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Heterocoumarins Are Selective Carbonic Anhydrase IX and XII Inhibitors with Cytotoxic Effects against Cancer Cells Lines

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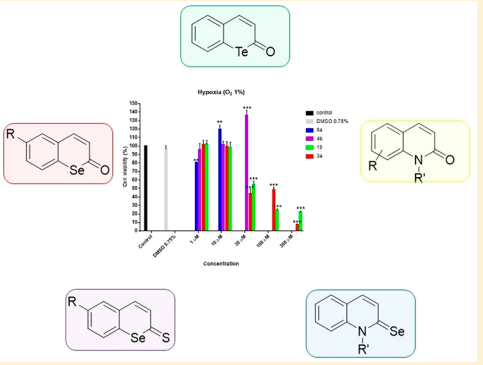
Supporting Information

ABSTRACT: We have synthesized a new series of coumarin-based compounds demonstrating high selectivity and potent effects with low nanomolar affinity against the tumor associated carbonic anhydrase (CA, EC 4.2.1.1) isoforms hCA IX and XII. A number of these compounds were evaluated *ex vivo* against human prostate (PC3) and breast (MDA-MB-231) cancer cell lines. Compounds **4b** and **15** revealed effective cytotoxic effects after 48 h of incubation in both normoxic and hypoxic conditions with PC3 cancer cell line. However, compound **3** showed selective cytotoxic effects against MDA-MB-231 in hypoxic condition. These results may be of particular importance for the choice of future drug candidates targeting hypoxic tumors and metastases, considering the fact that a selective carbonic anhydrase CA IX inhibitor (SLC-0111) is presently in phase II clinical trials.



KEYWORDS: *Carbonic anhydrase inhibitors (CAIs), selenium, metalloenzymes, coumarin, tumor, tellurium*

The coumarin scaffold is found in various natural products and bioactive compounds. For this reason, it is a biologically important and highly privileged structure also in the pharmaceutical field.[1] Coumarin derivatives showed a large number of biological activities, including anticoagulant,[2] antibacterial,[3] antiviral,[4] and recently many different derivatives have been reported as antitumor with interesting cytotoxic activity *in vitro* and *in vivo*.[5,6,23] The interesting heterocyclic system of coumarin derivatives, with their wide range of biological functions, has made them an excellent starting point for further chemical derivatization to identify novel therapeutic agents. In this particular contest, recently, several coumarin derivatives have been shown to constitute a new class of selective inhibitors against the human tumor-associate carbonic anhydrase (CA, EC 4.2.1.1) isoforms hCA IX and hCA XII.[7,8] These two isoforms are active extracellular enzymes involved in metabolism of CO_2, and they have been implicated in acidification of extracellular microenvironment and, at the same time, in protection of cancer cells from the acidosis.[9] For this reason, we developed potent and selective inhibitors from synthetic source with the aim to identify new CAIs structurally related to natural products as coumarins. In the last years, we have been engaged in the development of new synthetic methodologies involving different seleno functionalization of privileged structures[10,11] including their possible medical applications.[12,13] In continuation of our research on the preparation of potentially useful chalcogen derivatives, herein we report a facile and convenient access to synthetic strategies for the replacement of the endocyclic oxygen atom by selenium provides new heterocoumarins. The selenophenyl cinnamate (**2a−b**) needed for the synthesis of selenocoumarins was prepared by reduction of diselenides **1a−b** with $NaBH_4$ to the corresponding selenolate, which was treated in situ with cinnamoyl chloride, affording the compounds **2a−b** in excellent yield. Thus, in order to synthesize selenocoumarins **3a−b**, derivatives **2a−b** were treated with anhydrous $AlCl_3$ in chlorobenzene at 100 °C for 30 min to afford a red reaction mixture, which, after workup, furnished compounds **3a−b** in rather good yield,[14] as reported in Scheme 1.

In order to access further heterocoumarins, we evaluated the possibility to replace the carbonyl group with an isosteric thiocarbonylic moiety to employ an excess of Lawesson's reagent (2 equiv) in refluxing toluene for 4 h. Compounds **4a−b** were obtained in excellent yields as reported in Scheme 2.

We continued the possibility to replace exocyclic oxygen atom by selenium, but this time, our effort did not give the desired results. Thus, our study has been extended to the nitrogen endocyclic system. Cinnamoyl amides, differently substituted, **6a−f** were obtained from the corresponding amines **5a−f** with cinnamoyl chloride in dichloromethane at

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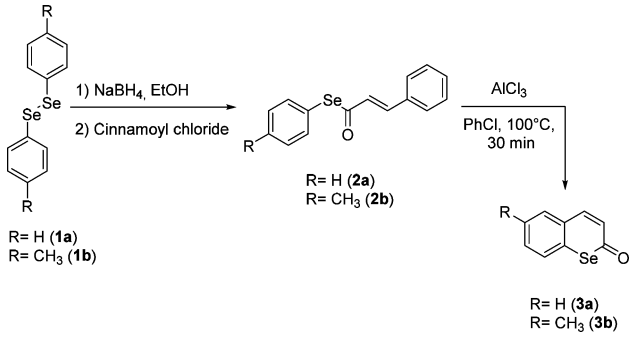
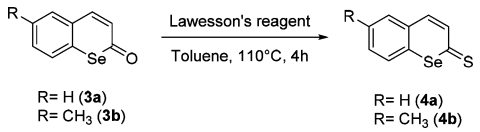
Scheme 1. Synthesis of Selenocoumarins 3a−b

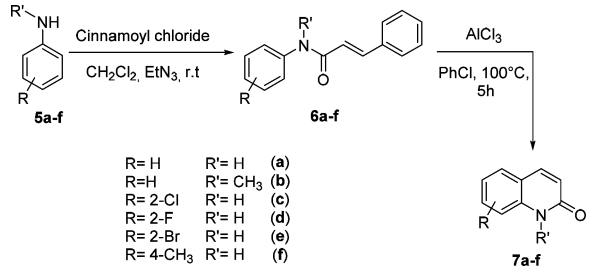


Scheme 2. Synthesis of Thioseleno Coumarins 4a−b



room temperature in quantitative yields. Treatment of compounds **6a−f** with anhydrous $AlCl_3$ in chlorobenzene at 100 °C for 5 h afforded the corresponding quinolin-2(1*H*)-one derivatives **7a−f** with similar mechanistic pathway of compounds **3a−b**. Moreover, the reaction proceeded efficiently with substituents on the nitrogen (**7b**) or on the aryl group (**7a,c−f**) as reported in Scheme 3.[15]

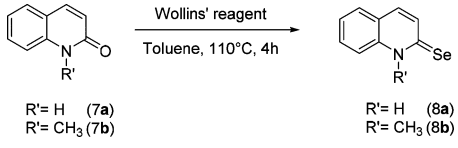
Scheme 3. Synthesis of Different Quinolin-2(1*H*)-one Derivatives 7a−f



In order to access an exocyclic selenium, this time, the isosteric replacement of oxygen was possible in a one-pot selenation reaction with Wollins' reagent. Compounds **7a−b** were readily converted to seleno quinolin-2(1*H*)-one derivatives **8a−b** with an excess of WR (1.5 equiv) in refluxing toluene for 4 h affording in good yield the desiderate compounds as outlined in Scheme 4.

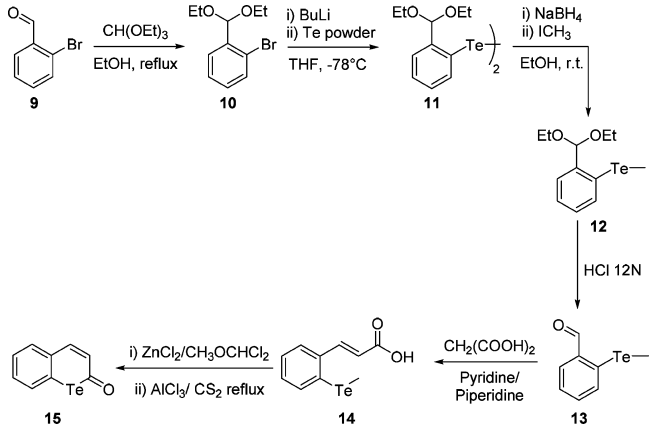
As a further investigation, we tried to synthesize with s similar mechanism of reaction also the endocyclic replacement of oxygen atom by tellurium; however, it proved to be difficult because telluro esters were more instable. In order to propose an alternative way to access tellurocoumarin, we sought to

Scheme 4. Synthesis of Seleno Quinolin-2(1*H*)-one Derivatives 8a−b



achieve it from cinnamic acid derivative **14**, thus avoiding the synthesis of telluro esters. The aldehyde group of compound **9** was protected by acetalization with triethyl orthoformate in refluxing ethanolic solution. Diaryl ditelluride **11** was prepared from aryl bromide **10** via lithiation, tellurium insertion, and oxidation in analogy with a literature procedure.[16] The ditelluride acetal derivative **11** was then hydrolyzed with concentrated HCl, and the resulting aldehyde **13** wa scondensed with a Knoevenagel reaction with malonic acid to afford the corresponding cinnamoyl acid derivative **14**. Finally, cyclization into tellurocoumarin **15** was obtained by converting compound **14** *in situ* in the corresponding acyl chloride and subsequently treated with $AlCl_3$ in dichloromehtyl methyl ether as reported previously in the literature[17] and outlined in Scheme 5.

Scheme 5. Synthesis of Tellurocoumarin 15



All synthesized compounds, **3a−b**, **4a-b**, **7a-f**, **8a-b** and **15**, were tested in vitro for their inhibitory properties against the physiologically relevant hCA isoforms (I, II, IX, and XII) by means of a stopped-flow carbon dioxide hydration assay[18] after a period of 6 h of incubation of the enzyme and inhibitor solutions.[19−21] Their activities were compared to the standard carbonic anhydrase inhibitor (CAI) acetazolamide (**AAZ**) (Table 1).

From the analysis of constants of inhibition values reported in Table 1, we observed that all compounds, according to previous reports,[19−21] were ineffective inhibitors of two dominant cytosolic hCA I and hCA II showing high selectivity against tumor-associated isoforms hCA IX and hCA XII. In terms of structure−activity relationships, it could be observed that methyl moiety in position 6 of seleno-coumarins **3a−b** and **4a−b** played a crucial role for activity. This moiety increased near two times the potency against hCA IX and over three times for hCA XII. Moreover, the isosteric substitution of carbonyl with a thiocarbonyl group did not influence significantly the activity. Methyl substituent on nitrogen of quinolin-2(1*H*)-one **7b** led to an increase in the activity twofold against hCA IX compared to compound **7a** (K_i 44 nM to 82 nM). However, different substituents in position 8 (**7c−e**) increase the potency for both tumor-associated isoforms. The replacement of carbonyl group with selenocarbonyl moiety (**8a−b**), this time, influenced the activity especially for compound **8b**, showing a decrease of near four times that of compound **7b** (K_i 44 nM to 172 nM). Finally, tellurocoumarin **15** showed an interesting inhibition pattern compared to

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Table 1. Inhibition Data of Human CA Isoforms I, II, IX, and XII with Compounds 3a−b, 4a−b, 7a−f, 8a−b, 15, and AAZ by a Stopped Flow CO_2 Hydrase Assay[18]

	K_i (nM)[a]			
compd	hCA I	hCAII	hCA IX	hCA XII
3a	>10000	>10000	26.3	22.9
3b	>10000	>10000	56.1	7.6
4a	>10000	>10000	39.5	25.8
4b	>10000	>10000	59.9	7.4
7a	>10000	>10000	82.0	93.0
7b	>10000	>10000	44.6	83.5
7c	>10000	>10000	23.0	6.8
7d	>10000	>10000	58.8	6.8
7e	>10000	>10000	23.4	8.4
7f	>10000	>10000	123.1	8.7
8a	>10000	>10000	54.4	7.6
8b	>10000	>10000	172.0	86.4
15	>10000	>10000	59.0	8.2
AAZ	250.0	12.1	25.8	5.7

[a]Mean from three different assays, by a stopped flow technique (errors were in the range of ±5−10% of the reported values).

selenocoumarin **3a**. The biggest chalcogen atom for compound **15** led to a decrease in potency near two times **3a** for hCA IX (K_i 59 nM to 26.3 nM), but for the other membrane isoform hCA XII, the activity increased near three times that of selenocoumarin (K_i 8.2 nM to 22.9 nM), thus showing tellurocoumarin is more selective against hCA XII.

The high isoform selectivity and upregulated expression of hCA IX and XII in a wide selection of hypoxic tumors make these compounds a desirable feature for compounds designed to target the tumor-associated enzymes. We focused our attention on the *ex vivo* activity of compounds **3a**, **4b**, **8a**, and **15**, which were evaluated for their effects on cell viability against the human prostate (PC3) and breast (MDA-MB-231) cancer cell lines. All compounds were highly selective hCA IX and XII inhibitors and were used at different concentrations, being incubated for 48 h in both normoxic and hypoxic conditions, when overexpression of high amounts of CA IX occurs.[22] In PC3 cells, seleno quinolin-2(1*H*)-one **8a** did not show any cytotoxic activity in normoxic and hypoxic conditions. However, selenocoumarin **3a** started to reduce the cell viability only at higher concentration (300 μM) to 68% in both conditions. Its efficacy increased significantly when selenium endocyclic was replaced by tellurium; this isosteric substitution, in normoxic condition, led to a reduced viability of 54% already at 30 μM and increased to 19% at higher concentrations (Figure 1). In the hypoxic condition, indeed, compound **15** showed less effect on cytotoxicity, which reached 71% at 30 μM and 38% at 100−300 μM. Moreover, the thioselenocoumarin derivative **4b** showed at 30 μM similar cytotoxicity effect to compound **15** in normoxic and hypoxic conditions (51% and 79%, respectively).

Derivative **8a** also in the MDA-MB231 cell line did not show any activity in these *ex vivo* normoxia and hypoxia assays. However, seleno-coumarin **3a** showed only at 300 μM a good cytotoxic activity in normoxic condition (28%). The potency against this cancer cell line increased drastically when compound **3a** was used in hypoxic condition. Indeed, this compound reduced cell viability already by more than 50% at 30 μM and arrived to kill over 90% at 300 μM (Figure 2), showing, thus, an interesting selectivity against this specific

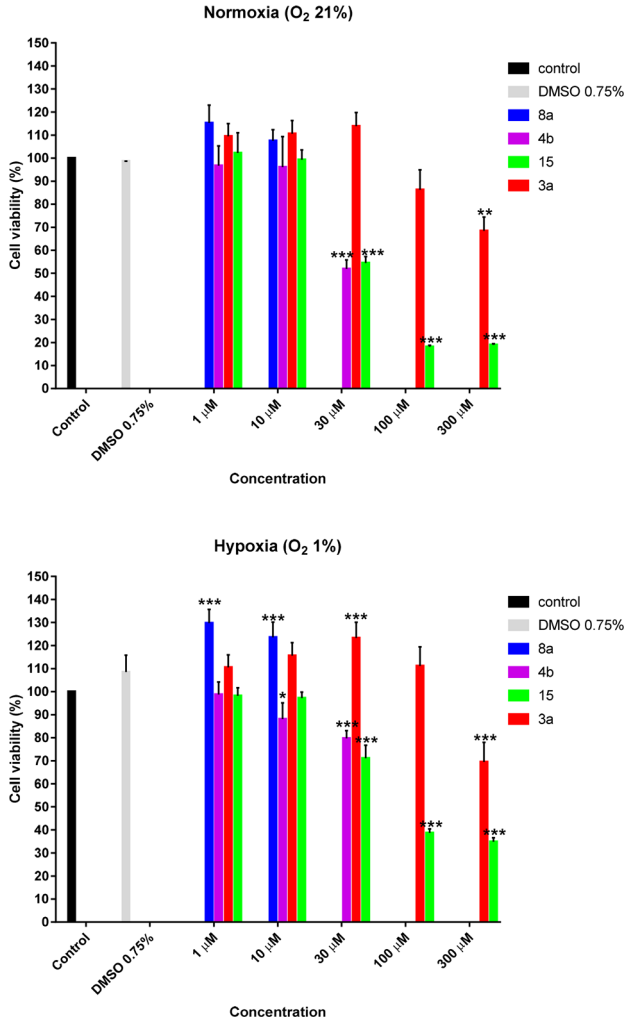

Figure 1. Effects of newly synthesized compounds **3a**, **4b**, **8a**, and **15** on viability of the human prostatic cancer cell line PC3 following 48 h treatment in normoxic and hypoxic (1% O_2) conditions. *p < 0.05, **p < 0.01, ***p < 0.001 versus control.

cancer cell line in hypoxic condition. Also this time, tellurocoumarin **15** exhibited a strong cytotoxicity in normoxic conditions, already at 30 μM (15.9%). The potency decreased over three times in hypoxic condition, reducing the cell viability to 22% with a concentration of 300 μM. A reduced cell viability (61%) was observed also for compound **4b** only at 30 μM. In the hypoxic condition, this compound did not show any significant activity.

In conclusion, we report a new series of different chalcogen-coumarins and quinolin-1(2*H*)-one. These compounds were evaluated for their inhibitory properties against the two dominant cytosolic isoforms hCA I and II and the tumor associated isoforms hCA IX and XII. All derivatives, here investigated, were ineffective against the off-target cytosolic hCA I and II, whereas they showed interesting selective and potent inhibition profiles (in the low nanomolar range) against the tumor associated isoforms hCA IX and XII. For this reason, different compounds were evaluated *ex vivo* against two cancer cell lines PC3 and MDA-MB231. In particular, compounds **3a** and **15** revealed an interesting cytotoxic effect after 48 h of incubation. These results may be of particular importance for the choice of future drug candidates targeting hypoxic tumors.

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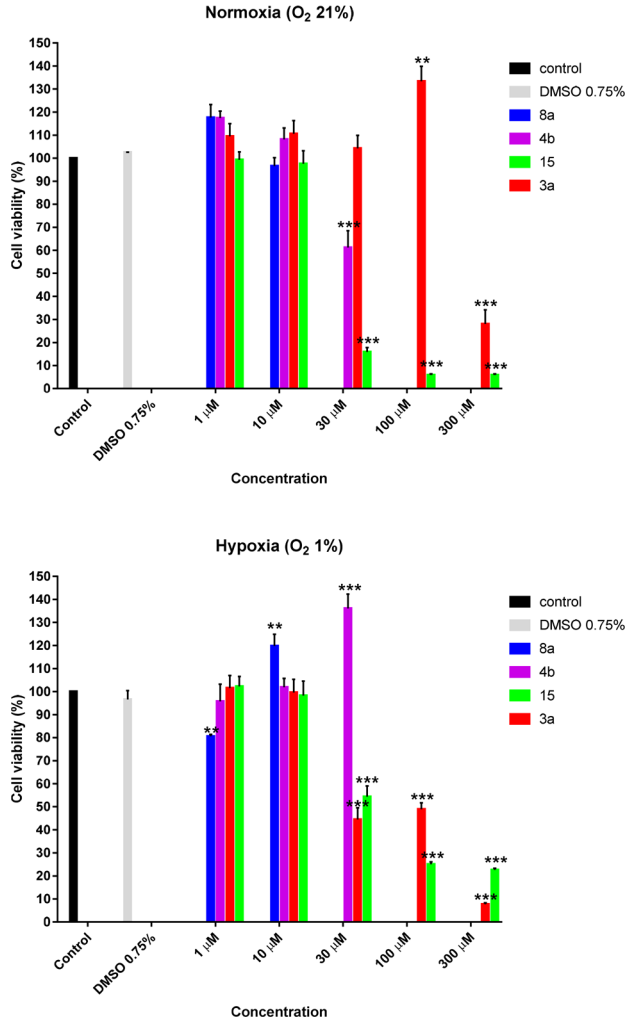

Figure 2. Effects of the newly synthesized compounds **3a**, **4b**, **8a**, and **15** on viability of the human adenocarcinoma breast cell line MDA-MB231 following 48 h treatment in normoxic and hypoxic (1% O_2) conditions. $^{**}p < 0.01$, $^{***}p < 0.001$ versus control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00362.

Synthetic procedures, characterization of compounds, in vitro kinetic procedure, and biological assay (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CAs, carbonic anhydrases; AAZ, acetazolamide

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