrationdependent cytotoxic effects against human tumor cell lines in a micromolar range (MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay). Hydrazone 4a proved to be the most potent antiproliferative agent of the series. Within the antioxidant screening 8b exhibited the highest radical scavenging activity (% RSA max) and the largest rate constant for the reaction with 2,2-diphenyl-1-picrylhydrazyl.

Keywords Antioxidant activity · Antiproliferative agents · 2*H*-Chromene · Coumarins · Hydrazides/Hydrazones · MTT-assay

Introduction

Pharmacological activities of hydrazones are related to antimicrobial (Kaplancıklı et al., 2014), antimycobacterial (Ellis et al., 2014), antidepressant (de Oliveira et al., 2011), anticonvulsant (Jain et al., 2011), analgesic/anti-inflammatory (Sondhi et al., 2006), antimalarial (Thuy et al., 2012), antineoplastic (Lovejoy and Richardson, 2003), and antioxidant (El-Tombary and El-Hawash, 2014) effects, and have been summarized in several review articles (Rollas and Küçükgüzel, 2007; Wang et al., 2011; Asif, 2014; Taha et al., 2013). Many studies have been exploring the antiproliferative activity of hybrid molecules with a hydrazone moiety [comprised of an azomethine (-NH-N=CH-) and acylhydrazones or arylhydrazones (-CON-HN=CH-) groups], presuming that hydrazone structure is probably accountable for the antineoplastic effects (Mohareb et al., 2010; Mohareb et al., 2011; Nikolova-Mladenova et al., 2011; Abdel-Aziz et al., 2012; Congiu and Onnis, 2013; Abdel-Aziz et al., 2013; Nasr et al., 2014; Jashari et al., 2014; Junjie Ma et al., 2014). Some of those compounds are potential metal chelating agents that may interfere with the redox metabolism (Edward et al., 1988; Hermes-Lima et al., 1998; Becker et al., 2003). Also, they prevent cancerous cell progression through different mechanisms: inhibition of kinases, generation of radicals, and dissipation of the mitochondrial membrane potential (Chaston et al., 2003; Chaston and Richardson, 2003; Yun Fu et al., 2014). Furthermore, some in vitro studies and clinical trials have demonstrated that some tumors, particularly neuroblastoma and leukemia, have susceptibility to iron (Fe) chelators greater than normal cells (Gupte and Mumper, 2009). The disparities in the generation and metabolism of reactive oxygen species in cancer cells vs. normal cells is the rationale for developing new, more selective anticancer agents.

Moreover, benzopyrane and benzopyrone-based compounds, such as coumarin, chromone, and flavone, have attracted the attention of many scientists as potential inhibitors of cellular proliferation in various carcinoma cell lines: malignant melanoma, leukemia, renal cell carcinoma, prostate, and breast cancer cell lines (Lacy and O'Kennedy, 2004; Jain and Joshi, 2012; Kontogiorgis et al., 2012; Rahmani-Nezhad et al., 2014). Besides, a comprehensive study (Bubols et al., 2013) reveals that coumarins exhibit a considerable antioxidant activity against free radicals what can be associated with their anticancer activity. Targeting for novel hydrazide/hydrazone hybrids with anticancer effect, we report herein the synthesis, in vitro antiproliferative, and antioxidant activity of a series of coumarin and 2*H*-chromene substituted hydrazones. Cytotoxic activity was tested against four human cancer cell lines, while antioxidant potency was examined by measuring their 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacities.

Material and methods

General

The commercial products used for the synthesis of the compounds were: 4-hydroxycoumarin, benzhydrazide, 4-hydroxy benzhydrazide, isonicotinovl hydrazide, and 96 % ethanol. All other chemicals were of analytical grade. The Fourier transform-infrared spectroscopy (FTIR) spectra were recorded on a Nicolet IS10 FT-IR Spectrometer from Thermo Scientific (USA) using ATR technique. All nuclear magnetic resonance (NMR) experiments were carried out on a Bruker Avance spectrometer II+600 MHz at 20 °C in dimethyl sulfoxide (DMSO)- d_6 as a solvent, using tetramethylsilane as an internal standard. The precise assignment of the ¹H and ¹³C NMR spectra was accomplished by measurement of 2D NMR correlation spectroscopy (COSY), DEPT-135, and 2D inverse detected heteronuclear (C-H) correlations heteronuclear singlequantum correlation spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC). Mass spectra were measured on a Bruker MAXIS IMPACT LC/MS equipped with an ESI (electrospray ionization) source. Elemental analyses were performed by Euro EA 3000 – Single, EuroVector SpA. The melting points were determined using a Buchi 535 apparatus. The purity of the new compounds was checked by thin-layer chromatography (TLC) on silica gel 60 GF254 Merck pre-coated aluminum sheets, eluted by hexane-chloroform-acetone-methanol 4:3:2:1 (vol. parts); the spots were visualized under ultraviolet irradiation ($\lambda = 254$ nm).

Synthesis

4-chlorocoumarin-3-carbaldehyde 2

The compound 4-chlorocoumarin-3-carbaldehyde **2** was prepared by Vilsmeier-Haack formylation of 4-hydroxycoumarin **1** (Heber et al., 1995) (Scheme 1). Yield 84 %, m. p. 120–122 °C (lit. m.p. 120–122 °C). The structure of the compound was proved by FTIR, ¹H NMR, ¹³C NMR (Sabatié et al., 2001).



Scheme 1 Synthetic route to the target compounds 4a-c, 8a-c, and 9a-c

General procedure for the synthesis of compounds 4a-cexemplified by the preparation of N'-[(E)-(4-chloro-2-oxo-2H-chromen-3-yl)methylidene] benzohydrazide 4a

An equimolecular amount of the appropriate hydrazide 3a (0.681 g, 5.0 mmol) in 5 mL of abs. Ethyl alcohol (EtOH) was added at room temperature to a solution of 4chlorocoumarin-3-carbaldehyde 2 (1.042 g, 5.0 mmol) in 5 mL of abs. EtOH under vigorous stirring. After 15 min the resulting precipitate was filtered, recrystallized from ethanol, and air-dried to give vellow crystals of compound 6a. Yellow solid, yield 86 %, 1.255 g; m.p. 197-199 °C; FTIR (ATR) ν_{max} 3295, 1718, 1663, 1620, 1599 cm⁻¹; ¹H NMR (DMSO-d₆, 600 MHz): 1:0.137 mixture of conformers; signals for major antiperiplanar conformer about the amide bond: $\delta = 7.52$ (2H, d, J = 6.6 Hz, H-8 + H-6), 7.55 (2H, t, J = 7.3 Hz, H-3' and H-5'), 7.62 (1H, d, J = 7.0 Hz, H-4'), 7.76 (1H, t, J = 7.5 Hz, H-7), 7.94 (1H, d, J = 7.4 Hz, H-2' and H-6'), 8.04 (1H, d, J = 7.7 Hz, H-5), 8.64 (1H, s, CH), 12.16 (1H, s, NH); 13 C-NMR (DMSO- d_6 , 150 MHz): δ (ppm) 116.7 (C-8), 118.4 (C-4a), 119.5 (C-3), 125.4 (C-6), 126.2 (C-5), 127.8 (C-2' and C-6'), 128.6 (C-3' and C-5'), 132.2 (C-4'), 133.1 (C-1'), 133.8 (C-7), 141.6 (CH), 145.0 (C-4), 151.4 (C-8a), 157.8 (C-2), 163.2 (C=O). ESI-MS m/z: 327.0530 [M+H]⁺, 349.0353 [M+Na]⁺; Anal. calcd. for C₁₇H₁₁ClN₂O₃: C, 62.49; H, 3.39; N 8.57. Found: C, 62.88; H, 3.34; N, 8.96.

N'-[(E)-(4-chloro-2-oxo-2H-chromen-3-yl)methylidene]-4hydroxybenzohydrazide **4b**

The reaction time was 30 min. Yellow solid, yield: 78 %, 1.335 g; m.p. 213–215 °C; FTIR (ATR) ν_{max} : 3265, 3126, 1705, 1659, 1624, 1596 cm⁻¹; ¹H NMR (DMSO-*d*₆, 600 MHz): 1:0.17 mixture of conformers; signals for major antiperiplanar conformer about the amide bond $\delta = 6.91$ (2H, d, J = 8.6 Hz, H-3' and H-5'), 7.52 (1H, t, J = 7.2 Hz,H-6), 7.52 (1H, d, J = 7.9 Hz, H-8), 7.77 (1H, t, J = 8.0 Hz, H-7), 7.81 (2H, d, J = 8.5 Hz, H-2' and H-6'), 8.04 (1H, d, J = 7.8 Hz, H-5), 8.58 (1H, s, CH), 10.37 (1H, s, OH), 12.41 (1H, s, NH); resolved signals for minor synperiplanar conformer: $\delta = 12.19$ (1H, s, NH); ¹³C-NMR (DMSO- d_6 , 150 MHz,): $\delta = 115.2$ (C-3' and C-5'), 116.6 (C-8), 118.4 (C-4a), 119.7 (C-3), 123.2 (C-1'), 125.4 (C-6), 126.2 (C-5), 129.8 (C-2' and C-6'), 133.8 (C-7), 140.9 (CH), 144.7 (C-4), 151.4 (C-8a), 157.7 (C-2), 161.0 (C-4'), 162.6 (C=O); ESI-MS *m/z*: 343.7340 [M+H]⁺, *m/z* 365.7333 [M+Na]⁺. Anal. calcd. for C₁₇H₁₁ClN₂O₄: C 59.57; H 3.23; N 8.17 %. Found: C 59.89; H 3.33; N 8.45.

N'-[(E)-(4-chloro-2-oxo-2H-chromen-3-yl)methylidene] pyridine-4-carbohydrazide **4c**

Yellow solid, yield 88 %, 1.44 g; m.p. 203–204 °C; FTIR (ATR) $\nu_{\rm max}$ 3272, 1706, 1666, 1624, 1596 cm⁻¹; ¹H NMR

(DMSO-d₆, 600 MHz): 1:0.31 mixture of conformers; signals for major antiperiplanar conformer about the amide bond: $\delta = 7.52$ (1H, t, J = 7.2 Hz, H-6), 7.52 (1H, d, J = 7.9Hz, H-8), 7.77 (1H, t, J = 8.0 Hz, H-7), 7.87 (2H, d, J = 5.4Hz, H-2' and H-6'), 8.04 (1H, d, J = 7.8 Hz, H-5), 8.660 (1H, s, CH), 8.81 (2H, d, J = 5.4 Hz, H-3' and H-5'), 12.37 (1H, s, NH); resolved signals for minor synperiplanar conformer: $\delta = 7.73$ (2H, d, J = 5.3 Hz, H-2' and H-6'), 7.96 (1H, d, J=7.9 Hz, H-5), 8.29 (1H, s, CH), 8.72 (2H, d, J = 5.3 Hz, H-3' and H-5'), 12.32 (1H, s, NH); ¹³C-NMR (DMSO- d_6 , 150 MHz): $\delta = 116.6$ (C-8), 118.3 (C-4a), 119.2 (C-3), 121.7 (C-2' and C-6'), 125.4 (C-6), 126.2 (C-5), 133.8 (C-7), 140.2 (C-1'), 142.9 (CH), 150.3 (C-3' and C-5'), 151.4 (C-8a), 157.7 (C-2), 161.6 (C=O); ESI-MS *m/z*: 328.0471 [M+H]⁺, 350.0290 [M+Na]⁺; Anal. calcd. for C₁₆H₁₀ClN₃O₃: C 58.64; H 3.08; N 12.82. Found: C 58.94; H 2.94; N 12.69.

2-Methyl-2H-chromene-3-carbaldehyde 7

To a mixture of 2-hydroxybenzaldehyde 5 (0.85 g, 0.8 mL, 7 mmol) and potassium carbonate (0.97 g, 7 mmol) in 1,4dioxane (12.5 mL) crotonaldehyde 6 (0.5 mL) was added dropwise under vigorous stirring. The mixture was heated at 100 °C for 48 h (monitored by TLC) and allowed to cool. The reaction was quenched with water and extracted several times with ether (Suslov et al., 2009; Azizmohammadi et al., 2013). The combined ether extracts were dried (Na₂SO₄) and evaporated to give raw compound as a yellow oil. The product was purified by silica gel column chromatography (hexane-EtOAc mixture 10:1) to give 2-methyl-2H-chromene-3-carbaldehyde 7. Oil, 0.7 g, yield 57 %; FTIR (ATR) ν_{max} 2975, 2809, 1664, 1627 cm⁻¹; ¹H NMR (DMSO- d_6 ; 600 MHz,): $\delta = 1.22$ (3H, d, J = 6.5 Hz, CH₃), 5.28 (1H, q, J = 6.5 Hz, H-2), 6.88 (1H, d, J = 8.2Hz, H-9), 6.99 (1H, dt, J = 1.1, 7.5 Hz, H-7), 7.34 (1H, ddd, J = 1.7, 8.2, 7.4 Hz, H-8), 7.38 (1H, dd, J = 1.6, 7.5 Hz, H-6), 7.57 (1H, s, H-4), 9.53 (1H, s, CHO); ¹³C-NMR $(DMSO-d_6, 150 \text{ MH}): \delta = 19.8 (CH_3), 69.1 (C-2), 117.0 (C-2)$ 9), 120.0 (C-5), 121.9 (C-7), 129.8 (C-6), 133.6 (C-8), 135.7 (C-3), 140.3 (C-4), 153.7 (C-10), 191.0 (CHO); ESI-MS *m/z*: 174.0676 [M⁺] (100).

General synthetic procedure for compounds 8a-c

An equimolecular amount of the appropriate hydrazide $3\mathbf{a-c}$ (2.0 mmol) in 10 mL abs. EtOH was added to a solution of 2-methyl-2*H*-chromene-3-carbaldehyde 7 (2.0 mmol) in 10 mL abs. EtOH under vigorous stirring at 25 °C for 30 min. The reaction mixture turned into yellow solution. After 2–8 h the clusters of yellow crystalline compounds **8a–c** deposited from the solution. They were

filtered, washed with aqueous ethanol $(2 \times 10 \text{ mL})$, and recrystallized from EtOH to give TLC pure crystals.

N'-[(E)-(2-methyl-2H-chromen-3-yl)methylidene] benzohydrazide **8a**

Yellow solid, yield: 88 %, 0.514 g; m.p. 120-121 °C; FTIR (ATR) ν_{max} 3341, 1651, 1629, 1601 cm⁻¹; ¹H NMR (DMSO- d_6 , 600 MHz): $\delta = 1.33$ (3H, d, J = 6.5 Hz, CH₃), 5.48 (1H, q, J = 6.5 Hz, H-2), 6.87 (1H, d, J = 8.0 Hz, H-8), 6.93 (1H, s, H-4), 6.94 (1H, t, J = 7.4 Hz, H-6), 7.22 (1H, t, J = 7.5 Hz, H-7), 7.24 (1H, d, J = 7.6 Hz, H-5), 7.53 (2H, t, J = 7.5 Hz, H-3' and H-5'), 7.60 (1H, t, J = 7.3 Hz, H-4'), 7.88 (2H, d, J = 7.4 Hz, H-2' and H-6'), 8.15 (1H, s, CH), 11.81 (1H, s, NH); ¹³C-NMR (DMSO-*d*₆, 150 MHz): $\delta = 19.3$ (CH₃), 69.7 (C-2), 116.5 (C-8), 121.4 (C-4a), 121.5 (C-6), 127.6 (C-4, C-5, C-2' and C-6'), 128.5 (C-3' and C-5'), 130.7 (C-7), 131.8 (C-4'), 133.4 (C-1'), 133.5 (C-3), 146.5 (CH), 152.2 (C-8a), 163.1 (C=O); ESI-MS *m/z*: 293.1273 [M+H]⁺; Anal. calcd. for C₁₈ H₁₆N₂O₂: C 73.95; H 5.52; N 9.58. Found: C 73.91; H 5.53; N 9.59.

4-hydroxy-N'-[(E)-(2-methyl-2H-chromen-3-yl) methylidene]benzohydrazide **8b**

Yellow solid, yield 86 %, 0.530 g; m.p. 257-258 °C; FTIR (ATR) ν_{max} 3508 3379, 1659, 1625, 1604 cm⁻¹; ¹H NMR (DMSO- d_6 , 600 MHz): $\delta = 1.31$ (3H, d, J = 6.4 Hz, CH₃), 5.46 (1H, q, J = 6.4 Hz, H-2), 6.85 (2H, d, J = 8.5Hz, H-3' and H-5'), 6.86 (1H, d, J = 8.3 Hz, H-8), 6.88 (1H, s, H-4), 6.93 (1H, dt, J=0.9, 7.4 Hz, H-6), 7.20 (1H, dt, J = 1.6, 7.7 Hz, H-7), 7.23 (1H, dd, J = 1.5, 7.5 Hz, H-5), 7.77 (2H, d, J = 8.5 Hz, H-2' and H-6'), 8.12 (1H, s, CH), 10.16 (1H, s, OH), 11.60 (1H, s, NH); ¹³C-NMR (DMSO- d_6 , 150 MHz,): $\delta = 19.3$ (CH₃), 69.7 (C-2), 115.1 (C-3' and C-5'), 116.5 (C-8), 121.5 (C-6), 121.5 (C-4a), 123.8 (C-1'), 127.0 (C-4), 127.6 (C-5), 129.7 (C-2' and C-6'), 130.6 (C-7), 133.7 (C-3), 145.6 (CH), 152.2 (C-8a), 160.8 (C-4'), 162.7 (C=O). ESI-MS m/z: 309.28 $[M+H]^+$, 331.20 $[M+Na]^+$; Anal. calcd. for $C_{18}H_{16}N_2O_3$: C 70.12, H 5.23, N 9.09. Found: C 70.14, H, 5.28, N 9.10.

N'-[(E)-(2-methyl-2H-chromen-3-yl)methylidene]pyridine-4-carbohydrazide **8c**

Yellow solid, yield 92 %, 0.539 g; m.p. 122–123 °C, FTIR (ATR) ν_{max} 3418, 1660, 1626, 1605 cm⁻¹; ¹H NMR (DMSO-*d*₆, 600 MHz): δ = 1.33 (3H, d, *J* = 6.5 Hz, CH₃), 5.47 (1H, q, *J* = 6.5 Hz, H-2), 6.88 (1H, d, *J* = 8.0 Hz, H-8), 6.94 (1H, t, *J* = 7.4 Hz, H-6), 7.23 (1H, t, *J* = 7.6 Hz, H-7), 7.25 (1H, d, *J* = 7.6 Hz, H-5), 6.98 (1H, s, H-4), 7.79 (2H, d, J = 6.0 Hz, H-2' and H-6'), 8.15 (1H, s, CH), 8.78 (2H, d, J = 6.0 Hz, H-3' and H-5'), 12.03 (1H, s, NH); ¹³C-NMR (DMSO- d_6 , 150 MHz): $\delta = 19.3$ (CH₃), 69.7 (C-2), 116.5 (C-8), 121.4 (C-4a), 121.6 (C-6), 121.6 (C-2' and C-6'), 127.8 (C-5), 128.5 (C-4), 131.0 (C-7), 133.2 (C-3), 140.5 (C-1'), 147.7 (CH), 150.4 (C-3' and C-5'), 152.3 (C-8a), 161.62 (C=O); ESI-MS m/z: 294.122 [M+H]⁺; Anal. calcd. for C₁₇H₁₅N₃O₂: C 69.61; H 5.15; N 14.33. Found: C 69.59; H 5.18; N 14.29.

General synthetic procedure for compounds 9a-c

An equimolecular amount of the appropriate hydrazide **3a–c** (2.0 mmol) in 20 mL abs. EtOH was added to a solution of 2-hydroxybenzaldehyde **5** (2.0 mmol) in 10 mL abs. EtOH under vigorous stirring at 25 °C for 30 min. The reaction mixture turned into an yellow solution. After 1–4 h the clusters of yellow crystalline compounds **9a–c** deposited from the solution. Upon filtration the crystals were washed with aqueous ethanol (2 × 10 mL) and recrystallized from ethanol give TLC pure crystals. The structure of the compounds was proved by FTIR, ¹H NMR, ¹³C NMR, elemental analyses.

N'-[(E)-(2-hydroxyphenyl)methylidene]benzohydrazide 9a

Yellow crystals, yield 91 %, m.p. 164–165 °C (lit. m.p. 163–164 °C) (Edward et al., 1988). ¹H NMR (DMSO- $d_{6,}$ 600 MHz): $\delta = 6.93$ (1H, t, J = 7.4 Hz, H-5), 6.94 (1H, d, J = 8.2 Hz, H-3), 7.31 (1H, ddd, J = 1.5, 7.0, 8.4 Hz, H-4), 7.55 (2H, t, J = 7.4 Hz, H-3' and H-5'), 7.56 (1H, d, J = 7.6 Hz, H-6), 7.62 (1H, t, J = 7.4 Hz, H-4'), 7.94 (2H, d, J = 7.3 Hz, H-2' and H-6'), 8.65 (1H, s, CH), 11.30 (1H, s, OH), 12.13 (1H, s, NH); ¹³C NMR (DMSO- $d_{6,}$ 151 MHz): $\delta = 116.5$ (C-3), 118.7 (C-1), 119.4 (C-5), 127.7 (C-2' and C-6'), 128.6 (C-3' and C-5'), 129.6 (C-6), 131.5 (C-4), 132.1 (C-4'), 132.8 (C-1'), 148.3 (CH), 157.5 (C-2), 162.9 (C=O).

4-Hydroxy-N'-[(E)-(2-hydroxyphenyl)methylidene] benzohydrazide **9b**

Yellow crystals, yield 88 %, m.p. 257–258 °C (lit. m.p. 255–256 °C) (Edward et al., 1988). ¹H NMR (600 MHz, DMSO- d_6): $\delta = 6.88$ (2H, d, J = 8.6 Hz, H-3' and H-5'), 6.92 (1H, t, J = 7.6 Hz, H-5), 6.93 (1H, d, J = 7.8 Hz, H-3), 7.29 (1H, t, J = 7.2 Hz, H-4), 7.51 (1H, dd, J = 1.0, 7.6 Hz, H-6), 7.82 (2H, d, J = 8.5 Hz, H-2' and H-6'), 8.59 (1H, s, CH), 10.20 (1H, s, OH), 11.42 (1H, s, OH), 11.93 (1H, s, NH); ¹³C NMR (151 MHz, DMSO- d_6): $\delta = 115.2$ (C-3' and C-5'), 116.4 (C-3), 118.7 (C-1), 119.3 (C-5), 123.2 (C-1'), 129.6 (C-6), 129.8 (C-2' and C-6'), 131.2 (C-4), 147.6 (CH), 157.5 (C-2), 161.0 (C-4'), 162.4 (C=O).

N'-[(E)-(2-hydroxyphenyl)methylidene]pyridine-4-carbohydrazide **9***c*

Yellow crystals, yield 85 %, m.p. 238–239 °C (lit. m.p. 238 °C) (Thorat et al., 2011). ¹H NMR (600 MHz, DMSO- d_6): $\delta = 6.93$ (1H, t, J = 7.4 Hz, H-5), 6.95 (1H, d, J = 8.2 Hz, H-3), 7.32 (1H, ddd, J = 1.6, 7.2, 8.2 Hz, H-4), 7.61 (1H, dd, J = 1.6, 7.7 Hz, H-6), 7.85 (2H, d, J = 6.1 Hz, H-2' and H-6'), 8.68 (1H, s, CH), 8.80 (2H, d, J = 6.1 Hz, H-3' and H-5'), 11.08 (1H, s, OH), 12.30 (1H, s, NH); ¹³C NMR (151 MHz, DMSO- d_6): $\delta = 116.5$ (C-3), 118.8 (C-1), 119.5 (C-5), 121.6 (C-2' and C-6'), 129.2 (C-6), 131.8 (C-4), 140.0 (C-1'), 148.9 (CH), 150.4 (C-3' and C-5'), 157.5 (C-2), 161.4 (C=O).

Biological evaluation

Cell lines and culture conditions

The study was carried out on the following human tumor cell lines: HL-60-acute myeloid leukemia; SKW-3-Tcell leukemia, a KE-37 derivative; K-562-chronic myeloid leukemia; MDA-MB-231—estrogen receptornegative breast adenocarcinoma. The cell lines were obtained from the DSMZ GmbH (Braunschweig, Germany). They were cultured under standard conditions-RPMI-1640 medium supplemented with 10 % fetal bovine serum and 2 mM L-glutamine, in cell culture flasks, housed at 37 °C in an incubator "BB 16-Function Line" Heraeus (Kendro, Germany) with humidified atmosphere and 5 % CO₂. The cell cultures were maintained in log phase by supplementation with fresh medium two or three times weekly.

Cytotoxicity assessment (MTT-dye reduction assay)

After exposure to the tested compounds, the cell viability was assessed by using the MTT-dye reduction assay, based on the biotransformation of the yellow dye 3-(4,5-dime-thylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide into a violet formazan product via the mitochondrial succinate dehydrogenase of viable cells. The procedure and its modifications was already described (Mosmann, 1983; Konstantinov et al., 1999).

DPPH radical scavenging activity

The DPPH radical scavenging capacity assay was assayed spectrophotometrically according to Brand-Williams et al. (1995), with modifications made by Gaspar et al. (2010). In brief, 0.1 mL of 1 mM solution of the compounds in DMSO was added to 3.9 mL of $100 \,\mu\text{M}$ of DPPH radical

(Sigma-Aldrich, Germany) solution in methanol. Upon, the reaction mixture was incubated in the dark, at room temperature for 30 min the absorbance was measured at 517 nm. The impact of DMSO and the scavenging capacity of 1 mM vitamin C (J.T. Baker, The Netherlands) were also tested. Tests were carried out in triplicate. The results were expressed as a percentage of the radical scavenging capacity, calculated using the following formula:

$$\operatorname{RSC} \% = (A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$$
(1)

where A_{control} : absorption of blank sample; A_{sample} : absorption of tested solution. Methanol served as a control.

Reducing power assay

The reducing power was assessed according to the spectrophotometric method of Oyaizu (1986). Briefly, 1 mL of the sample (DMSO, 1 mM solutions of compounds, and vitamin C) was mixed with 2.5 mL of a 0.2 mM phosphate buffer (pH = 6.6) and 2.5 mL of a 1 % (w/v) K₃[Fe(CN)₆]. The reaction mixture was incubated at 50 °C for 20 min. Afterwards 2.5 mL of 10 % (w/v) trichloroacetic acid were added and the mixture was centrifuged at 1750 × g for 10 min. Then 2.5 mL of the upper layer were added to 2.5 mL of distilled water and 0.5 mL of 0.1 % (w/v) FeCl₃ × 6H₂O. The absorbance was measured at 700 nm. Distilled water was used as a blank, instead of the tested samples. Higher absorbance values indicated increased reducing power (reducing capability).

Statistical analysis of antioxidant assays and antiproliferative activity

The cell survival data were fitted to sigmoidal dose response curves and the corresponding IC₅₀ values were calculated using nonlinear regression analysis (GraphPad Prizm Software for PC). The statistical processing of MTT data included the Student's *t*-test with significance level of $p \leq 0.05$.

Statistical analysis of the antioxidant assays was performed using Excel 2010. All experiments were performed in triplicate and the results were expressed as a mean \pm standard deviation. The correlation between DPPH and reducing power assay, as well as the correlation of antioxidant and antiproliferative activity, were determined by calculating the Pearson's *r* and *p*-value. With alpha (α) value of 0.05, $p \le 0.05$ was considered statistically significant. Linear and multiple regressions were performed.

Results and discussion

Synthesis

The substituted chromene-based hydrazones **4a–c** and **8a–c** were synthesized via a two-step sequence starting from compounds **1** and **5**, respectively (Scheme 1). At the first step the refluxing of 4-hydroxycoumarin **1** with freshly distilled phosphoryl chloride (POCl₃) in the presence of dimethylformamide yielded the corresponding 2*H*-chromene-3-carbaldehyde **2**, which was used without further purification (Heber et al., 1995).

Domino oxa-Michael addition/aldol condensation reaction was selected as the base module in the preparation of a second key starting material 2-methyl-2H-chromen-3-carbaldehyde 7. Condensations between Michael acceptors and 2-hydroxybenzaldehydes proved to be a versatile route to benzopyrans (Yong-Ling and Min, 2007; Masesane and Yibralign, 2012). As outlined in Scheme 1, the reaction of 2-hydroxybenzaldehyde 5 and crotonaldehyde 6 in the presence of potassium carbonate afforded 2-methyl-2Hchromene-3-carbaldehyde 7. According to the reaction mechanism (Masesane and Yibralign, 2012) a benzopyran moiety was formed by a tandem oxa-Michael addition, intramolecular aldol reaction, and dehydration pathway. The reaction was a simple route to racemic carbonyl compounds containing a benzopyran moiety and was used for the first time to synthesize compound 7.

The key step in the synthesis of target compounds 4chlorocoumarin benzoylhydrazones 4a-c and 2-methyl-2Hchromen benzoylhydrazones 8a-c involved the condensation reaction of an equimolecular amount of 4-chloro-2oxo-2H-chromene-3-carbaldehyde 2 or 2-methyl-2H-cromene-3-carbaldehyde 7 with an appropriate hydrazidesbenzohydrazide 3a or 4-hydroxy-benzohydrazide 3b, or pyridine-4-carbohydrazide 3c. The reactions were carried out at room temperature for different reaction times (15 min to 8 h) and their progress was monitored by TLC. The products were obtained in good yields and excellent purity. Hydrazones 9a-c, synthesized according to a similar method (see Experimental part), were used to estimate the contribution and the efficiency of coumarin and 2H-chromene rings to the antioxidant activity of tested compounds 4a-c and 8a-c. All final compounds were well proved by elemental analysis and by FTIR, ¹H NMR, ¹³C NMR, and mass spectroscopy. The ions observed by mass spectrometry were quasi-molecular ions created by the addition of a hydrogen cation [M+H]⁺ and a sodium ion [M+Na]⁺. In the present work, the structural elucidation was mainly focused on the analysis of NMR spectra. In DMSO- d_6 , new hydrazones may exist as C=N double bond stereoisomers (E/Z) and as syn/antiperiplanar conformers around the amide CO-NH bond (Scheme 2).

Scheme 2 (*E/Z*) C=N double bond stereoisomers and *syn/ antiperiplanar* conformers around the amide CO–NH bond of **4a–c** in DMSO-*d*₆



The two sets of resonances observed in the ¹H NMR spectra of compounds 4a-c in DMSO- d_6 proved the hindered rotation in the CO-NH group. The separation of signals of methylidene protons and NH protons in the two isomers is well resolved. The population ratio of the major and the minor isomer increases in the order: 4a (1:0.137) > 4b (1:0.17) > 4c (1:0.31). According to the available literature data (Lopes et al., 2013; Abdel-Aziz et al., 2013) the most stable isomers are E around C=N double bond and the antiperiplanar one around the amide CO-NH bond. That is in agreement with the observed NOE between the methylidene protons and the NH protons in both isomers. On the other hand, there are exchange signals in NOESY spectra between each pair of signals of both isomers. The studied compounds are not stable upon heating, hence the study of the exchange process in a wide temperature range has been impossible. Nevertheless, we calculated the rate constants from the exchange signals in NOESY spectra at 20, 25, 30, and 35 °C and estimated the rotational barriers in compound 4c (in Fig. ESM1 and Table ESM1 in the Supplementary file). The calculated barriers are typical rotational barriers for amide systems (Stewart and Siddall, 1970; Wiberg et al., 1995).

Biological evaluation

Cytotoxic activity

The antiproliferative/cytotoxic effects of the tested series of 4-chlorocoumarin benzoylhydrazones **4a-c** and 2-methyl-

Table 1 Cytotoxic effect of the newly synthesized compounds

Compounds	$IC_{50} (\mu M)^1$			
	HL-60 ²	KE-37 ³	K-562 ⁴	MDA-MB-231 ⁵
4a	2.9 ± 0.4	5.3 ± 1.1	7.2 ± 0.9	9.1 ± 1.2
4b	6.8 ± 1.2	10.2 ± 2.0	17.5 ± 1.7	18.4 ± 2.3
4c	3.1 ± 0.9	9.2 ± 2.7	10.5 ± 3.2	11.2 ± 2.1
8a	19.8 ± 3.5	29.4 ± 5.1	31.6 ± 2.8	27.7 ± 5.2
8b	27.1 ± 4.4	32.6 ± 4.6	42.8 ± 5.9	57.1 ± 9.4
8c	21.2 ± 6.1	27.7 ± 3.2	29.8 ± 5.6	31.5 ± 6.3
Melphalan	14.9 ± 3.3	17.3 ± 2.7	25.7 ± 2.5	21.4 ± 4.2

 1 means ± sd from eight independent experiments. Cell lines: 2 acute myeloid leukemia, 3 T-cell leukemia, 4 chronic myeloid leukemia, 5 ER-negative breast cancer

2*H*-chromen-3 benzoylhydrazones **8a–c** in different human tumor cell lines were evaluated using the standard MTT-dye reduction assay. The clinically applied anticancer drug melphalan was employed as a positive control throughout the cytotoxicity screenings. The bioassay data for the series of hydrazones **4a–c** and **8a–c** are summarized in the Table 1. As evident from IC₅₀ values, the tested compounds exhibit cytotoxic properties at low micromolar concentrations.

The evaluated compounds with a coumarin moiety **4a–c** exhibit invariably superior antiproliferative activity, as compared to the analogs bearing a 2-methyl-2*H*-chromene moiety **8a–c**. Importantly both potent coumarins and the less effective unsubstituted 2-methyl-2*H*-chromene

derivatives exhibit antiproliferative activity, if compared to melphalan. Such high cytotoxicity of both series is attributed to the high nitrogen content of the hydrazone moieties that are present. Compound 4a exhibits the most potent antiproliferative activity with IC₅₀ $2.9 \pm 0.4 \mu$ M in leukemia HL-60 cell line, followed by compounds 4c and 4b. The antiproliferative activity of the other synthesized compounds is in the following decreasing order: 8a > 8c > 8b. Noteworthy, among all synthesized compounds N'-[(E)-(4-chloro-2-oxo-2H-chromen-3-yl)methylidene] benzohydrazide 4a is the most active candidate against proliferation of breast MDA-MB-231 cell line, chronic myeloid leukemia K-562 cell line, and acute lymphoid leukemia KE-37 cell line, while compound 8b exhibits the weakest antiproliferative activity in breast MDA-MB-231 cell line with IC_{50} 57.1 ± 9.4 µM. Compound **8b** has 2-methyl-2*H*-chromene moiety, and a substituent hydroxyl group at the para position of benzene nucleus. The lowest in vitro antiproliferative profile of compounds 4a-c and 8a-c is showed against breast MDA-MB-231 cell line. The decreasing order of antiproliferative activity of the compounds toward chronic myeloid leukemia K-562 and acute lymphoid leukemia KE-37 cell lines is as follows: 4a > 4c > 4b > 8c >8a > 8b. Although the number and relatively close chemical structures of the tested analogs limit drawing broad and definitive conclusions, the study has outlined three distinct features: (1) the hydrazide/hydrazone moiety is a significant factor in antiproliferative activity; (2) the substitution with the isonicotinovl and p-hydroxyphenvl moieties decreases the antiproliferative activity both of 2-methyl-2Hchromenes and coumarin derivatives; (3) the coumarin moiety is responsible for its higher cytotoxicity effect than the 2-methyl-2H-chromene moiety. The hydrazide-hydrazone moiety at the third position of the coumarin and 2H-chromene rings seems to afford optimal activity.

Antioxidant activity

Salicylaldehyde isonicotinoyl hydrazone 9c is a lipophilic, tridentate iron chelator agent with a marked antioxidant and modest cytotoxic activity against neoplastic cells (Kondo et al., 1999). It has low in vitro and in vivo toxicity and good tolerability, even following prolonged administration to animals (Shadnia and Wright, 2009). The main purpose of our current study has been to enhance the ability of the newly synthesized hydrazones to protect cells against oxidative injury by incorporation of a chromene or coumarin moiety. Antioxidant activity of the synthesized compounds was tested by the DPPH free radical scavenging method. The DPPH method is rapid, simple, and widely used to test antioxidant activity of compounds by free radical scavenging or hydrogen donating. The solvent, DMSO, was tested

 Table 2
 Antioxidant capacity of 1 mM solution of the synthesized compounds, DMSO and vitamin C

Compounds	RSC _{DPPH} (%)	Reducing power (absorbance)
DMSO	0.45 ± 0.07	0.001 ± 0.000
Std-Vitamin C	26.88 ± 0.07	0.582 ± 0.016
4a	0.45 ± 0.09	0.059 ± 0.002
4b	1.95 ± 0.08	0.123 ± 0.002
4c	4.93 ± 0.09	0.234 ± 0.002
8a	0.56 ± 0.06	0.026 ± 0.001
8b	10.80 ± 0.15	0.869 ± 0.064
8c	7.44 ± 0.16	0.102 ± 0.002
9a	5.72 ± 0.28	0.135 ± 0.002
9b	3.96 ± 0.24	0.146 ± 0.002
9c	3.19 ± 0.34	0.138 ± 0.002

DMSO was not taken away from the value. Values are presented as means stdev of three individual experiments

for its antioxidant characteristics, as well as vitamin C, which were used for comparison. The results are presented in Table 2.

Vitamin C exhibits the highest RSC_{DPPH}, while the lowest values are those for DMSO and compound **4a** (0.45 \pm 0.09 %). Regarding the newly synthesized compounds, hydrazone **8b** possesses the highest RSC_{DPPH} value (10.80 \pm 0.15 %). Compared to DPPH, the hydrazones with a chromene moiety **8a–c** accomplish antioxidant activity higher than that of the other tested groups **4a–c** and **9a–c**. A significant difference in RSC_{DPPH} values has been observed for **4a–c** and **8a–c** groups.

The scavenging activity of compounds 4a-c and 8a-c is possibly due to the presence of an N-H group in the hydrazine moiety, which can donate a hydrogen atom to a DPPH radical. After donating a hydrogen atom, the compounds may exist in the radical form stabilized by a resonance hybrid structure, due to delocalization. Probably benzoylhydrazones could exert its antioxidant activity according to the mechanism (Alam and Lee, 2015) shown in Fig. 1. The electron conjugation in the structure is supposed to stabilize the radical and prevent its participation in a destructive biochemical reaction. Having donated the hydrogen atom from the hydroxyl group of a phenol form, 8b possibly exists in its radical form stabilized by electron conjugation. That is consistent with our experimental data and with the higher free radical scavenging potential of 8b. The low antioxidant activity of hydrazones 4a-c may be a result of the altered spatial arrangement of the groups in the molecules caused by the hindered rotation in the CO-NH group or influence of 3chlorosubstituted coumarin moiety. These activity differences exist in 9a-c group as well, but they are not pronounced for **9b** and **9c**. In conclusion, the presence of a hydrazide NH group and a phenolic hydroxyl one are regarded as important structural features, both having the ability of transferring a Fig. 1 The proposed DPPH radical scavenging mechanism of **8c**



hydrogen atom to the DPPH free radical to give a resonance stabilized radical. The mesomeric stabilization of this radical, in particular, through the addition of 2H-chromene scaffold could contribute to the radical scavenging ability with notable improvements, as observed for **8b**.

The reducing capacity is a significant indicator of potential antioxidant activity. Compound **8b** bearing a free hydroxyl group on the phenyl ring of the hydrozone moiety has the highest reducing power (0.869) among all the tested samples. The reducing power of this compound may be due to the hydroxyl substitution in the aromatic ring with potent hydrogen donating abilities. The reducing power of vitamin C of 0.582 is insignificantly higher than that of compounds **9a–c**, and significantly higher, if compared to that of the compounds from **4a–c** and **8a–c** groups emphasizing the structure-related influence. Hydrazones **4a** and **4c** have reducing power of DMSO is insignificant. The results of the correlation analysis are presented in Fig. 2.

The linear regression indicates a significant positive relationship between DPPH and the reducing power assay, since the Pearson's coefficient is 0.71 and *p*-value is 0.01. (The results of antioxidant and antiproliferative activity correlation are given in Fig. ESM2 and Fig. ESM3 in Supplementary Material.) Multiple regression analysis showed a strong positive correlation between DPPH



Fig. 2 Correlation scatter graph of DPPH and reducing power assay of the synthesized compounds **4a–c**, **8a–c**, and **9a–c**, vitamin C, and DMSO

scavenging capacity and antiproliferative activity (Pearson's r = 0.90, *F*-value = 0.62) (Fig. ESM2 in Supplementary Material). The adjusted value of $R^2 = 0.03$ indicates 3 % of the total variability. All *p*-values obtained for IC₅₀ of HL-60, KE-37, K-562, and MDA-MB-231 are higher than 0.05 (0.86, 0.69, 0.54, and 0.43, respectively). Reducing power and antiproliferative activity correlated positively, as determined by multiple regression analysis (Fig. ESM3 in Supplementary Material). Value of Pearson's *r* is 0.999 and *F*-value (0.02) indicating that the model is statistically

significant. Adjusted R^2 suggests that the model accounts for 99.90 % of the total variability. The *p*-values obtained for IC₅₀ of HL-60 (0.03), KE-37 (0.04), and MDA-MB-231 (0.02) are lower than 0.05, while *p*-value of IC₅₀ for K-562 is equal to 0.05. The *p*-values also indicate the statistical significance of the correlation.

Conclusion

Synthesis and in vitro antiproliferative activity of new hydrazones 4a-c and 8a-c toward leukemia HL-60, breast MDA-MB-231, chronic myeloid leukemia K-562, acute lymphoid leukemia KE-37 cell lines was reported. The title compounds 4a-c and 8a-c exhibited good antiproliferative profile activity, where compound 4a emerged as the most active candidate among all the synthesized compounds with $IC_{50} 2.9 \pm 0.4 \,\mu\text{M}$ in leukemia HL-60 cell line, followed by compounds 4c and 4b. The introduction of hydroxysubstituted benzovlhydrazone in compounds 4c, 8c, and isonicotinovl hydrazone moieties in compounds 4b and 8b did not improve the antiproliferative activity, but in general the Ph-CONHN=CH- moiety at the third position of the coumarin 4a and chromene 8a nucleus seems to afford optimal activity. The hydrazones containing 2-methyl-2Hchromene moiety **8a-c** exhibit a DPPH radical scavenging activity higher than that of hydrazones with coumarin moiety 4a-c. The activity of these compounds has been attributed chiefly to the hydrazide/hydrazone functionality, but in fact it is clearly modulated and enhanced by the incorporation of the 2H-chromene motif. We was found benzoylhydrazone 8b to be the most active compound possessing the highest DPPH radical scavenging ability, comparable to that of vitamin C. Multiple regression analysis suggests a strong positive correlation between DPPH scavenging capacity and antiproliferative activity (Pearson's r = 0.90, F = 0.62).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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