Bioorganic & Medicinal Chemistry 22 (2014) 2655–2661

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and cellular characterization of novel isoxazolo- and thiazolohydrazinylidene-chroman-2,4-diones on cancer and non-cancer cell growth and death

Ahmed Jashari [a,†], Faik Imeri [b,†], Lulzime Ballazhi [a,b], Agim Shabani [a], Bozhana Mikhova [c], Gerald Dräger [d], Emil Popovski [e], Andrea Huwiler [b,*]

[a] Group of Chemistry, Faculty of Natural Sciences & Mathematics, State University of Tetova, 1200 Tetova, Macedonia
[b] Institute of Pharmacology, University of Bern, Friedbühlstrasse 49, CH-3010 Bern, Switzerland
[c] Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Build. 9, 1113 Sofia, Bulgaria
[d] Institute of Organic Chemistry, Leibniz Universität Hannover, D-30167 Hannover, Germany
[e] Institute of Chemistry, Faculty of Natural Sciences & Mathematics, Ss. Cyril & Methodius University, PO Box 162, 1000 Skopje, Macedonia

ARTICLE INFO

Article history:
Received 17 December 2013
Revised 12 March 2014
Accepted 16 March 2014
Available online 24 March 2014

Keywords:
Chromandiones
Cancer cells
Apoptosis
PARP-1
Akt

ABSTRACT

Coumarins are extensively studied anticoagulants that exert additional effects such as anticancerogenic and even anti-inflammatory. In order to find new drugs with anticancer activities, we report here the synthesis and the structural analysis of new coumarin derivatives which combine the coumarin core and five member heterocycles in hydrazinylidene-chroman-2,4-diones. The derivatives were prepared by derivatization of the appropriate heterocyclic amines which were used as electrophiles to attack the coumarin ring. The structures were characterized by spectroscopic techniques including IR, NMR, 2D-NMR and MS. These derivatives were further characterized especially in terms of a potential cytotoxic and apoptogenic effect in several cancer cell lines including the breast and prostate cancer cell lines MCF-7, MDA-MB-231, PC-3, LNCaP, and the monocytic leukemia cell line U937. Cell viability was determined after 48 h and 72 h of treatment with the novel compounds by MTT assay and the 50% inhibitory concentrations (EC_{50} values) were determined. Out of the 8 novel compounds screened for reduced cell viability, **4c**, **4d** and **4e** were found to be the most promising and effective ones having EC_{50} values that were several fold reduced when compared to the reference substance 4-hydroxycoumarin. However, the effects were cancer cell line dependent. The breast cancer MDA-MB-231 cells, the prostate cancer LNCaP cells, and U937 cells were most sensitive, MCF-7 cells were less sensitive, and PC-3 cells were more resistant. Reduced cell viability was accompanied by increased apoptosis as shown by PARP-1 cleavage and reduced activity of the survival protein kinase Akt.

In summary, this study has identified three novel coumarin derivatives that in comparison to 4-hydroxycoumarin have a higher efficiency to reduce cancer cell viability and trigger apoptosis and therefore may represent interesting novel drug candidates.

1. Introduction

Coumarins are a group of heterocyclic compounds synthesized by numerous plant species as well as by some bacteria and fungi.[1,2] According to their chemical structure, they belong to the family of benzopyrones. So far, more than 1300 different coumarins have been identified. The most representative molecule, that is coumarin, has been extensively studied both in biochemical and pharmaceutical fields.[3–5]

Over the past decades, many studies have reported that coumarins and derivatives exert a plethora of biological activities including anti-microbial, anti-viral, anti-coagulant, anti-inflammatory, and anti-cancer effects.[6–13] Best known is the anti-coagulant effect of the 4-hydroxycoumarin derivative warfarin ((*RS*)-4-hydroxy-3-(3-oxo-1-phenyl-butyl)-coumarin) that reached market approval early on.[14–16] A beneficial effect of warfarin in cancer patients leading to prolonged survival was shown by Zacharski and colleagues.[17] Meanwhile, many studies have reported a beneficial effect of coumarins on other cancer types including malignant

* Corresponding author. Tel.: +41 31 632 32 14.
E-mail address: Huwiler@pki.unibe.ch (A. Huwiler).
† These authors contributed equally.

http://dx.doi.org/10.1016/j.bmc.2014.03.026
0968-0896/

melanoma, leukemia, renal cell carcinoma, prostate and breast cancer cells progression.[18–20] Also, certain platinum(II) complexes of aminocoumarins showed very good in vitro cytotoxicity.[21] A variety of mechanisms have been proposed such as interfering with estrogen synthesis, interfering with cell cycle progression or even acting as inhibitors of cytochrome P450 1.[22]

Also, a number of coumarins with substituents in position 7 (usually some electron-releasing group) and position 3, especially imines,[23,24] were reported and, in general their photosensitivity was tested. Finally, a number of nitrogen-rich compounds were found to be good chemotherapeutic agents[25] especially if a thiazole ring was introduced as shown by Gouda et al.[26] Recent studies have suggested that several coumarin derivatives showed antiproliferative activity in various tumor cells.[13,20,27] Considering those inputs, coumarin as a very versatile biological agent, and nitrogen-rich heterocyclic compounds as good chemotherapeutic agents, it was tempting to combine these moieties and evaluate their activity. Therefore, in this study, we have synthesized several novel 3-substituted thiazolo and isoxazolo hydrazinylidene-chroman-2,4-dione compounds. These compounds were tested for cell viability in different cancer and non-cancer cell lines. We found that three of the novel compounds effectively reduced cell viability in a concentration-dependent manner. A decrease in the level of phospho-Akt and an increase in the level of PARP-1 cleavage strongly argues for the induction of the intrinsic pathway of apoptosis.

2. Materials and methods

2.1. General synthetic procedure

The heterocyclic amines (**1a–h**, 10 mmol) were dissolved in 10 mL water followed by addition of 40 mL of 6 M HCl and the systems were cooled in the ice-salt bath down to −10 °C. Afterwards, an aqueous solution of $NaNO_2$ (10 mmol, 0.7 g/5 mL H_2O) was added slowly drop by drop and stirred vigorously on a magnetic stirrer. After 15 min, fresh solution of 4-hydroxycoumarin (**3**, 10 mmol, 1.62 g) in 10 mL NaOH (10 wt.) was added. Intensively colored and voluminous precipitates (**4a–h**) were obtained immediately which were stirred 15 min. in the bath and 30 min. on room temperature. Finally, they were filtrated by vacuum, washed 3 times with distilled water and dried on air. The purification was carried out by the technique of recrystallization using ethanol as solvent.

2.1.1. 3-[2-(4*H*-1,2,4-Triazol-3-yl)hydrazinylidene]chroman-2,4-dione (4a)

Yellow powder (93%), mp 225–227 °C. FTIR (KBr, ν/cm^{-1}): 3616-3175 (NH, stretching, broad), 3120 (CH, aromatic stretching), 1736 (C=O, stretching), 1617, 1544 (aromatic deformations). ^{1}H NMR (DMSO-d_6, δ/ppm, *J*/Hz): 8.01 dd (7.5, 1.5, H5-coum.), 7.30–7.45 m (H6&H8-coum.), 7.80 dd (7.5, 1.5, H7-coum.), 8.63 br s (H5-triazole), 10.77 s (NH-triazole). ^{13}C NMR (DMSO-d_6, δ/ppm): 157.9 (C2-coum.), 124.4 (C3-coum.), 178.6 (C4-coum.), 126.7 (C5-coum.), 124.8 (C6-coum.), 136.2 (C7-coum.), 117.3 (C8-coum.), 120.3 (C4a-coum.), 154.0 (C8a-coum.), 164.0 (C2-triazole), 145.3 (C5-triazole). TOF-MS-ES+ (*m*/*z*): 280.0582 [M+Na]$^+$, $C_{11}H_7N_5O_3$.

2.1.2. 3-[2-(5-Methylisoxazol-3-yl)hydrazinylidene]chroman-2,4-dione (4b)

Yellow small needles (92%), mp 203–205 °C. FTIR (KBr, ν/cm^{-1}): 3620–3053 (NH, stretching, broad), 1740 (C=O, stretching), 1602, 1525 (aromatic deformations). ^{1}H NMR (DMSO-d_6, δ/ppm, *J*/Hz): 8.00 dd (7.8, 1.7, H5-coum.), 7.30–7.45 m (H6&H8-coum.), 7.80 ddd (8.0, 8.0, 1.5, H7-coum.), 6.63 (H4-isoxazole), 2.46 s (CH_3-isoxazole). ^{13}C NMR (DMSO-d_6, δ/ppm): 157.6 (C2-coum.), 125.7 (C3-coum.), 178.4 (C4-coum.), 126.8 (C5-coum.), 124.8 (C6-coum.), 136.9 (C7-coum.), 117.4 (C8-coum.), 120.4 (C4a-coum.), 154.1 (C8a-coum.), 162.7 (C3-isoxazole), 94.1(C4-isoxazole), 172.0 (C5-isoxazole), 12.3 (CH_3-isoxazole). TOF-MS-ES+ (*m*/*z*): 272.0677 [M+H]$^+$, 294.0440 [M+Na]$^+$, $C_{13}H_{10}N_3O_4$.

2.1.3. 3-[2-(Thiazol-2-yl)hydrazinylidene]chroman-2,4-dione (4c)

Orange-red crystals (82%), mp 209–211 °C. FTIR (KBr, ν/cm^{-1}): 3625-3250 (NH, stretching, broad), 3125, 3080 (CH, aromatic stretching), 1765 (C=O, stretching), 1623, 1606, 1521 (aromatic deformations). ^{1}H NMR (DMSO-d_6, δ/ppm, *J*/Hz): 8.00 dd (8.2, 1.6, H5-coum.), 7.30–7.45 m (H6&H8-coum.), 7.81 ddd (8.0, 8.0, 1.8, H7-coum.), 7.70 d (2.5, H4-thiazole), 7.57 d (2.5, H5-thiazole). ^{13}C NMR (DMSO-d_6, δ/ppm): 157.3 (C2-coum.), 125.0 (C3-coum.), 177.9 (C4-coum.), 126.7 (C5-coum.), 124.8 (C6-coum.), 136.8 (C7-coum.), 117.4 (C8-coum.), 120.4 (C4a-coum.), 154.0 (C8a-coum.), 166.3 (C2-thiazole), 140.4 (C4-thiazole), 117.6 (C5-thiazole). TOF-MS-ES+ (*m*/*z*): 274. 0345 [M+H]$^+$, 296.0160 [M+Na]$^+$, $C_{12}H_7N_3O_3S$.

2.1.4. 3-[2-(5-Methylthiazol-2-yl)hydrazinylidene]chroman-2,4-dione (4d)

Red crystals (63%), mp 218–220 °C. FTIR (KBr, ν/cm^{-1}): 3616-3175 (NH, stretching, broad), 3120 (CH, aromatic stretching), 1736 (C=O, stretching), 1617, 1544 (aromatic deformations). ^{1}H NMR (DMSO-d_6, δ/ppm, *J*/Hz): 8.00 dd (8.0, 1.7, H5-coum.), 7.30–7.45 m (H6&H8-coum. &H4-thiazole), 7.78 ddd (8.0, 8.0, 1.5, H7-coum.), 2.44 d (1.2, CH_3-thiazole). ^{13}C NMR (DMSO-d_6, δ/ppm): 157.3 (C2-coum.), 124.6 (C3-coum.), 177.6 (C4-coum.), 126.6 (C5-coum.), 124.7 (C6-coum.), 136.7 (C7-coum.), 117.3 (C8-coum.), 120.4 (C4a-coum.), 153.9 (C8a-coum.), 164.5 (C2-thiazole), 137.5 (C4-thiazole), 131.7 (C5-thiazole), 11.9 (CH_3-thiazole). TOF-MS-ES+ (*m*/*z*): 288.0403 [M+H]$^+$, 310.0294 [M+Na]$^+$, $C_{13}H_9N_3O_3S$.

2.1.5. 3-[2-(4,5-Dimethylthiazol-2-yl)hydrazinylidene]chroman-2,4-dione (4e)

Carmine-red crystals (65%), mp 206–208 °C. FTIR (KBr, ν/cm^{-1}): 3629-3075 (NH, stretching, broad), 1752 (C=O, stretching), 1616, 1558 (aromatic deformations). ^{1}H NMR (DMSO-d_6, δ/ppm, *J*/Hz): 8.01 dd (7.8, 1.4, H5-coum.), 7.25-7.45 m (H6&H8-coum.), 7.77 ddd (8.0, 8.0, 1.8, H7-coum.), 2.34 s (5-CH_3-thiazole), 2.23 s (4-CH_3-thiazole). ^{13}C NMR (DMSO-d_6, δ/ppm): 157.3 (C2-coum.), 124.0 (C3-coum.), 177.2 (C4-coum.), 126.7 (C5-coum.), 124.7 (C6-coum.), 136.5 (C7-coum.), 117.3 (C8-coum.), 120.6 (C4a-coum.), 153.9 (C8a-coum.), 163.9 (C2-thiazole), 145.0 (C5-thiazole), 144.5 (C4-thiazole), 11.2 (5-CH_3-thiazole), 14.0 (4-CH_3-thiazole). TOF-MS-ES+ (*m*/*z*): 302.0834 [M+H]$^+$, 324.0802 [M+Na]$^+$, $C_{14}H_{11}N_3O_3S$.

2.1.6. 3-[2-(5-*tert*-Butylisoxazol-3-yl)hydrazinylidene]chroman-2,4-dione (4f)

Yellow crystals (86%), mp 225–227 °C. FTIR (KBr, ν/cm^{-1}): 3616–3175 (NH, stretching, broad), 3120 (CH, aromatic stretching), 1736 (C=O, stretching), 1617, 1544 (aromatic deformations). ^{1}H NMR (DMSO-d_6, δ/ppm, *J*/Hz): 8.00 dd (8.0, 1.5, H5-coum.), 7.30–7.45 m (H6&H8-coum.), 7.80 ddd (8.0, 8.0, 1.5, H7-coum.), 6.58 s (H4-isoxazole), 1.35 s (*tert*-CH_3-isoxazole). ^{13}C NMR (DMSO-d_6, δ/ppm): 157.6 (C2-coum.), 125.7 (C3-coum.), 178.3 (C4-coum.), 126.8 (C5-coum.), 124.9 (C6-coum.), 137.0 (C7-coum.), 117.4 (C8-coum.), 120.4 (C4a-coum.), 154.1 (C8a-coum.), 162.4 (C3-isoxazole), 91.2 (C4-isoxazole), 182.8 (C5-isoxazole), 32.1 (*tert*-$C(CH_3)_3$-isoxazole), 28.2 (*tert*-$C(CH_3)_3$-isoxazole). TOF-MS-ES+ (*m*/*z*): 314.1189 [M+H]$^+$, 336.0926 [M+Na]$^+$, 649.2048 [2M+Na]$^+$ $C_{16}H_{15}N_3O_4$.

2.1.7. 3-[2-(5-Bromothiazol-2-yl)hydrazinylidene]chroman-2,4-dione (4g)

Orange crystals (69%), mp 217–219 °C. FTIR (KBr, ν/cm^{-1}): 3624–3164 (NH, stretching, broad), 1738 (C=O, stretching), 1617, 1560 (aromatic deformations). ^{1}H NMR (DMSO-d_6, δ/ppm, J/Hz): 7.99 dd (8.0, 1.7, H5-coum.), 7.30–7.45 m (H6&H8-coum.), 7.77 dd (7.5, 1.7, H7-coum.), 7.77 s (H4-thiazole). ^{13}C NMR (DMSO-d_6, δ/ppm): 157.2 (C2-coum.), 125.3 (C3-coum.), 177.3 (C4-coum.), 126.8 (C5-coum.), 124.7 (C6-coum.), 136.6 (C7-coum.), 117.3 (C8-coum.), 120.7 (C4a-coum.), 154.0 (C8a-coum.), 163.1 (C2-thiazole), 142.3 (C4-thiazole), 105 (C5-thiazole). TOF-MS-ES+ (m/z): 353.9615 $[M+H]^+$, 375.9648 $[M+Na]^+$, $C_{12}H_6BrN_3O_3S$.

2.1.8. 3-[2-(Isoxazol-3-yl)hydrazinylidene]chroman-2,4-dione (4h)

Yellow crystals (74%), mp 218–221 °C. FTIR (KBr, ν/cm^{-1}): 3611-3198 (NH, stretching, broad), 3107 (CH, aromatic stretching), 1741 (C=O, stretching), 1626, 1572 (aromatic deformations). ^{1}H NMR (DMSO-d_6, δ/ppm, J/Hz): 8.00 dd (7.8, 1.7, H5-coum.), 7.32–7.42 m (H6&H8-coum.), 7.80 ddd (8.0, 8.0, 1.8, H7-coum.), 6.92 d (1.6, H4-isoxazole), 8.99 d (1.6, H5-isoxazole). ^{13}C NMR (DMSO-d_6, δ/ppm): 157.6 (C2-coum.), 126.0 (C3-coum.), 178.6 (C4-coum.), 126.8 (C5-coum.), 124.9 (C6-coum.), 137.0 (C7-coum.), 117.4 (C8-coum.), 120.4 (C4a-coum.), 154.1 (C8a-coum.), 162.2 (C3-isoxazole), 97.3 (C4-isoxazole), 162.4 (C5-isoxazole). TOF-MS-ES+ (m/z): 258.0629 $[M+H]^+$, 280.0565 $[M+Na]^+$, $C_{12}H_7N_3O_4$

2.2. Characterization of synthesized compounds

Melting points were determined on a Reichert heating plate and were uncorrected. Infrared spectra (KBr pellets) were measured on a Perkin–Elmer System 2000 FT IR. The NMR spectra were run on a Bruker-250 DRX Spectrometer using standard Bruker Topspin software. DMSO-d_6 was used as a solvent and the chemical shifts were referenced to the residual solvent signal (2.5 ppm for ^{1}H and 39.5 ppm for ^{13}C spectra). The signals were assigned with the aim of ^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC spectra. The digital resolution of the 1D-spectra was 0.12 Hz/Pt for ^{1}H and 1.4 Hz/Pt for ^{13}C. Mass spectra were measured with Q-TOF premier (MICROMASS) spectrometer (ESI mode) in combination with a WATERS Acquity UPLC system equipped with a WATERS Acquity UPLC BEH C18 1.7 μm column (solvent A: water + 0.1% {v/v} formic acid, solvent B: MeOH + 0.1% {v/v} formic acid; flow rate = 0.4 mL/min; gradient {t [min]/solvent B [%]}: {0/5} {2.5/95} {6.5/95} {6.6/5} {8/5}). All the reagents and solvents were obtained from commercial sources and were used without further purification.

2.3. Chemicals for cell culture studies

All synthesized compounds were dissolved in DMSO as 10 mM stock solutions and stored at -20 °C. Further dilutions were made in complete Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). MTT 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide were purchased from Sigma (St. Louis, MO, US). All cell media and supplements were from Invitrogen, Basel, Switzerland.

2.4. Cell culturing

The human breast cancer cell line MCF-7 was maintained in DMEM containing 10% FBS, supplemented with glutamax (0.03%) and 100 μg/mL benzyl penicillin, 100 U/mL streptomycin. The human endothelial cell line (EA.hy 926), the breast cancer cell line MDA-MB-231 and U937 (human leukemic monocyte lymphoma cell line) were cultured in RPMI medium containing 4.5 g/L glucose, 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), 2 mM N-acetyl-L-alanyl-L-glutamine and 10 mM HEPES. Prostate cancer cells PC-3 were maintained in DMEM medium with 10% FCS, HEPES 1M (5 mL), PS (5 mL). LNCaP cells were maintained in RPMI medium with 10% FCS, HEPES 1M (5 mL), PS (5 mL). All cell lines were incubated at 37 °C in an atmosphere containing 5% CO_2. For experiments, cells from exponentially growing culture were used.

2.5. Cell viability assay

The effect of 4-hydroxycoumarin and novel derivatives on the viability of the different cell lines was determined by an MTT (3[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. In brief, 5000 cells were plated into each well of a 96-well plate in a volume of 100 μL growth medium. Cells were allowed to attach overnight and then treated with the different compounds in increasing concentrations (1–500 μM). After 48 h and 72 h of incubation, 20 μL of a 5 mg/mL MTT solution was added to each well and incubated for further 4 h at 37 °C. Thereafter, 100 μL of 4 mM HCl, 0.1% Nonidet P-40 (in isopropanol) was added to each well to solubilize MTT crystals. The plates were covered with a foil and vortexed on an orbital shaker for 15 min. Then, absorbance at 590 nm was measured in a SpectraMax microplate reader. All experiments were performed at least 3 times, with 4 wells for each concentration of the tested agents. Cell survival (% of control) was calculated relative to untreated controls. The 50% inhibitory concentration (EC_{50}) was determined as the anticancer drug concentration causing 50% reduction in cell viability and calculated from the viability curves by using the Bliss's software (Bliss Co, CA). Cell survival was calculated using the following formula: survival rate = (mean experimental absorbance/mean control absorbance) × 100%. The viability of U937 cells was measured by Alamar blue.

2.6. Western blot analysis

Stimulated cells were homogenized in lysis buffer[28] and centrifuged for 10 min at 14,000g. The supernatant was taken for protein determination. 30 μg of protein were separated by SDS–PAGE, transferred to a nitrocellulose membrane and Western blot analysis was performed as previously described[29] using antibodies as indicated in the figure legends.

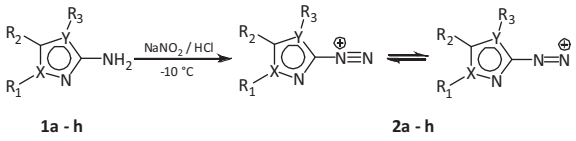
2.7. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by a Bonferroni's post hoc test for multiple comparisons (GraphPad InStat version 3.00 for Windows NT, GraphPad Software, San Diego, CA, USA).

3. Results

In order to synthesize novel derivatives of coumarines, we used the fact based on HSAB[30,31] that electrophiles are attacking the coumarin core at the position 3. We expected that diazonium ions as electrophilic particles will attack right at this position.

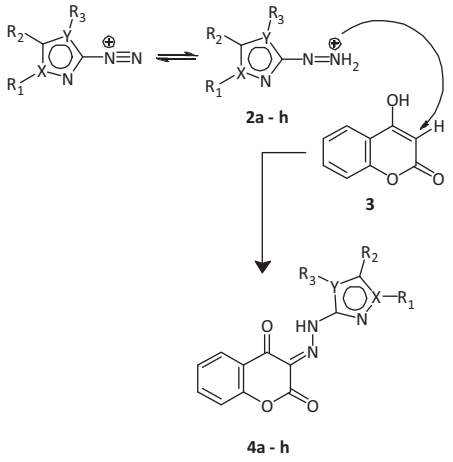
For this aim, 8 salts (**2a**–**h**) from their corresponding five member heterocyclic amines (**1a**–**h**, Scheme 1) were prepared. A basic aqueous solution ($NaOH_{aq,\ 10\%}$) of 4-hydroxycoumarin (**3**, schema 2) was prepared and added dropwise to the freshly prepared salts (**2a**–**h**). Precipitates of hydrazinylidene-chroman-2,4-diones (**4a**–**h**) with characteristic intensive colors were obtained (Scheme 2). The reactions were monitored by the TLC technique using different eluents. Total spectral assignation, by IR, ^{1}H NMR, ^{13}C NMR, 2D-NMR and MS was performed.



1a - h 2a - h

1a: X=N; Y=NH; R_2=H
1b: X=O; Y=C; R_2=CH_3; R_3=H
1c: X=C; Y=S; R_1=R_2=H
1d: X=C; Y=S; R_1=H; R_2=CH_3
1e: X=C; Y=S; R_1=R_2=CH_3
1f: X=O; Y=C; R_2=*terc*-Bu; R_3=H
1g: X=C; Y=S; R_1=H; R_2=Br
1h: X=O; Y=C; R_2=R_3=H

Scheme 1. Derivatization of heterocyclic amines.



2a - h 3 4a - h

4a: X=N; Y=NH; R_2=H
4b: X=O; Y=C; R_2=CH_3; R_3=H
4c: X=C; Y=S; R_1=R_2=H
4d: X=C; Y=S; R_1=H; R_2=CH_3
4e: X=C; Y=S; R_1=R_2=CH_3
4f: X=O; Y=C; R_2=*terc*-Bu; R_3=H
4g: X=C; Y=S; R_1=H; R_2=Br
4h: X=O; Y=C; R_2=R_3=H

Scheme 2. Synthesis of hydrazinylidene-chroman-2,4-diones.

In the IR spectra's of those derivatives it can be noticed characteristic C=O band in the narrow region from 1766–1736 cm^{-1}. In the ^1H NMR in general doublet of doublets, triplet of doublets and multiplet at about 8.07, 7.80 and 7.35 ppm on 1:1:2 ratio can be seen, which is similar to that of **3** for the four aromatic protons of the benzene ring. On the other side, the signal at 5.37 ppm from the hydrogen at the position 3 of compound **3** is absent in all derivatives which confirms that the substitution had taken please right in this position, as expected. Additionally, the HMBC, HMQC and MS were successfully performed to confirm that the structures of those coupled derivatives matches the proposed structures (**4a**–**h**, Scheme 2). Additionally, mono-crystals from one of the derivatives (**4f**) were successfully obtained that allowed single-crystal X-ray diffractogram.[32] By the means of diffractometry the hydrazinylidene structure of the synthesized derivatives was revealed.

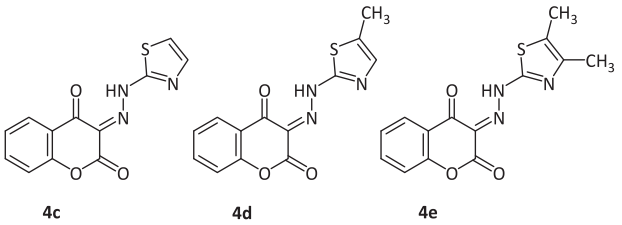
To see whether these novel isoxazolo- and thiazolo derivatives of coumarin had a superior cellular effect compared to the 4-hydroxycoumarin, we tested their effects on cell viability of a panel of different human cell lines including cancer cell lines of different histotypes and non-cancer cell lines such as the human endothelial cell line EA.hy 926. All compounds were treated with increasing concentrations from 1 to 500 μM and the 50% inhibitory concentration was thereafter calculated from the viability curves. EC_{50} values at 48 h and at 72 h are summarized in Table 1A and B, respectively.

Results show that the lead compound 4-hydroxycoumarin is not very cytotoxic in any of the cell types tested. EC_{50} values are in a very high concentration range of 100–200 μM in all cell types tested. The derivatives **4a** and **4b** which had a nitrogen or oxygen in the position 5 of the five-membered heterocycle did not reveal any higher cytotoxic effect than 4-HC (**3**). Interestingly, the derivatives **4c**, **4d** and **4e** having sulfur in position 3 of the five membered heterocycle (Scheme 3) showed a more potent effect on cell viability than 4-HC (**3**) although this was strikingly dependent on the cell type tested. Whereas the estrogen-dependent breast cancer cell line MCF-7, the androgen-independent prostate cancer cell line PC-3 and the human endothelial cell line EA.hy926 were rather resistant to treatment with **4c**, **4d**, and **4e** and EC_{50} values remained between 100 and 200 μM, the breast cancer cell line MDA-MB231 cell line, the prostate cancer cell line LNCaP, and the human monocytic leukemia cell line U937 were several time more sensitive to treatment. EC_{50} values dropped to 20–60 μM after 48 h of treatment, and to 10–50 μM after 72 h of treatment.

Table 1
Inhibitory effects of 4-hydroxycoumarin and of the synthesized derivatives on the viability of various human cancer and non-cancer cell lines

	MCF-7	MDA-MB-231	EA.hy926	LNCaP	PC-3	U937
A						
48 h						
4-HC	>200	>200	>200	100.51 ± 10.26	150.46 ± 12.92	>200
4a	>200	>200	>200	>200	>200	>200
4b	>200	>200	>200	>200	>200	>200
4c	99.94 ± 0.53	64.84 ± 6.65	128.27 ± 6.54	29.96 ± 8.41	98.08 ± 6.73	49.72 ± 3.51
4d	100.40 ± 0.41	59.14 ± 6.18	153.93 ± 1.74	20.46 ± 8.92	118.10 ± 3.14	49.97 ± 0.42
4e	99.94 ± 0.51	50.43 ± 5.74	120.35 ± 1.04	20.07 ± 11.47	82.93 ± 4.55	50.05 ± 0.54
4f	>200	>200	163.45 ± 8.32	>200	>200	>200
4g	99.78 ± 0.25	99.58 ± 1.33	150.43 ± 3.12	>200	>200	117.57 ± 3.53
B						
72 h						
4-HC	199.82 ± 2.88	100.26 ±18.40	>200	120.27 ± 9.06	173.31 ± 11.27	164.12 ± 2.78
4a	49.69 ± 0.88	49.68 ± 5.18	95.12 ± 1.21	>200	>200	154.41 ± 7.68
4b	100.29 ± 2.96	50.26 ± 0.92	150.34 ± 6.71	>200	>200	128.90 ± 7.01
4c	19.95 ± 0.90	49.96 ± 6.50	96.54 ± 5.03	19.69 ± 5.27	95.32 ± 6.92	49.89 ± 0.25
4d	19.60 ± 5.96	9.91 ± 0.38	77.27 ± 2.15	20.12 ± 4.07	118.10 ± 9.68	28.38 ± 2.17
4e	20.15 ± 0.80	9.82 ± 0.41	72.77 ± 3.28	20.09 ± 3.67	109.32 ± 9.66	29.68 ± 1.72
4f	100.03 ± 3.18	50.26 ± 0.92	149.96 ± 1.51	>200	>200	119.71 ± 6.75
4g	50.21 ± 2.18	49.60 ± 2.01	156.06 ± 1.02	>200	>200	107.99 ± 5.41

The different cell lines were treated for either 48 h (1A) or 72 h (1B) with the indicated compounds at concentrations between 1 μM and 500 μM and cell viability was determined by the MTT method as described in the Section 2. EC_{50} values were calculated for each compound. Values are means ± S.D. of at least three independent experiments.



Scheme 3. Structures of the most potent compounds.

After 72 h of treatment the MCF-7 cells also turned sensitive to **4c**, **4d**, and **4e** treatment underlining the concentration- and time-dependent cytotoxic effect of the compounds. Derivative **4f** resembling structure **4b**, that is oxygen in position 5 of the pentacycle, but replacing methyl for *tert*-butyl at position 4, was hardly effective. Finally, derivative **4g** which resembled the active sulfur-containing **4c**, **4d**, and **4e** compounds but had a bromide in position 4, was also less effective than the series of **4c**, **4d** and **4e** on all cell lines tested although after 72 h of treatment MCF-7 and MDA-MB231 cells responded more to **4g** (EC_{50} value at 50 μM).

We further tested whether the reduced viability of cells was due to increased apoptosis. To this end, the breast cancer cell lines MDA-MB231 and MCF-7, and the prostate cancer cell lines LNCaP and PC-3, and the monocytic leukemia cell line U937 were treated with different concentrations of **4c**, **4d**, **4e**, and in comparison with 4-HC (**3**). PARP-1 cleavage, which is considered a marker of apoptosis, was determined by Western blot analysis. Figure 1A shows that in MDA-MB231 cells, all concentrations of **4c**, **4d** and **4e** led to a reduced protein expression of 116 kDa full-length PARP with a concentration-dependent appearance of a cleavage product at 86 kDa. In parallel, we measured the protein expression and phosphorylation level of the protein kinase Akt. Akt is a well known protein kinase involved in cell growth and survival and reduced activity often correlates with reduced proliferation and increased apoptosis. The level of phospho-Thr^{308} Akt is well accepted to

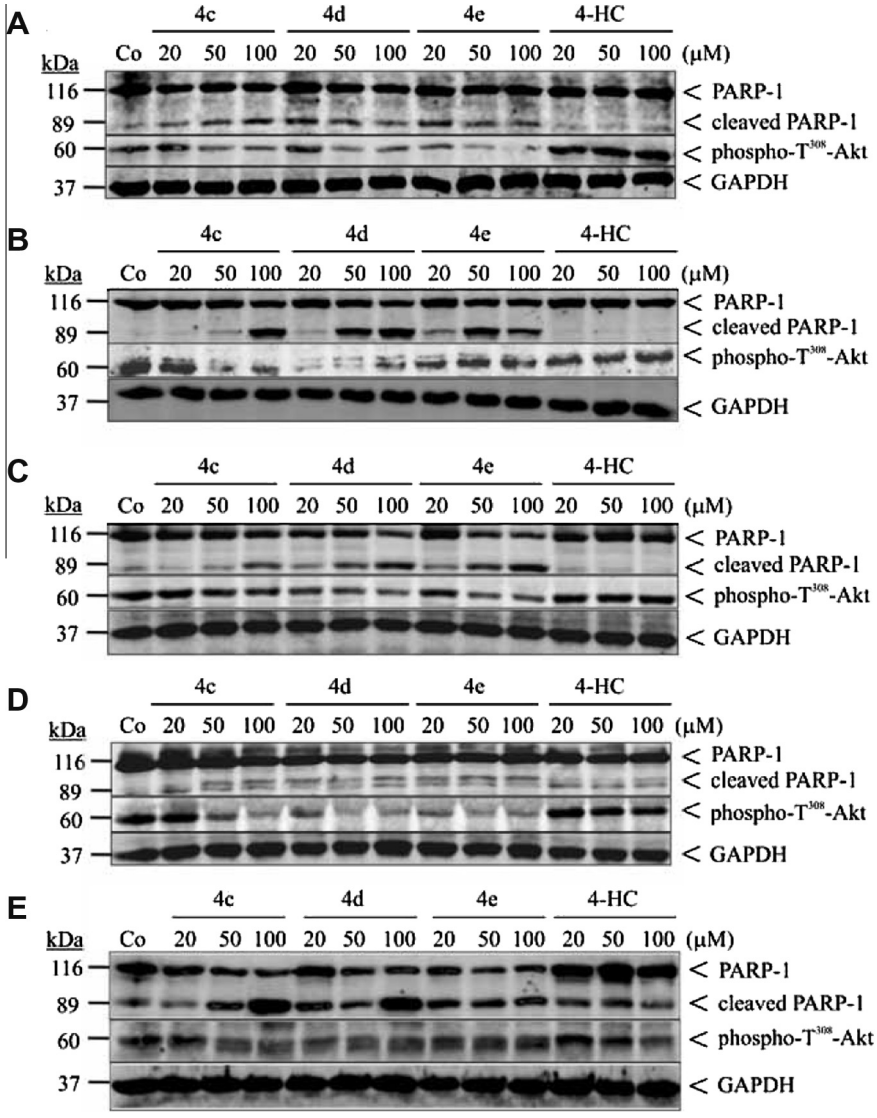


Figure 1. Effect of novel 4-hydroxycoumarin derivatives on PARP-1 cleavage and activity of the survival kinase Akt in various cancer cell lines. The breast cancer cell lines MDA-MB-231 (A) and MCF-7 (B), the prostate cancer cell lines LNCaP (C) and PC-3 (D) and the monocytic leukemia cell line U937, (E) cells were treated for 48 h with either vehicle (0.1% DMSO, co) or the indicated concentrations of **4c**, **4d**, **4e**, and 4-HC (in μM). Protein extracts were prepared and equal amounts of protein (30 μg) were separated by 8% SDS–PAGE and transferred to nitrocellulose membranes. Western blot analysis was performed by using antibodies against PARP-1 (upper panels), phospho-Thr^{308}-Akt (middle panels) and the house-keeping protein GAPDH (lower panels). Data show one representative blot.

reflect Akt activity. We found that phospho-Thr^{308} Akt levels were downregulated by **4c**, **4d** and **4e** when compared to control and to 4-HC (**3**) treated cells. In the more resistant cell line MCF-7, **4c**, **4d** and **4e** were less effective in downregulating phospho-Akt. Only **4c** and **4d** at higher concentrations downregulated phospho-Akt, whereas **4e** and 4-HC (**3**) had no major effect (Fig. 1B). Still, PARP-1 cleavage occurred in MCF-7 by all three compounds **4c**, **4d** and **4e**, but not by 4-HC (**3**).

The androgen-dependent prostate cancer cell line LNCaP showed PARP-1 cleavage upon treatment with all compounds with the exception of 4-HC (Fig. 1C). In parallel, phospho-Thr^{308} Akt was decreased in a concentration-dependent manner by the three compounds **4c**, **4d** and **4e**, but not by 4-HC (**3**). In contrast to the LNCaP cells, the androgen-independent prostate cancer cell line PC-3, which was found to be resistant to **4c**, **4e** and **4d** treatment in the viability assay (Table 1), was also more resistant to apoptosis induction as shown in Figure 1D. Hardly any cleaved PARP-1 fragment was seen upon **4c**, **4d** and **4e** treatment (Fig. 1 D). However, phospho-Thr^{308} Akt was downregulated by **4c**, **4d** and **4e** when compared to control and 4-HC treated cells. Finally, the monocytoic leukemia cell line U937, which was sensitive to **4c**, **4d** and **4e** treatment in the viability assay (Table 1), consistently showed enhanced PARP-1 cleavage and reduced phospho-Thr^{308} Akt upon treatment (Fig. 1E).

4. Discussion

Some coumarins including the 4-hydroxycoumarin derivative warfarin are well-known anticoagulants that act as a vitamin K antagonists. However, many other biological activities have also been appointed to coumarins and certain derivatives such as anti-bacterial, anti-inflammatory, and anti-viral effects.

Their anti-coagulant property has proven useful also in cancer patients to reduce venous thromboembolism which is a frequent complication of cancer.[17,33] In addition to this beneficial effect in cancer patients, a direct anti-tumor and anti-metastatic activity has also been described.[33,34] The potential mechanisms of action may occur on multiple levels. Thus, it has been reported that coumarins may interfere with proper cell cycle progression and trigger cell cycle arrest and subsequent apoptosis. In a human cervical cancer cell line (HeLa), coumarin derivatives were shown to induce cell-cycle arrest and apoptosis by downregulating the anti-apoptotic factors Bcl-2 and Bcl-x_L, and upregulating the pro-apoptotic factor Bax.[35] Additionally, coumarins are prodrug inhibitors of the metalloenzyme carbonic anhydrase (CA).[36] This enzyme efficiently hydrates carbon dioxide to protons and bicarbonate and thereby promotes acidification of solid tumors. 15 different subtypes of CA exist and certain subtypes such as CA IX and XII were reported to be overexpressed in many tumor types and to be involved in cancer progression and metastases formation.[37] Coumarins were also shown to directly inhibit sulfatase and aromatase activities, two enzymes taking part in estrogen synthesis[38,39] and to interfere and modulate estrogen receptor activation.[40] Moreover, it was recently proposed that the cytochrome P450 1 (CYP1) enzymes are targets of coumarins. These enzymes take part in the metabolic activation of procarcinogens and deactivation of certain anticancer drugs[22] and therefore, inhibition of these enzymes may exert an anti-cancer effect.

In our study, the novel coumarin derivatives, especially **4c**, **4d** and **4e**, showed a clear pro-apoptotic effect evident by enhanced PARP-1 cleavage, and a diminished survival capacity evident by reduced Akt-1 activity. Whether one of the above indicated possible targets are directly inhibited by these novel coumarin derivatives remains to be proven in future studies. When comparing the effect of the compounds in the two breast cancer cell lines, the effect was more pronounced in the p53- and ER-negative MDA-MB-231 cell line than in the p53- and ER-positive MCF-7 cell line. Although the MDA-MB-231 cell line is considered highly tumorigenic, neither p53 nor ER seem to be predictive for drug susceptibility. Notably, MDA-MB-231 cells were also reported to be more sensitive to taxane treatment than MCF-7.[41] Recently, a study reported on the identification of a group of genes that regulated the susceptibility of breast cancer cells to antineoplastic drugs.[42] Whether these genes also regulate the susceptibility towards **4c**, **4d**, and **4e** remains an interesting point for future studies.

Our data further show that **4c**, **4d**, and **4e** differentially affected the androgen-dependent and androgen-independent prostate cancer cell lines LnCaP and PC-3 in that only LnCaP were sensitive to the compounds and responded with increased PARP-1 cleavage and apoptosis. These two cell lines are known to exhibit different capabilities in angiogenesis and tumor aggressiveness, and especially the PC-3 cell line has acquired a more aggressive phenotype that is highly angiogenic.[42] Molecular profiling of the two cell lines revealed that several thousand genes are differentially expressed which may account for the aggressive and drug-resistant phenotype of the PC-3 cells.[43]

5. Conclusions

This study has revealed three novel coumarin derivatives with hydrazinylidene-chromandione structure which exert more potent anti-proliferative and pro-apoptotic effects on various cancer cell lines than the reference compound 4-hydroxycoumarine. These novel compounds may therefore represent interesting novel lead compounds for further anti-cancer studies.

Acknowledgments

We wish to thank the South-East European Pact for stabilization in the frame of the 'Deutscher Akademischer Austauschdienst' (DAAD) for financial support. We also thank Prof. Dr. Evamarie Hey-Hawkins and Dr. Alexandra Hildebrand from the University of Leipzig for helpful discussions.

References and notes

1. Raistrick, H.; Stickings, C. E.; Thomas, R. *Biochem. J.* **1953**, *55*, 421.
2. Hinman, J. W.; Caron, E. L.; Hoeksema, H. *J. Am. Chem. Soc.* **1957**, *79*, 3789.
3. Lacy, A.; O'Kennedy, R. *Curr. Pharm. Des.* **2004**, *10*, 3797.
4. Musa, M. A.; Badisa, V. L.; Latinwo, L. M.; Waryoba, C.; Ugochukwu, N. *Anticancer Res.* **2010**, *30*, 4613.
5. Manolov, I.; Danchev, N. D. *Eur. J. Med. Chem. Chim. Ther.* **1995**, *30*, 531.
6. Wallick, H.; Haris, D. A.; Reagan, M. A.; Ruger, M.; Woodruff, H. B. *Antibiot. Ann.* **1956**. Medical Encyclopedia, Inc., NY, p909.
7. Parmar, V. S.; Bisht, K. S.; Jain, R.; Singh, S.; Sharma, S. K.; Gupta, S.; Malhotra, S.; Tyagi, O. D.; Vardhan, A.; Pati, H. N.; Berghe, D. V.; Vlietinck, A. J. *Ind. J. Chem. Sec. B* **1996**, *35*, 220.
8. Kirkiacharian, S.; Thuy, D. T.; Sicsic, S.; Bakhchinian, R.; Kurkjian, R.; Tonnaire, T., II *Farmaco* **2002**, *57*, 703.
9. Huebner, C. F.; Link, K. P. *J. Biol. Chem.* **1941**, *138*, 513.
10. Emmanuel-Giota, A. A.; Fylaktakidou, K. C.; Hadjipavlou-Litina, D. J.; Litinas, K. E.; Nicolaides, D. N. J. *Heterocycl. Chem.* **2001**, *38*, 717.
11. Zhang, L.; Jiang, G.; Yao, F.; He, Y.; Liang, G.; Zhang, Y.; Hu, B.; Wu, Y.; Li, Y.; Liu, H. *PLoS One* **2012**, *7*, e 37865.
12. Wang, H.; Zheng, W.; Xie, F.; Song, H. *Youji Huaxue* **2011**, *31*(5), 708.
13. Musa, M. A.; Zhou, A.; Sadik, O. A. *Med. Chem.* **2011**, *7*, 112.
14. Pengo, V.; Denas, G.; Jose, S. P.; Pengo, M. F. *Thromb. Res.* **2010**, *125*, S103.
15. Bobek, V.; Kovařík, J. *Biomed. Pharmacother.* **2004**, *58*, 213.
16. Watanabe, J.; Nishiyama, H.; Matsui, Y.; Ito, M.; Kawanishi, H.; Kamoto, T.; Ogawa, O. *Oncogene* **2006**, *25*, 2500.
17. Zacharski, L. R.; Henderson, W. G.; Rickles, F. R.; Forman, W. B.; Cornell, C. J., Jr.; Forcier, R. J.; Edwards, R. L.; Headley, E.; Kim, S. H.; O'Donnell, J. F.; O'Dell, R.; Tornyos, K.; Kwaan, H. C. *Cancer* **1984**, *53*, 2046.
18. Musa, M. A.; Cooperwood, J. S.; Khan, M. O.; Rahman, T. *Arch. Pharm. (Weinheim)* **2011**, *344*, 102.
19. Musa, M. A.; Badisa, V. L.; Latinwo, L. M.; Waryoba, C.; Ugochukwu, N. *Anticancer Res.* **2010**, *30*, 4613.
20. Musa, M. A.; Khan, M. O.; Cooperwood, J. S. *Lett. Drug Des. Discov.* **2009**, *6*, 133.

21. Kokotos, G.; Theodorou, V.; Tzougraki, C.; Deforce, D.; Van den Eeckhout, E. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2165.
22. Cui, J.; Li, S. *Curr. Med. Chem.* **2014**, *21*, 519.
23. Karcı, F.; Ertan, N. *Dyes Pigm.* **2005**, *64*, 243.
24. Yazdanbakhsh, M. R.; Ghanadzadeh, A.; Moradi, E. *J. Mol. Liq.* **2007**, *136*, 165.
25. Abdel-Wahab, B. F.; Awad, G. E. A.; Badria, F. A. *Eur. J. Med. Chem.* **2011**, *46*, 1505.
26. Gouda, M. A.; Berghot, M. A.; Baz, E. A.; Hamama, W. S. *Med. Chem. Res.* **2012**, *21*, 1062.
27. Kathuria, A.; Jalal, S.; Tiwari, R.; Shirazi, A. N.; Gupta, S.; Kumar, S.; Parang, K.; Sharma, S. K. *Chem. Biol. Interface* **2011**, *1*, 279.
28. Döll, F.; Pfeilschifter, J.; Huwiler, A. *Biochim. Biophys. Acta* **2005**, *1738*, 72.
29. Huwiler, A.; Stabel, S.; Fabbro, D.; Pfeilschifter, J. *Biochem. J.* **1995**, *305*, 777.
30. Tabakovic, K.; Tabakovic, I.; Trkovnik, M.; Trinajstic, N. *Liebigs Ann. Chem.* **1983**, *11*, 1901.
31. Jashari, A.; Hey-Hawkins, E.; Mikhova, B.; Draeger, G.; Popovski, E. *Molecules* **2007**, *12*, 2017.
32. Jashari, A.; Popovski, E.; Mikhova, B.; Nikolova, R. P.; Shivachev, B. L. *Acta Crystallogr.* **2013**, *E69*, o258.
33. Brown, J. M. *Cancer Res.* **1973**, *33*, 1217.
34. Gil-Bernabé, A. M.; Lucotti, S.; Muschel, R. J. *Br. J. Haematol.* **2013**, *162*, 433.
35. Chuang, J. Y.; Huang, Y. F.; Lu, H. F.; Ho, H. C.; Yang, J. S.; Li, T. M.; Chang, N. W.; Chung, J. G. *In Vivo* **2007**, *21*, 1003.
36. Maresca, A.; Temperini, C.; Vu, H.; Pham, N. B.; Poulsen, S. A.; Scozzafava, A.; Quinn, R. J.; Supuran, C. T. *J. Am. Chem. Soc.* **2009**, *131*, 3057.
37. Winum, J.-Y.; Rami, M.; Scozzafava, A.; Montero, J.-L.; Supuran, C. *Med. Res. Rev.* **2007**, *28*, 445.
38. Chen, S.; Cho, M.; Karlsberg, K.; Zhou, D.; Yuan, Y. C. *J. Biol. Chem.* **2004**, *27*, 48071.
39. Musa, M. A.; Cooperwood, J. S.; Khan, M. O. F. *Curr. Med. Chem.* **2008**, *15*, 2664.
40. Jacquot, Y.; Bermont, L.; Giorgi, H.; Refouvelet, B.; Adessi, G.; Daubrosse, E.; Xicluna, A. *Eur. J. Med. Chem.* **2001**, *36*, 127.
41. Braunhut, S. J.; McIntosh, D.; Vorotnikova, E.; Zhou, T.; Marx, K. A. *Assay Drug Dev. Technol.* **2005**, *3*, 77.
42. Mallory, J. C.; Crudden, G.; Oliva, A.; Saunders, C.; Stromberg, A.; Craven, R. J. *Mol. Pharmacol.* **2005**, *68*, 1747.
43. Dozmorov, M. G.; Hurst, R. E.; Culkin, D. J.; Kropp, B. P.; Frank, M. B.; Osban, J.; Penning, T. M.; Lin, H.-K. *Prostate* **2009**, *69*, 1077.