Potential antiproliferative effect of isoxazolo- and thiazolo coumarin derivatives on breast cancer mediated bone and lung metastases

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The study highlights the current progress in the development of coumarin scaffolds for drug discovery as novel anticancer agents in metastatic breast cancer. Eight compounds, combining the coumarin core and five membered heterocycles (isoxazoles and thiazoles) in hydrazinyldiene--chroman-2,4-diones, were characterized in terms of a potential antiproliferative effect on bone (SCP1833) and lung (SCP4175) metastatic breast cancer cell lines using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Cell viability was evaluated after 48 and 72 h of treatment and the 50 % inhibitory concentrations were determined. The results demonstrated dose- and time-dependent activity, with the most potent molecules having a thiazole moiety, without or with additional methyl group(s) attached to the carbon(s) at position(s) 5 and/or 4 in the thiazole ring. These molecules possessed significantly higher potency against both test cell lines compared to 4-hydroxycoumarin.

Keywords: chromandiones, breast cancer, metastatic cells, antiproliferative effect

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Anticancer drugs have traditionally been targeted to damage the aberrantly dividing cell by interrupting the cell division process. The reagents used for this purpose include DNA intercalating agents, topoisomerase inhibitors, cytoskeleton-disrupting agents and antimetabolites. Among these coumarin and coumarin-related compounds, members of the benzo-α-pyrone family, have been proven to exert antitumour effects and cause significant changes in the regulation of immune responses, cell growth and differentiation (1). Antitumour activity is believed to be due to coumarin metabolites (*e.g.,* 7-hydroxycoumarin). Some coumarins display cytostatic properties while others have cytotoxic activities (2). Their inhibitory effect on cell proliferation was confirmed in various carcinoma cell lines, including those of melanoma, leukaemia, renal carcinoma, prostate and breast cancer (1, 3–5). This activity was further validated by other investigators in human subjects as well (6, 7). A signalling pathway component was pointed out as a potential mechanism/cellular target for antitumour activity of coumarins. 7-Hydroxycoumarin has been shown to inhibit tyrosine phosphorylation in EGF-stimulated tumour cells in a time- and dose-dependent manner suggesting that this effect may be achieved by reducing the tyrosine kinase activity of the EGF-receptor (8). In a separate study, in the absence of changes in the cyclin D1 level, mRNA/post-transcriptional effect was observed pointing to the PI-3K/AKT pathway (9). Some coumarins and their active metabolite 7-hydroxycoumarin analogs have shown sulfatase and aromatase inhibitory activities, which is of particular interest in breast cancer chemotherapy. Coumarin based selective estrogen receptor modulators and coumarin-estrogen conjugates have also been described as potential anti-breast cancer agents (10).

Breast cancer is the most common malignancy among females and affects approximately one in every ten women worldwide, being the second cancer type responsible for mortality in women after lung cancer (11). It represents a group of highly heterogeneous lesions consisting of about 20 morphologically distinct subtypes with substantially different molecular and/or biochemical signatures, clinical courses, and prognoses (12). A subset of normal or hyperplastic breast duct clusters showing malignancy-associated changes, including focal disruptions in the surrounding myoepithelial cell layer and basement membrane, expression of p53 and HER-2, and morphological signs of stromal and vascular invasion, can progress directly into invasive or metastatic lesions to other organs in the body, the favourite sites being the bones, lungs, liver or brain (12, 13). Although an incurable condition, metastatic breast cancer treatment, chemotherapy drugs, antiestrogen and biologic therapy, can prolong life, delay the progression of cancer, relieve cancer-related symptoms and improve the quality of life (14–17). Despite an ever-expanding armamentarium of cytotoxics, endocrine therapies, biologics and small-molecule inhibitors, metastatic breast cancer is still the leading cause of death in women aged 40 to 55, which emphasizes the need for new drugs and combination therapies that will selectively act on specific tumour targets and improve the overall survival with low toxicity.

In order to find new coumarin structure based drugs with anticancer activity, with special focus on (metastatic) breast cancer, we have synthesized compounds that combine the coumarin core and five membered heterocycles (isoxazoles and thiazoles) in hydrazinylidiene-chroman-2,4-diones (18). Based on the »hard and soft (Lewis) acids and bases« (HSAB) concept that electrophiles attack the coumarine ring (19), the eight heterocyclic amines, isoxazoles and thiazoles, were derivatized to obtain diazonium ions which would be further used as electrophiles to attack the coumarine ring at position 3. Isoxazole and

thiazole substituents were used based on the literature data pointing to the antiproliferative and tumour vascular-disrupting activity of their derivatives (20–23), with isoxazole and thiazole rings being important pharmacophores. Their anticancer effects were demonstrated *in vivo* (23) and *in vitro* (24–26) using various cancer cell lines, including breast cancer cells. Hence, a superior cellular effect was expected in comparison with 4-hydroxy-coumarin as a reference. Therefore, these isoxazolo- and thiazolo coumarin derivatives were further characterized for potential cytotoxic and apoptogenic effects in the selected breast cancer cell lines, MCF-7 and MDA-MB-231. Three of the eight novel compounds were found to have cancer cell line dependent activity in MDA-MB-231, having IC_{50} values several-fold reduced compared to 4-hydroxycoumarin. Similar results were obtained when antiproliferative effects on prostate cancer (LnCaP) and monocytic leukemia (U937) cell lines were evaluated. Reduced cell viability accompanied by increased apoptosis was shown by PARP cleavage and reduced activity of survival kinase Akt (18).

Considering the cell line dependent activity of the investigated 3-substituted thiazolo and isoxazolo hydrazinylidene-chroman-2,4-diones, the aim of this study was to test their antiproliferative effects in SCP1833 and SCP4175 cell lines. These human MDA-MB-231 derived breast cancer cell lines have different metastatic potentials in terms of their tissue tropisms and aggressiveness. Cell line 1833 is specifically metastatic to the bone, while the highly aggressive cell line 4175 is specific to the lung (27, 28). We found that three of the compounds reduced cell viability in a concentration-dependent manner and exhibited stronger antiproliferative activity than 4-hydroxycoumarin.

EXPERIMENTAL

Synthetic procedure and characterization of synthesized compounds

The synthetic procedure involved derivatization of the appropriate heterocyclic amines to obtain diazonium ions, which were further used as electrophiles to attack the coumarine ring (29). This procedure has already been described in detail in a previous study (18). Briefly, cooled solutions (–10 °C) of the heterocyclic amines (10 mmol) in a mixture of water (10 mL) and HCl (6 mol L^{-1}, 40 mL) were slowly added to the aqueous solution of NaNO$_2$ (0.14 g mL^{-1}) and stirred vigorously. Afterwards, a fresh solution of 4-hydroxy-coumarin (4-HC, 10 mmol, 1.62 g) in 10 mL aqeous solution of NaOH (0.1 g mL^{-1}) was added. The precipitates obtained were vacuum filtered after stirring for 15 min in an ice-salt bath at –10 °C and 30 min at room temperature, washed three times with distilled water and dried in air. The recrystallization with ethanol as a solvent was used to purify the compounds. The reactions were monitored by TLC using different solvents.

Melting points of the compunds were determined on a Reichert heating plate (Reichert-Jung Optische Werke AG, Austria) and were uncorrected. Their structures were evaluated using spectroscopic techniques, including FTIR (Perkin-Elmer System 2000 FTIR, USA), ^{1}H NMR, ^{13}C NMR and 2D NMR (Bruker-250 DRX Spectrometer, USA, using standard Bruker Topspin software) and MS (MS –Q-TOF premierspectrometer (ESI mode), Waters (USA). X-ray crystallography was done on Agilent SuperNova Dual, Cu at zero, Atlas diffractometer (Agilent Technologies, USA) (18, 30).

Chemicals for cell culture studies

Synthesized compounds were dissolved in dimethyl sulfoxide (DMSO) to get 10 mmol L^{-1} 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-diphenyltetrazolium bromide) was purchased from Sigma (USA). FBS, DMEM, antibiotics and trypsin-ethylenediaminetetraacetic acid (EDTA) were obtained from Invitrogen, Switzerland, while 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) was from Roth (Germany).

Cell culturing

Human bone (SCP1833) and lung (SCP4175) metastatic cell lines were derived from the parental breast cancer cell line MDA-MB-231 and the procedure for their generation was described by Kang *et al.* (27) and Minn *et al.* (28). The SCP1833 and SCP4175 cells were cultured in Roswell Park Memorial Institute (RPMI) medium (Life Technologies Europe B.V., Switzerland) containing 4.5 g L^{-1} glucose, 10 % FBS, penicillin (100 units mL^{-1}), streptomycin (100 μg mL^{-1}), 2 mmol L^{-1} N-acetyl-L-alanyl-L-glutamine and 10 mmol L^{-1} HEPES. All cell lines were incubated at 37 °C in an atmosphere containing 5 % CO_2. Cells from exponentially growing cultures were used for experiments.

Cell viability assay

Antiproliferative effects of the novel coumarin derivatives and 4-hydroxycoumarin (as a reference, purity ≥ 98.0%, Merck KGaA, Germany) on SCP1833 and SCP4175 cells were determined using the MTT assay. Briefly, 100 μL of the growth medium (DMEM) was poured into each well of a 96-well plate, plated with 5000 cells per well. Cells were allowed to attach overnight and were then treated with the reference and synthesized compounds in increasing concentrations. After 48 and 72 h incubation at 37 °C, 5 % CO_2 and relative humidity 95 %, 20 μL of MTT reagent (5 mg mL^{-1}) was added to each well and incubated further for 4 h at 37 °C. Afterwards, 100 μL of the solvent consisting of 4 mmol L^{-1} HCl and 0.1 % octylphenoxypolyethoxyethanol (Nonidet P-40, AppliChem, Germany) in isopropanol was added to each well to solubilize the MTT crystals. The plates were covered with a foil and the cells were agitated on an orbital shaker for 15 min. Then, the absorbance was read at 590 nm in a microplate reader (SpectraMax M2 Fluorometer, BucherBiotec Inc., USA). All experiments were performed at least 3 times, with 4 wells for each concentration of the tested agent. Control cells were grown under the same conditions without addition of the test compounds.

Data presentation and statistical analysis

Cell survival was calculated relative to the untreated (vehicle-treated) controls. The 50 % inhibitory concentration (IC_{50}) was determined as the anticancer drug concentration causing 50 % reduction in cell viability and was calculated from the viability curves by linear interpolation between the values immediately above and below the 50 % inhibition using the Bliss software (Bliss Co, Castro Valley, CA 94552, USA).
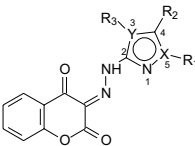
The results were presented as mean ± SD and statistical analysis was performed using the one-way analysis of variance (ANOVA), followed by Bonferroni's *post hoc* test for multiple comparisons (GraphPad InStat version 3.00 for Windows NT, GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Structure and characteristics of the isoxazolo- and thiazolo derivatives of coumarin

Eight 3-substituted coumarin derivatives that combine the coumarin core and five membered heterocycles in hydrazinyldiene-chroman-2,4-diones (Table I, **1a–h**) were previously synthesized by Jashari *et al.* (18). FTIR and NMR studies confirmed the presence of C=O at position 2 in the coumarin ring and substitution of the hydrogen at position 3 with isoaoxozoles and thiazoles *via* a hydralazinylidene linker. The proposed structures were additionally confirmed by MS studies (Table I) (18). Based on the structure of **1f**, for which mono-crystals were succesfully obtained, the hydralazinylidene structure of the synthesized compounds was confirmed using X-ray crystallography (30).

Table I. Structure, yield and characteristics of the novel hydrazinylidiene-chroman-2,4-diones obtained after the reaction of coumarin with the corresponding salts of the heterocyclic amines (18, 30)

Compound	X	Y	R_1	R_2	R_3	Yield (%)	TOF-MS-ES+ (*m/z*)	M. p. (°C)
1a	N	N	H	H	H	93	280[M+Na]$^+$	225–227
1b	O	C		CH_3	H	92	272[M+H]$^+$, 294[M+Na]$^+$	203–205
1c	C	S	H	H		82	274[M+H]$^+$, 296[M+Na]$^+$	209–211
1d	C	S	H	CH_3		63	288[M+H]$^+$, 310[M+Na]$^+$	218–220
1e	C	S	CH_3	CH_3		65	302[M+H]$^+$, 324[M+Na]$^+$	206–208
1f	O	C		*t*-Bu	H	86	314[M+H]$^+$, 336[M+Na]$^+$	225–227
1g	C	S	H	Br		69	353[M+H]$^+$, 375[M+Na]$^+$	217–219
1h	O	C		H	H	74	258[M+H]$^+$, 280[M+Na]$^+$	218–221

Antiproliferation activity towards metastatic cells from breast cancer

Anticancer activities of the synthesized coumarin derivatives (**1a–h**) and 4-hydroxy-coumarin (4-HC) as a reference were evaluated against SCP1833 and SCP4175 metastatic cancer cell lines from breast cancer after 48 h (Figs. 1a and 2a) and 72 h (Figs. 2a and 2b) of

the treatment. Both cancer cell lines were treated with increasing concentrations of the compounds from 1 to 500 µmol L^{-1} (1, 10, 20, 50, 100, 200 and 500 µmol L^{-1}) and the 50 % inhibitory concentrations were then determined from the viability curves (Table II). One can see that cell viabilities were generally concentration- and time-dependent, which was confirmed by the sensitivity of the cancer cell lines, *i.e.*, by the decrease of IC_{50} after 72 h of treatment for almost all of the compounds. Namely, IC_{50} of almost all compounds, including the reference 4-HC, dropped from 50–200 µmol L^{-1} in the 48th hour to 19–100 µmol L^{-1}, after 72 h (Table II). Exceptions were compound **1f** (resembling structure **1b**, *i.e.*, oxygen at position 5 of the pentacycle and methyl replaced with *t*-butyl attached to the carbon at
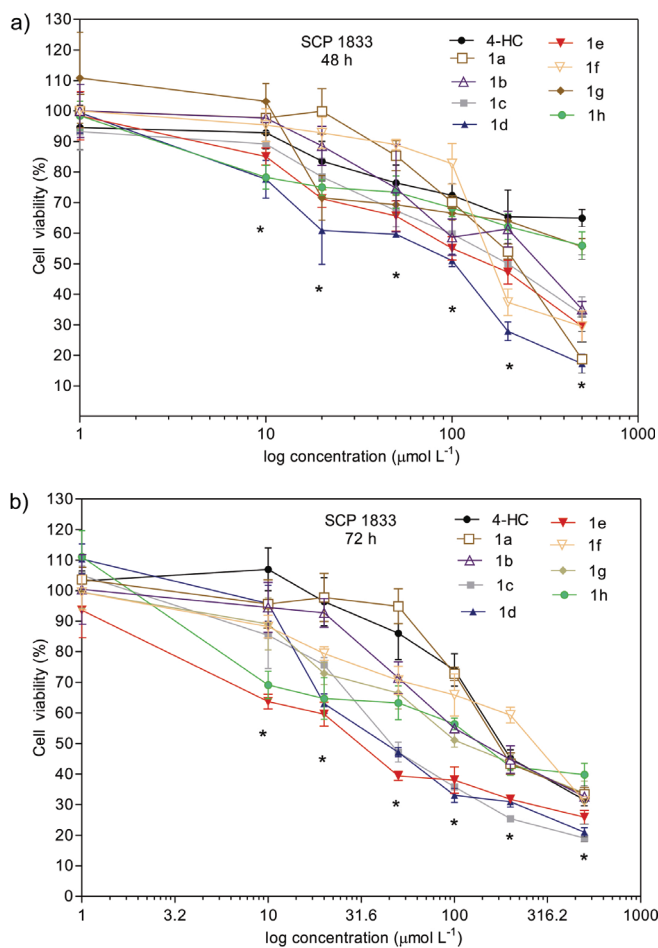

Fig. 1. Viability of the human bone metastatic cell lines from breast cancer, SCP1833, after: a) 48 h and b) 72 h of treatment with different concentrations of the novel chromandiones and the reference 4-hydroxycoumarin, assessed by MTT assay. Mean (boxes) ± SEM (error bars), *n* = 4. *Statistically significant difference from the control (*p* < 0.05) using Dunnett's multiple comparison test.
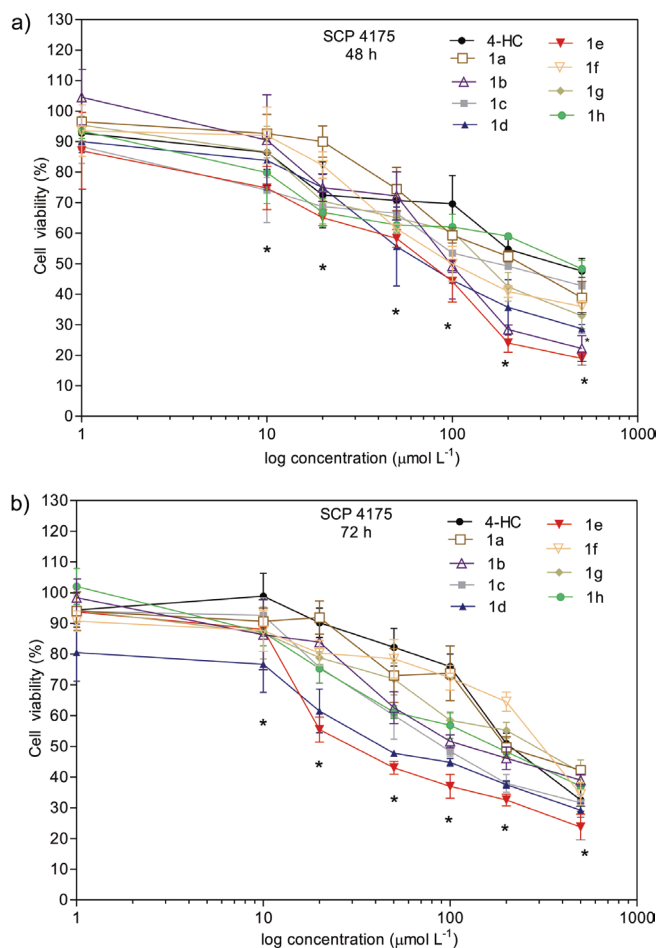
Fig. 2. Viability of the human lung metastatic cell lines from breast cancer, SCP4175, after: a) 48 h and b) 72 h of treatment with different concentrations of the novel chromandiones and the reference 4-hydroxycoumarin, assessed by the MTT assay. Mean (boxes) ± SEM (error bars), $n = 4$. *Statistically significant difference from the control ($p < 0.05$) using Dunnett's multiple comparison test.

position 4) and **1c** (having a thiazole moiety), which showed no time-dependent effect on SCP4175. The potency of compound **1f** for this cell line was lower than that of 4-HC (IC_{50} 200 µmol L^{-1} *vs.* 100 µmol L^{-1}, respectively), while **1c** showed IC_{50} of 50 µmol L^{-1}, which was almost equal to the activity observed after 48-h treatment against the same cell cancer line (Table II).

As reported in the previous study, in which breast cancer cell lines MCF-7 and MDA-MB-231 were used (18), 4-HC did not show significant toxicity in this study either in any of the tested metastatic breast cancer cell lines and its IC_{50} was high, from 100–200 µmol L^{-1} after 72- and 48-h treatment, respectively (Table II).

Table II. Cytotoxicity of 4-hydroxycoumarin and its novel derivatives against metastatic cancer cell lines from breast cancer as determined by the MTT assay[a]

Compound	IC_{50} (µmol L^{-1})			
	Cell line after 48 h		Cell line after 72 h	
	SCP1833	SCP4175	SCP1833	SCP4175
4-HC	> 200	> 200	100.23 ± 2.59	100.79 ± 6.17
1a	> 200	> 200	100.13 ± 4.94	49.96 ± 4.29
1b	> 200	> 200	81.39 ± 3.40	49.79 ± 6.29
1c	99.90 ± 6.81	50.23 ± 1.71	49.57 ± 0.59	49.88 ± 4.55
1d	50.14 ± 1.40	49.85 ± 3.95	19.78 ± 4.46	20.10 ± 1.26
1e	50.05 ± 1.12	50.22 ± 1.69	20.13 ± 1.35	19.78 ± 3.31
1f	> 200	> 200	100.04 ± 4.58	200.23 ± 1.73
1g	> 200	202.52 ± 7.41	50.05 ± 5.89	50.13 ± 5.64
1h	> 200	> 200	95.74 ± 5.85	90.49 ± 2.23

[a] Mean ± SD from at least three independent experiments.

After 48 h of treatment, a similar antiproliferative activity to that of 4-HC against SCP1833 and SPC4175 was observed for compounds **1a**, **1b**, **1f**, **1h** with a nitrogen or oxygen at position 5 of the heterocycle, and **1g** with a sulfur at position 3 of the heterocycle. Derivatives **1c**, **1d** and **1e**, having a sulfur at position 3 of the five membered heterocycle, were more potent against viability of both cell lines after 48 h compared to 4-HC. Within this period of treatment, no significant difference between the effects of these compounds on both cell lines (IC_{50} around 50 µmol L^{-1}) was observed, except for the effect of compound **1c** to which the SCP1833 cell line was less sensitive (IC_{50} 99.90 ± 6.81 µmol L^{-1}, Table II).

The SCP1833 cell line showed similar sensitivity to compounds **1a**, **1f** and **1h** as to reference 4-HC after 72 h of treatment, while sensitivity to other compounds was more pronounced, with compounds **1g** and **1c** being moderately potent (IC_{50} around 50 µmol L^{-1}) and **1d** and **1e** being the most potent (IC_{50} around 20 µmol L^{-1}) compared to 4-HC (IC_{50} around 100 µmol L^{-1}).

Similarly, the sensitivity of SPC4175 after 72 h of treatment was similar to the reference for **1h** and **1f** (IC_{50} between 90 and 100 µmol L^{-1}), while all other compounds had higher cytotoxic effects than 4-HC, with the IC_{50} between 49.79 ± 6.29 (**1b**) and 19.78 ± 3.31 µmol L^{-1} (**1e**).

Specifically, the inhibitory effect of compound **1g**, resembling the active sulfur containing compounds **1c**, **1d**, and **1e**, and having a bromine attached to the carbon at position 4, was time–dependent and this compound was less effective than **1c**, **1d** and **1e** after 48 h of treatment. However, both cell lines responded more intensively after 72 h with **1g** than with **1d** and **1e**, but comparable to **1c**.

Interestingly, compounds **1a** and **1b** having a nitrogen and an oxygen at position 5 of the heterocycle, respectively, showed almost two times higher potency against SCP4175 (IC_{50} around 50 µmol L^{-1}) than against SCP1833 (IC_{50} 100.13 ± 4.94 µmol L^{-1} for **1a** and 81.39 ± 3.40 µmol L^{-1} for **1b**) within 72 h of treatment, pointing to the cell type-dependent

sensitivity. For all other compounds, **1c**, **1d**, **1e** and **1g**, no significant difference in the sensitivity was observed between the two metastatic cell lines from breast cancer.

CONCLUSIONS

Eight compounds that combine the coumarin core and isoxazoles or thiazoles in hydrazinyldiene-chroman-2,4-diones were evaluated for their inhibitory activity against human lung and bone cancer cell lines derived from breast cancer. Of all the evaluated compounds, compounds **1d** and **1e**, followed by **1c**, showed stronger effect on the viability of both cell lines during the whole study period than 4-hydroxycoumarin. The most potent molecules had a thiazole moiety attached to the coumarin ring *via* hydrazinylidene linker at position 3, without or with additional methyl group(s) attached to the carbon at position(s) 5 and/or 4 in the thiazole ring. These results allow structural improvements in the 4-hydroxycoumarin nucleus and encourage efforts towards optimization of their chemo-therapeutic profiles.

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