Article

Novel 6- and 7-Substituted Coumarins with Inhibitory Action against Lipoxygenase and Tumor-Associated Carbonic Anhydrase IX

Aikaterini Peperidou 1,2, Silvia Bua 1, Murat Bozdag 3, Dimitra Hadjipavlou-Litina 2 and Claudiu T. Supuran 1,*

1 Dipartimento Neurofarba, Sezione di Scienze Farmaceutiche e Nutraceutiche, Università degli Studi di Firenze, Via Ugo Schiff 6, 50019 Sesto Fiorentino (Firenze), Italy; peperidou@pharm.auth.gr (A.P.); silvia.bua@unifi.it (S.B.)
2 Department of Pharmaceutical Chemistry, School of Pharmacy, Faculty of Health Sciences, 54124 Thessaloniki, Greece; hadjipav@pharm.auth.gr
3 Dipartimento di Chimica, Laboratorio di Chimica Bioinorganica, Università degli Studi di Firenze, Via della Lastruccia 3, 50019 Sesto Fiorentino (Firenze), Italy; bozdag.murat@unifi.it

* Correspondence: claudiu.supuran@unifi.it; Tel./Fax: +39-055-4573729

Received: 11 December 2017; Accepted: 11 January 2018; Published: 12 January 2018

Abstract: A series of carboxamide derivatives of 6- and 7-substituted coumarins have been prepared by an original procedure starting from the corresponding 6- or 7-hydroxycoumarins which were alkylated with ethyl iodoacetate, and the obtained ester was converted to the corresponding carboxylic acids which were thereafter reacted with a series of aromatic/aliphatic/heterocyclic amines leading to the desired amides. The new derivatives were investigated as inhibitors of two enzymes, human carbonic anhydrases (hCAs) and soy bean lipoxygenase (LOX). Compounds 4a and 4b were potent LOX inhibitors, whereas many effective hCA IX inhibitors (K$_{i}$s in the range of 30.2–30.5 nM) were detected in this study. Two compounds, 4b and 5b, showed the phenomenon of dual inhibition. Furthermore, these coumarins did not significantly inhibit the widespread cytosolic isoforms hCA I and II, whereas they were weak hCA IV inhibitors, making them hCA IX-selective inhibitors. As hCA IX and LOX are validated antitumor targets, these results are promising for the investigation of novel drug targets involved in tumorigenesis.

Keywords: coumarins; carboxamides; carbonic anhydrase; lipoxygenase; enzyme inhibitor

1. Introduction

Vertebrates, including humans, encode for a multitude of metalloenzymes belonging to the carbonic anhydrase (CA, EC 4.2.1.1) family of proteins [1–4]. Although seven CA genetic families are known to date (α-, β-, γ-, δ-, ε-, η- and θ-CAs) [2,5], only α-CAs are present in humans, but as 15 different isoforms, 12 of which are catalytically active and involved in a multitude of physiologic functions [3–9]. By catalyzing the reversible hydration of CO$_2$ to bicarbonate, with the release of a hydronium ion, in humans CAs are involved in pH regulation, biosynthetic reactions, electrolyte secretion, excretion, tumorigenesis, etc. [3,4,6–9]. CA inhibitors (CAIs) are in pharmacological/clinical use for decades for the treatment of glaucoma [6,7], for the imaging and treatment of hypoxic tumors [3,4,8,9], as anti-obesity agents [10], or as diuretics [11]. Recently these pharmacological agents were validated for the management of neuropathic pain [12], but the sulfonamides, which are the main class of CAIs [11–13] possess a rather large number of side effects, as they indiscriminately inhibit all catalytically active CA isoforms, and not only the ones targeted for a specific application [1–3,13–17]. Thus, alternative classes of CAIs to the sulfonamides and their isosteres were explored in the last
period [14], which led to the discovery of several totally different inhibition mechanisms and families of inhibitors [14]. Among them, the coumarins are among the most relevant ones for several reasons [18]. Discovered initially in a natural product library isolated from an Australian biota [18], the coumarins were demonstrated to possess a very particular inhibition mechanism [18,19]. Indeed, they act as prodrug, suicide inhibitors which undergo a hydrolytic process within the enzyme active site with generation of 2-hydroxycinnamic acid derivatives [18,19]. These relatively bulky compounds cannot bind to the catalytic metal ion, which is a Zn(II) ion in α-CAs, and is situated deep within the active site [1–3]. Instead, the hydrolyzed coumarins were observed (by means of X-ray crystallography) to be bound at the entrance of the active site cavity, which is rather large for the hCAs [1–3,18,19]. Furthermore, that is the only region of the active site which is the most variable between the 12 catalytically active isoforms, which may explain why the coumarins and their derivatives are among the most isoform-selective CAIs known to date [19–25]. Indeed, extensive drug design campaigns in which various parts of the coumarin moiety were changed, showed the useful as well as the detrimental substitution patterns as well as the tolerated or less tolerated substituents that can be appended to the ring system in order to obtain effective and isoform-selective CAIs [18–25]. Among the most effective coumarin CAIs detected in this way it has been observed that 6- and 7- or 6,7-disubstituted derivatives possess an effective inhibition of the tumor-associated isoforms CA IX and XII, whereas they are poor inhibitors or do not significantly inhibit the widespread “house-keeping” isoforms hCA I and II (the inhibition of which is responsible for the side effects of the sulfonamide CAIs [1–4]). Thus, here we continue our research in developing non-sulfonamide CAIs and report a new series of coumarins possessing 6- and 7 moieties which have not been explored earlier, of the ether-carboxamide type.

Lipoxygenase (LOX) plays a major role in many inflammatory diseases including chronic obstructive pulmonary disease (COPD), asthma, chronic bronchitis, cancer including pancreatic, gastric and brain tumors. Similarly to different isozymes of CA, such as CA II, LOX is expressed in pancreatic, gastric as well as brain tumors [26]. It should be mentioned that morphological cells changes and CA activity are used to determine the effect of LOX inhibitors on cancer cell differentiation [26]. LOX is upregulated in cancer cells and arachidonic acid as well as its metabolites, 5-HETE and 12-HETE, stimulate mitogenesis of human pancreatic cancer cells. Furthermore, blockade of LOX pathways abolishes cancer cell proliferation in vitro and induces cancer cell apoptosis [27]. The development of coumarins as antioxidant agents, anticancer and LOX inhibitors has attracted much attention recently [27–29]. Several reviews and research papers have updated and expanded the knowledge in this field [27–29]. In recent years, intensive research has been conducted on creating new polyfunctional drugs [30,31]. For the treatment of complex diseases e.g., neurological disorders, cancer and inflammation, in which more than one target is implicated, a combination of drugs is frequently used. Therefore, novel potent inhibitors of both LOX and CA II are required to explore the role of these enzymes further and to enable the drug discovery efforts. Thus, we considered of interest to prepare and test new compounds as dual CA and LOX inhibitors.

2. Results and Discussion

A large number of variously substituted coumarins were reported to act as CAIs [18–25] and to also possess diverse other biological/pharmacological actions [30]. For example, the development of coumarins as antioxidant agents, anticancer and LOX inhibitors has attracted much attention [27,29,31–33]. It has also been reported that antioxidant polyphenols structurally related to coumarins effectively inhibited CAs [34]. In recent years, intensive research has been conducted for creating new polyfunctional drugs for the treatment of complex diseases, in which more than one target is implicated [30–35].

No carboxamide derivatives of coumarins at the 6- or 7-position of the ring were explored so far. Thus, the drug design strategy was to obtain the carboxymethyl-oxy derivatives 3a and 3b, which possess a reactive COOH moiety, easy to derivatize with aromatic, aliphatic or heterocyclic amines, in order to generate chemical diversity. Thus, commercially available 6- or
7-hydroxy-coumarins 1a–b were reacted with ethyl iodoacetate leading to esters 2a and 2b, which were then hydrolysed in alkaline medium to the corresponding acids 3a and 3b (Scheme 1).

Scheme 1. Preparation of the key intermediate carboxylic acids 3a and 3b.

The two carboxylic acids 3a and 3b were converted to the corresponding amides by reaction with aromatic, aliphatic and heterocyclic primary amines, as shown in Scheme 2, by using carbodiimide chemistry. The nature of the various amines was chosen in such a way as to generate the widest possible chemical diversity (Scheme 2). All compounds were extensively characterized by spectral and other physico-chemical procedures which proved their structure (see Experimental part for details). The new coumarins (5a,b–6a,b, 9a,b–13a,b) and previously reported coumarins (2a–b, 3a–b, 7a–b, 8a–b) were investigated here for the inhibition of four physiologically relevant CA isoforms, hCA I and II (cytosolic, widespread isoforms, involved in glaucoma and other eye diseases [1,3,6], hCA IV (membrane-bound isoform highly abundant in the kidney and lungs and involved in diuresis, respiration and retinitis [11] as well as hCA IX (tumor-associated, transmembrane isoforme, a newly validated antitumor target [3,4,9]. A stopped-flow CO₂ hydrase assay has been used for monitoring the inhibition of these CAs with the new coumarins and acetazolamide (AAZ, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, a clinically used CAI) as standard inhibitor [1,3].

As seen from the data in Table 1, like other coumarins investigated by our group these derivatives also do not inhibit the cytosolic isoforms hCA I and II up to 10 µM concentration of inhibitor in the assay system. hCA IV was also poorly inhibited, with most compounds being inactive whereas few of them showed activity in the high nanomolar range (e.g., 8b, 9a,b and 11a, with Kᵢ in the range of 350.4–848.3 nM). Several other coumarins, including 2a, 3a and 7b, were micromolar hCA IV inhibitors, with Kᵢ in the range of 2.65–8.48 µM. Thus, the 4-fluoroanilides of
both 2-((2-oxo-2H-chromen-7-yl)oxy)acetic acid as well as its 6-isomer led to the best inhibitors of this isoform.

hCA IX on the other hand was effectively inhibited by most new coumarins reported here, except for 12a and 12b which were not hCA IX inhibitors up to 10 µM (Table 1). These compounds incorporate the morpholine-ethylamide moiety which is obviously inappropriate for obtaining effective CAIs in that position of the coumarin ring and with this type of substitution pattern. The remaining compounds showed an interesting hCA IX inhibitory patterns, with several compounds being quite effective inhibitors, with $K_I$s in the range of 30.2–30.5 nM, similar to AAZ ($K_I$ of 25 nM).

Table 1. Inhibition data of CA I, II, IV and IX with compounds reported here and the standard sulfonamide inhibitor acetazolamide (AAZ) by a stopped flow CO$_2$ hydrase assay [36].

| Compound | $K_I$ (nM) * |
|----------|-------------|
|          | hCA I | hCA II | hCA IV | hCA IX |
| 2a       | >10,000 | >10,000 | 5572   | 247.1 |
| 2b       | >10,000 | >10,000 | >10,000 | 2044  |
| 3a       | >10,000 | >10,000 | 8480   | 290.2 |
| 3b       | >10,000 | >10,000 | >10,000 | 194.9 |
| 4a       | >10,000 | >10,000 | >10,000 | 165.7 |
| 4b       | >10,000 | >10,000 | >10,000 | 30.5  |
| 5a       | >10,000 | >10,000 | >10,000 | 83.7  |
| 5b       | >10,000 | >10,000 | >10,000 | 30.2  |
| 6a       | >10,000 | >10,000 | >10,000 | 2536  |
| 6b       | >10,000 | >10,000 | >10,000 | 2785  |
| 7a       | >10,000 | >10,000 | >10,000 | 200.6 |
| 7b       | >10,000 | >10,000 | 2649   | 201.9 |
| 8a       | >10,000 | >10,000 | 350.4  | 136.1 |
| 8b       | >10,000 | >10,000 | 848.3  | 145.6 |
| 9a       | >10,000 | >10,000 | >10,000 | 2732  |
| 9b       | >10,000 | >10,000 | >10,000 | 2041  |
| 10a      | >10,000 | >10,000 | >10,000 | 2377  |
| 10b      | >10,000 | >10,000 | >10,000 | 2147  |
| 11a      | >10,000 | >10,000 | 766.4  | 122.3 |
| 11b      | >10,000 | >10,000 | >10,000 | 1962  |
| 12a      | >10,000 | >10,000 | >10,000 | >10,000 |
| 12b      | >10,000 | >10,000 | >10,000 | >10,000 |
| 13a      | >10,000 | >10,000 | >10,000 | 1969  |
| 13b      | >10,000 | >10,000 | >10,000 | 273.7 |
| AAZ      | 250    | 12     | 74     | 25    |

* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ±5–10% of the reported values, data not shown).

These compounds, 4b and 5b, are the phenethylamide and benzylamide derivatives of 2-((2-oxo-2H-chromen-6-yl)oxy)acetic acid 3b and they are much more effective CA IX inhibitors compared to the corresponding 6-isomers 4a and 5a (Table 1). However, this was not always the case, as for other pairs of isoforms, the 7-isomer was a better hCA IX inhibitor compared to the corresponding 6-isomer (e.g., 2a, which is a better inhibitor than 2b; 11a, a much more effective CA IX inhibitor compared to its isomer 11b, etc.). Many other coumarins were slightly less effective hCA IX inhibitors, with $K_I$s in the range of 83.7–290.2 nM. They include derivatives 2a, 3a, 3b, 4a, 5a, 7a, 7b, 8a, 8b, 11a, 13b (Table 1). It is thus obvious that apart from the position in the coumarin ring where the substituent is appended, the most important factor influencing hCA IX inhibition is the nature of the moiety present on the amide part of the functionality. Indeed, the effective hCA IX inhibitors incorporate amides obtained from phenethylamine, benzylamine, aniline and substituted anilines. The only heterocyclic derivative leading to effective inhibitors was 4-pyridylmethylamine.
and piperidin-1-yl-ethylamine. The remaining amides (2b, 6a, 6b, 9a, 9b, 10a, 10b, 11b, 13a) were micromolar hCA IX inhibitors, with Kᵢ values in the range of 1.96–2.73 µM (Table 1).

An important feature of many coumarins reported here is that they are highly selective hCA IX versus hCA I/II/IV inhibitors, and in many cases also very effective in inhibiting the tumor-associated isoform hCA IX without inhibition of the widespread cytosolic/membrane-bound isoforms I; II and IV. For example 4b and 5b are equipotent to acetazolamide as hCA IX inhibitors but do not inhibit all hCA I, II and IV, whereas AAZ inhibits these three isoforms significantly (Table 1).

In vitro inhibition of soybean lipoxygenase (LOX) has also been investigated with the new coumarins reported here (Table 2). Eicosanoids are oxygenated metabolites of arachidonic acid with a broad implication in a diversity of diseases among which are included the pathogenesis of neutrophil-mediated inflammatory diseases with a marked relation to the severity of cardiovascular diseases, asthma and cancer [36].

Table 2. In vitro inhibition of soybean LOX (IC₅₀ µM or % LOX inhibition) [34] and lipophilicity values of 6- or 7-substituted coumarin derivatives 1–13 and their clogP values.

| Compounds | ClogP a | % LOX Inhibition at 100 Mµ b | IC₅₀ (µM) b |
|-----------|---------|----------------------------|-----------|
| 1a (7-HC) | 1.62    | Reference compound         | 43 µM c   |
| 1b (6-HC) | 1.62    | 40%                        | nt e      |
| 2a d      | 1.89    | 16%                        | nt e      |
| 2b d      | 1.89    | 41%                        | nt e      |
| 3a d      | 1.03    | 50%                        | 100 µM    |
| 3b d      | 1.03    | 50%                        | 100 µM    |
| 4a        | 2.60    | 96%                        | 10 µM     |
| 4b        | 2.60    | 85%                        | 10 µM     |
| 5a        | 2.39    | 45%                        | nt e      |
| 5b        | 2.39    | 42%                        | nt e      |
| 6a        | 2.53    | 63%                        | 47 µM     |
| 6b        | 2.53    | 26%                        | nt e      |
| 7a        | 2.36    | 50%                        | 100 µM    |
| 7b        | 2.36    | 45%                        | nt e      |
| 8a        | 2.76    | 43%                        | nt e      |
| 8b        | 2.76    | 11%                        | nt e      |
| 9a        | 2.80    | 33%                        | nt e      |
| 9b        | 2.80    | 50%                        | 100 µM    |
| 10a       | 1.11    | 79%                        | 15 µM     |
| 10b       | 1.11    | 40%                        | nt e      |
| 11a       | 0.89    | 77%                        | 27 µM     |
| 11b       | 0.89    | 37%                        | nt e      |
| 12a       | 0.91    | 56%                        | 42.5 µM   |
| 12b       | 0.91    | 64%                        | 16.5 µM   |
| 13a       | 2.32    | 50%                        | 100 µM    |
| 13b       | 2.32    | 37.6%                      | nt e      |
| NDGA      | 93%     | 0.45 µM                    |           |

Means within each column differ significantly (p < 0.05). a ClogP values were measured by using the Biobyte C-QSAR [37]. b Values are means (±SD < 10%) of three or four different determinations. c From reference [38]. d Values are referred to 2a, 3a, 2b and 3b coumarin acetic acid derivatives [38]. e nt, not tested (IC₅₀ values not found due to the fact that it may be >100 µM).

In this context, we evaluated the synthesized compounds of Table 2 for their ability to inhibit soybean LOX by the UV absorbance based enzyme assay [34] using compounds samples with concentrations from 0.1–100 µM. Most of the LOX inhibitors are antioxidants or free radical scavengers. LOXs contain a non-heme iron per molecule in the enzyme active site as high-spin Fe²⁺ in the native state and the high spin Fe³⁺ in the activated state [35]. Some studies suggest a relationship between LOX inhibition and the ability of the inhibitors to reduce Fe³⁺ at the active site to the catalytically inactive Fe²⁺, whereas several LOX inhibitors are excellent ligands.
for Fe$^{3+}$ [35]. Nordihydroguaiaretic acid (NDGA), a known inhibitor of soybean LOX, has been used as a reference compound (IC$_{50}$ 0.45 µM/93% at 100 µM) and as a positive control in our experiments [35]. We determined the IC$_{50}$ inhibition values for compounds 1a, 3a–b, 4a–b, 6a, 7a, 9b, 10a, 11a, 12a–b, 13a. We did not succeed to evaluate the IC$_{50}$ values for the rest of the compounds, since they were not active LOX inhibitors at 100 µM (11–46%). The most potent % inhibition at 100 µM is shown by compound 4a (4a > 4b > 10a~11a > 12b~6a).

Perusal of the IC$_{50}$’s inhibition values (Table 2) shows that the most potent, and equipotent, inhibitors are compounds 4a and 4b (10 µM) followed by 10a (15 µM) and 12b (16.5 µM). It is interesting to note that attachment on the coumarin ring, e.g., in the 6-/7- for compounds 4a and 4b, does not seem to play any role. Replacement of phenyl (4a) by a 2-pyridyl group (10a) or by a morpholinyl group (12a) leads to a reduction of the inhibitory activity, which is highly significant for 12a (42.5 µM). The presence of a 2-pyridyl group in compound 10b significantly decreased activity (by 40%) compared to 4b. In a similar manner, the presence of a 4-pyridyl group (11a) resulted in significant loss of inhibitory activity (27 µM) compared to compound 10a. The replacement by a morpholinyl group (12b) does not induce a considerable loss in activity.

The length of the chain between the aromatic ring and the NHCO-group [(CH$_2$)$_n$], influenced the biological response, since compound 4a (10 µM) with n = 2, is more potent compared to 7a (100 µM) in which n = 0 and 5a (45%) in which n = 1. The same is seen for 7b and 5b. The F-substitution allows an improved inhibitory activity compared to the unsubstituted compound: for example 6a has an IC$_{50}$ of 47 µM, whereas 5a only presents 45% at a concentration of 100 µM (Table 2). As concerns the acids 3a and 3b they appear to present some inhibitory activities (Table 2). Although lipophilicity is referred to as an important physicochemical property for LOX inhibitors [35], herein the theoretically calculated log P values did not always support this observation. The most potent compounds 4a and 4b showed the third higher lipophilicity values (2.60) in this series (Table 2). Furthermore, compounds with comparable lipophilicities showed in many cases striking different LOX inhibitory activities (Table 2).

3. Experimental Section

3.1. General Information

All biochemical reagents were of analytical grade and purchased from commercial sources. Soybean lipoxigenase, sodium linoleate, and NDGA were obtained from Sigma Chemical, Co. (St. Louis, MO, USA).

3.2. Chemistry

3.2.1. General Procedure for the Synthesis of Compounds 3a–b [39]

A mixture of 7-hydroxycoumarin (1a) or 6-hydroxycoumarin (1b) (1 eq.) and potassium carbonate (3 eq.) was dissolved in dry DMF (5 mL) and the mixture was stirred at room temperature for 15 min. Then, ethyl 2-iodoacetate (a, 1.5 eq.) was added dropwise to the mixture under nitrogen atmosphere and heated to 100 °C for 30 min. After completion of the reaction (TLC monitoring) the mixture was cooled to room temperature and quenched with water and 1M aqueous HCl solution. The precipitated products 2a–b were collected by filtration and washed with water, and used without further purification.
Ethyl 2-((2-oxo-2H-chromen-7-yl)oxy)acetate (2a). Using 7-hydroxycoumarin and, ethyl 2-iodoacetate as starting materials and the general procedure described above compound 2a was obtained in 94% yield; m.p. 112.9–113.0 °C; δH (400 MHz, CDCl3) 1.31 (3H, t, J = 7.2 Hz), 4.28 (2H, q, J = 7.2 Hz), 4.68 (2H, s), 6.27 (1H, d, J = 9.5 Hz), 6.78 (1H, d, J = 2.4 Hz), 6.88 (1H, dd, J = 2.4, 8.6 Hz), 7.39 (1H, d, J = 8.6 Hz), 7.63 (1H, d, J = 9.5 Hz); δC (100 MHz, CDCl3) 14.3, 61.9, 65.5, 101.9, 113.0, 113.5, 113.9, 129.1, 143.3, 155.8, 161.0, 161.0, 168.0; m/z (ESI positive) (C13H12O5) 249.2 [M + H]+. Experimental data are in agreement with those reported in [40].

Ethyl 2-((2-oxo-2H-chromen-6-yl)oxy)acetate (2b). Using 6-hydroxycoumarin and, ethyl 2-iodoacetate as starting materials and the general procedure described above compound 2a was obtained in 86% yield; m.p. 120–122 °C; δH (400 MHz, DMSO-d6) 1.26 (3H, t, J = 9.5 Hz), 4.21 (2H, q, J = 7.2 Hz), 4.88 (2H, s), 6.55(1H, d, J = 9.6 Hz), 7.27 (1H, dd, J = 3.0, 9.0 Hz), 7.33 (1H, d, J = 3.0 Hz), 7.39 (1H, d, J = 9.0 Hz), 8.03 (1H, d, J = 9.6 Hz); δC (100 MHz, DMSO-d6) 15.0, 61.6, 66.1, 112.9, 117.6, 118.3, 120.1, 120.6, 144.8, 149.2, 154.9, 161.0, 169.4; m/z (ESI positive) (C13H12O5) 249.2 [M + H]+. Experimental data are in agreement with those reported in [41].

The crude products 2a or 2b (2.7 mmol) were dissolved in an aqueous solution of 5% NaOH (5 mL) in ethanol (15 mL) and the mixture was stirred at room temperature for 5 min. The residue quenched with water and acidified with aqueous 6 M solution HCl. The precipitated white solid was filtered off and subsequently washed with cool water and DCM to give compounds 3a–b, respectively.

2-((2-Oxo-2H-chromen-7-yl)oxy)acetic acid (3a). Compound 3a was obtained in 98% yield; m.p. 180–182 °C; δH (400 MHz, DMSO-d6) 4.86 (2H, s), 6.34(1H, d, J = 9.2 Hz), 7.98–7.00 (2H, m), 7.68 (1H, d, J = 9.6 Hz), 8.03 (1H, d, J = 9.2 Hz); δC (100 MHz, DMSO-d6) 65.8, 102.4, 113.5, 113.7, 113.7, 130.4, 145.2, 156.1, 161.2, 161.8, 170.5; m/z (ESI positive) (C11H8O5) 221.0 [M + H]+. Experimental data are in agreement with those reported in [40].

2-((2-Oxo-2H-chromen-6-yl)oxy)acetic acid (3b). Compound 3b was obtained in 87% yield; m.p. 163.5–163.7 °C; δH (400 MHz, DMSO-d6) 4.78 (2H, s,), 6.53(1H, d, J = 9.6 Hz), 7.27 (1H, dd, J = 3.0, 9.0 Hz), 7.30 (1H, d, J = 3.0 Hz), 7.38 (1H, d, J = 9.0 Hz), 8.03 (1H, d, J = 9.5 Hz); δC (100 MHz, DMSO-d6) 65.9, 112.7, 117.6, 118.2, 120.1, 120.6, 144.9, 149.0, 155.0, 160.1, 170.8; m/z (ESI positive) 221. [M + H]+, m/z (ESI negative) 219.0 [M − H]−. Experimental data are in agreement with those reported in [41].

3.2.2. General Procedure for the Synthesis of 4a,b–13a,b

The appropriate coumarin acetic acid derivative 2-((2-oxo-2H-chromen-7-yl)oxy)acetic acid (3a) or 2-((2-oxo-2H-chromen-6-yl)oxy)acetic acid (3b) (1.0 eq.), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDCI-HCl, 1.5 eq.) and 1-hydroxy-7-azabenzotriazole (HOAT, 1.5 eq.), were dissolved in dry DMA (3.0 mL) and stirred for 10 min at r.t., followed by addition of the corresponding amine 4–13 (1.0 eq.) and N,N-diisopropylethylamine (DIPEA, 4.0 eq.) or triethylamine (Et3N, 5.0 eq.) in the same solvent (2.0 mL). The reaction mixture was stirred until the consumption of starting materials (TLC monitoring) and quenched with water and 6.0 M aqueous HCl solution at 0–5 °C. The crude products were collected by filtration and washed with cool water, DCM and diethyl ether to obtain desired products 4a,b–13a,b.
2-((2-Oxo-2H-chromen-7-yl)oxy)-N-phenethylacetamide (4a). Using 3a and 4 as starting materials and the general procedure described above compound 4a was obtained as a white solid in 80% yield; m.p. 178–179 °C; silica gel TLC Rf = 0.73 (MeOH/DCM 10% v/v); IR (KBr, cm⁻¹) 1710.1, 1604.1, 1558.2; δH (400 MHz, DMSO-d₆) 2.79 (2H, t, J = 7.2 Hz, 2′-H₂), 3.39 (2H, q, J = 7.2 Hz, 1′-H₂), 4.63 (2H, s, OCH₂CO), 6.35 (1H, d, J = 9.5 Hz, 3-H), 7.00 (2H, m, 6, 8-H); 7.20–7.24 (3H, m, 4′, 6′-H), 7.29–7.33 (2H, m, 5′-H), 7.40 (1H, d, J = 8.8, 8-H), 8.03 (1H, d, J = 9.6 Hz, 4-H), 8.21 (1H, t, J = 7.2 Hz, exchangeable with D₂O, NH); δC (100 MHz, DMSO-d₆) 35.9, 68.1, 102.6, 113.6, 113.7, 113.8, 127.0, 129.2, 129.5, 130.4, 140.1, 145.1, 156.0, 161.0, 161.7, 167.7; m/z (ESI positive) (C₁₉H₁₇NO₄) 324.3 [M + H⁺].

2-((2-Oxo-2H-chromen-6-yl)oxy)-N-phenethylacetamide (4b). Using 3b and 4 as starting materials and the general procedure described above compound 4b was obtained as a white solid 64% yield; m.p. 150–151 °C; silica gel TLC Rf = 0.5 (MeOH/DCM 10% v/v); IR (KBr, cm⁻¹) 1699.2, 1670.5, 1610.1, 1560.2; δH (400 MHz, DMSO-d₆) 2.74 (2H, t, J = 7.2 Hz, 2′-H₂), 3.39 (2H, q, J = 7.2 Hz, 1′-H₂), 4.50 (2H, s, OCH₂CO), 6.52 (1H, d, J = 9.6 Hz, 3-H); 7.68 (1H, d, J = 9.5 Hz, 5-H), 8.04 (1H, d, J = 9.5 Hz, 4-H), 8.27 (1H, t, J = 7.2 Hz, exchangeable with D₂O, NH); δC (100 MHz, DMSO-d₆) 35.9, 68.1, 102.6, 113.6, 113.7, 113.8, 127.0, 129.2, 129.5, 130.4, 140.1, 145.1, 156.0, 161.0, 161.7, 167.7; m/z (ESI positive) (C₁₉H₁₇NO₄) 324.3 [M + H⁺].

N-Benzyl-2-((2-oxo-2H-chromen-7-yl)oxy)acetamide (5a). Using 3a and 5 as starting materials and the general procedure described above compound 5a was obtained as a white solid 67% yield; m.p. 167–168 °C; silica gel TLC Rf = 0.52 (MeOH/DCM 10% v/v); IR (KBr, cm⁻¹) 1699.5, 1672.7, 1612.8, 1550.4; δH (400 MHz, DMSO-d₆) 4.40 (2H, d, J = 6.1 Hz, 1′-H), 4.74 (2H, s, OCH₂CO), 6.35 (1H, d, J = 9.5 Hz, 3-H), 7.03–7.06 (2H, m, 6, 8-H); 7.25–7.36 (5H, m, 3′, 4′, 5′-H), 7.69 (1H, d, J = 8.4 Hz, 5-H), 8.03 (1H, d, J = 9.6 Hz, 4-H), 8.78 (1H, t, J = 6.1 Hz, exchange with D₂O, NH); δC (100 MHz, DMSO-d₆) 42.8, 68.2, 102.7, 113.7, 113.8, 113.9, 127.8, 128.2, 129.2, 130.5, 140.2, 145.2, 156.1, 161.2, 161.7, 168.1; m/z (ESI positive) (C₁₉H₁₅NO₄) 310.2 [M + H⁺].
N-Benzyl-2-((2-oxo-2H-chromen-6-yl)oxy)acetamide (5b). Using 3b and 5 as starting materials and the general procedure described above compound 5b was obtained as a white solid in 67% yield; m.p. 161–162 °C; silica gel TLC Rf = 0.63 (MeOH/DCM 10% v/v); IR (KBr, cm⁻¹) 1705.2, 1680.5, 1605.7, 1555.2; δH (400 MHz, DMSO-d6) 4.38 (2H, d, J = 6.2 Hz, 1'-H), 4.65 (2H, s, OCH2CO), 6.52 (1H, d, J = 9.6 Hz, 3-H), 7.25–7.36 (7H, m, 3', 4', 5'-H), 7.40 (1H, d, J = 8.6 Hz, 8-H), 8.02 (1H, d, J = 9.6 Hz, 4-H), 8.07 (1H, t, J = 6.2 Hz, exchange with D2O, N-D); δC (100 MHz, DMSO-d6) 42.9, 68.5, 113.0, 117.7, 118.4, 120.2, 121.1, 127.8, 128.2, 129.2, 140.3, 145.2, 149.2, 155.1, 161.1, 168.6; m/z (ESI positive) (C18H13NO4) 310.2 [M + H]+.

N-(4-Fluorobenzyl)-2-((2-oxo-2H-chromen-7-yl)oxy)acetamide (6a). Using 3a and 6 as starting materials and the general procedure described above compound 6a was obtained as a white solid in 54% yield; m.p. 160–161 °C; silica gel TLC Rf = 0.62 (MeOH/DCM 10% v/v); IR (KBr, cm⁻¹) 1711.6, 1673.1, 1554.8, 1549.6; δH (400 MHz, DMSO-d6) 4.37 (2H, d, J = 6 Hz, 1'-H), 4.73 (2H, s, OCH2CO), 6.36 (1H, d, J = 9.6 Hz, 3-H), 7.06–7.10 (2H, m, 6, 8-H), 7.18–7.23 (2H, m, 4, 3'-H), 7.69 (1H, d, J = 8.4 Hz), 8.04 (1H, d, J = 9.6 Hz, 4-H), 8.77 (1H, t, J = 6 Hz, exchange with D2O, N-D); δC (100 MHz, DMSO-d6) 42.1, 68.1, 102.6, 113.7, 113.8, 113.8, 115.8 (d, 2J_C,F 22), 130.2 (d, 3J_C,F 8), 130.4, 136.3 (d, 4J_C,F 3), 145.2, 156.0, 161.7, 162.1 (d, 1J_C,F 238.8), 161.61, 168.0; δF (376 MHz, DMSO-d6) -115.9 (1F, s); m/z (ESI positive) (C18H14FNO4) 328.2 [M + H]+.

N-(4-Fluorobenzyl)-2-((2-oxo-2H-chromen-6-yl)oxy)acetamide (6b). Using 3b and 6 as starting materials and the general procedure described above compound 6b was obtained as a white solid in 45% yield; m.p. 158–159 °C; silica gel TLC Rf = 0.42 (MeOH/DCM 10% v/v); IR (KBr, cm⁻¹) 1698.6, 1672.3, 1615.0, 1554.9; δH (400 MHz, DMSO-d6) 4.36 (2H, d, J = 6 Hz, 1'-H), 4.65 (2H, s, OCH2CO), 6.54 (1H, d, J = 9.6 Hz, 3-H), 7.07–7.21 (2H, m, 4', 5'-H), 7.33–7.42 (3H, m, 5, 7, 8-H), 7.67–7.70 (2H, m, 3'-H), 8.04 (1H, d, J = 9.6 Hz, 4-H), 8.76 (1H, t, J = 6 Hz, exchange with D2O, NH); δC (100 MHz, DMSO-d6) 43.2, 68.4, 112.9, 115.8 (d, 2J_C,F 21), 117.6, 118.3, 120.0, 121.0, 130.2 (d, 3J_C,F 8), 136.4 (d, 4J_C,F 3), 144.9, 149.1, 154.9, 161.0, 162.06 (d, 1J_C,F 240), 168.4. δF (376 MHz, DMSO-d6) -116.1 (1F, s); m/z (ESI positive) (C18H14FNO4) 328.2 [M + H]+.

2-((2-Oxo-2H-chromen-7-yl)oxy)-N-phenylacetamide (7a). Using 3a and 7 as starting materials and the general procedure described above compound 7a was obtained as a white solid in 70% yield; m.p.
182–183 °C; silica gel TLC Rf = 0.57 (MeOH/DCM 10% v/v); δH (400 MHz, DMSO-d6) 4.96 (2H, s, COCH2O), 6.35 (1H, d, J = 8 Hz, 3-H), 7.10 (3H, m), 7.36 (2H, m), 7.69 (3H, m), 8.04 (1H, d, J = 8 Hz), 10.22 (1H, s, exchange with D2O, NH); δC (100 MHz, DMSO-d6) 68.2, 102.6, 113.6, 113.8, 113.9, 120.6, 124.7, 129.6, 130.4, 139.2, 145.1, 156.0, 161.1, 161.9, 166.7; m/z (ESI positive) (C17H12FNO4) 296.2 [M + H]+. Experimental data are in agreement with those reported in [39].

2-(2-Oxo-2H-chromen-6-yl)oxy)-N-phenylacetamide (7b) [39]. Using 3b and 7 as starting materials and the general procedure described above compound 7b was obtained as a white solid in 80% yield; m.p. 182–183 °C; silica gel TLC Rf 0.56 (MeOH/DCM 10% v/v); δH (400 MHz, DMSO-d6) 4.80 (2H, s), 6.54 (1H, d, J = 9.6 Hz), 7.12 (1H, t, J = 7.4 Hz), 7.36 (4H, m), 7.43 (1H, d, J = 8.7), 7.68 (2H, d, J = 7.4 Hz), 8.07 (1H, d, J = 9.6 Hz), 10.14 (1H, s, exchange with D2O, NH); δC (100 MHz, DMSO-d6) 68.6, 113.0, 117.6, 118.3, 120.1, 120.6, 120.8, 124.6, 129.7, 139.2, 144.9, 149.1, 155.1, 161.0, 167.1. m/z (ESI positive) (C17H13NO4) 296.2 [M + H]+. Experimental data are in agreement with those reported in [39].

N-(4-Fluorophenyl)-2-(2-oxo-2H-chromen-7-yl)oxy)acetamide (8a) [39]. Using 3a and 8 as starting materials and the general procedure described above compound 8a was obtained as a white solid in 80% yield; m.p. 208–209 °C; silica gel TLC Rf 0.75 (MeOH/DCM 10% v/v); δH (400 MHz, DMSO-d6) 4.87 (2H, s), 6.35 (1H, d, J = 9.5 Hz), 7.08 (2H, m), 7.21 (2H, m), 7.69 (3H, m), 8.03 (1H, d, J = 9.5 Hz), 10.25 (1H, s, exchange with D2O, NH); δC (100 MHz, DMSO-d6) 82.2, 106.2, 113.7, 113.9, 113.9, 116.4 (d, 2J_C-F = 22), 122.6 (d, 3J_C-F = 8), 130.5, 135.6 (d, 4J_C-F = 3), 145.2, 156.1, 159.3 (d, 4J_C-F = 239), 161.2, 161.9, 166.8; δF (376 MHz, DMSO-d6) –118.7 (1F, s); m/z (ESI positive) (C17H12FNO4) 314.2 [M + H]+. Experimental data are in agreement with those reported in [39].

N-(4-Fluorophenyl)-2-(2-oxo-2H-chromen-6-yl)oxy)acetamide (8b) [39]. Using 3b and 8 as starting materials and the general procedure described above compound 8b was obtained as a white solid in 80% yield; m.p. 206–207 °C; silica gel TLC Rf 0.86 (MeOH/DCM 10% v/v); δH (400 MHz, DMSO-d6) 4.79 (2H, s), 6.52 (1H, d, J = 9.6 Hz), 7.18 (2H, t, J = 8.8), 7.38 (3H, m), 7.68 (2H, m), 8.03 (1H, d, J = 9.6 Hz), 10.22 (1H, s, exchange with D2O, NH); δC (100 MHz, DMSO-d6) 68.6, 113.1, 116.3 (d, 2J_C-F = 22), 117.7, 118.5, 120.2, 121.0, 122.8 (d, 3J_C-F = 8), 135.6 (d, 4J_C-F = 3), 145.1, 149.3, 155.2, 159.3 (d, 1J_C-F = 238.8), 161.2, 167.3. δF (376 MHz, DMSO-d6) –118.78 (1F, s); m/z (ESI positive) (C17H12FNO4) 314.2 [M + H]+. Experimental data are in agreement with those reported in [39].
Methyl 2-(2-((2-oxo-2H-chromen-7-yl)oxy)acetamido)benzoate (9a). Using 3a and 9 as starting materials and the general procedure described above compound 9a was obtained as a white solid in 45% yield; m.p. 213–214 °C; silica gel TLC $R_f = 0.69$ (MeOH/DCM 10% v/v); IR (KBr, cm$^{-1}$) 1710.3, 1668.7, 1612.3, 1562.0; $\delta_H$ (400 MHz, DMSO-$d_6$) 3.96 (3H, s, COOCH$_3$), 4.93 (2H, s, OCH$_2$CO), 6.39 (1H, d, $J = 9.6$ Hz, 3-H), 7.15–7.20 (2H, m, 6, 8-H), 7.28 (1H, t, $J = 7.5$, 4'-H), 7.70 (1H, t, $J = 7.5$, 5'-H), 7.76 (1H, d, $J = 9.2$, 5-H), 8.03–8.09 (2H, m, 4, 6'-H), 8.62 (1H, d, $J = 7.5$ Hz, 3'-H), 11.71 (1H, s, exchange with D$_2$O, NH); $\delta_C$ (100 MHz, DMSO-$d_6$) 53.5, 68.5, 102.8, 113.7, 114.1, 114.2, 117.2, 121.1, 124.4, 130.7, 131.7, 135.4, 140.3, 145.1, 156.1, 160.9, 161.0, 167.3, 168.4. m/z (ESI positive) (C$_{19}$H$_{15}$NO$_6$) 354 [M + H]$^+$. 

\[
\begin{align*}
\text{9a} \\
\end{align*}
\]

Methyl 2-(2-((2-oxo-2H-chromen-6-yl)oxy)acetamido)benzoate (9b). Using 3b and 9 as starting materials and the general procedure described above compound 9b was obtained as a white solid in 50% yield; m.p. 213–214 °C; silica gel TLC $R_f = 0.67$ (MeOH/DCM 10% v/v); IR (KBr, cm$^{-1}$) 1712.9, 1670.4, 1611.9, 1563.8; $\delta_H$ (400 MHz, DMSO-$d_6$) 3.94 (3H, s, COOCH$_3$), 4.85 (2H, s, OCH$_2$CO), 6.56 (1H, d, $J = 9.6$ Hz, 3-H), 7.27 (1H, t, $J = 7.8$, 4'-H), 7.43 (1H, dd, $J = 2.8$, 9.1 Hz, 7-H), 7.47–7.50 (2H, m, 5, 8-H), 7.71 (1H, t, $J = 7.8$, 5'-H), 8.05 (1H, d, $J = 7.8$, 6'-H), 8.08 (1H, d, $J = 9.6$ Hz, 4-H), 8.65 (1H, d, $J = 7.8$ Hz, 3'-H), 11.74 (1H, s, exchange with D$_2$O, NH); $\delta_C$ (100 MHz, DMSO-$d_6$) 51.6, 66.2, 110.4, 113.2, 113.9, 115.6, 118.3, 119.4, 119.5, 124.2, 130.3, 133.6, 141.3, 143.5, 147.8, 157.6, 163.2, 167.7, 168.3. m/z (ESI negative) (C$_{19}$H$_{15}$NO$_6$) 352 [M – H]$^-$. 

\[
\begin{align*}
\text{9b} \\
\end{align*}
\]

2-((2-Oxo-2H-chromen-7-yl)oxy)-N-(2-(pyridin-2-yl)ethyl)acetamide (10a). Using 3a and 10 as starting materials and the general procedure described above compound 10a was obtained as a white solid in 68% yield; m.p. 138–139 °C; silica gel TLC $R_f = 0.60$ (MeOH/DCM 10% v/v); IR (KBr, cm$^{-1}$) 1710.5, 1672.3, 1612.8, 1554.7; $\delta_H$ (400 MHz, DMSO-$d_6$) 2.95 (2H, t, $J = 7.2$ Hz, 2'-H), 3.54 (2H, q, $J = 7.2$ Hz, 1'-H), 4.63 (2H, s, OCH$_2$CO), 6.36 (1H, d, $J = 9.6$ Hz, 3-H), 6.97 (1H, d, $J = 2.4$ Hz, 8-H), 7.00 (1H, dd, $J = 2.4$, 8.6 Hz, 6-H), 7.22–7.24 (1H, m, 6'-H), 7.26 (1H, d, $J = 8.6$ Hz, 5-H), 7.67–7.73 (2H, m, 5',6'-H), 8.03 (1H, d, $J = 9.6$ Hz, 4-H), 8.27 (1H, t, $J = 7.2$ Hz, exchange with D$_2$O, NH), 8.50–8.52 (1H, m, 7'-H); $\delta_C$ (100 MHz, DMSO-$d_6$) 38.2, 39.8, 68.5, 103.2, 114.4, 114.5, 114.6, 123.5, 125.1, 131.3, 138.7, 146.2, 150.4, 156.6, 160.2, 162.3, 162.6, 169.4. m/z (ESI positive) (C$_{18}$H$_{16}$N$_2$O$_4$) 325.3 [M + H]$^+$. 

\[
\begin{align*}
\text{10a} \\
\end{align*}
\]
2-((2-Oxo-2H-chromen-6-yl)oxy)-N-(2-(pyridin-2-yl)ethyl)acetamide (10b). Using 3b and 10 as starting materials and the general procedure described above compound 10b was obtained as a white solid in 60% yield; m.p. 141–142 °C; silica gel TLC Rf = 0.60 (MeOH/DCM 10% v/v); IR (KBr, cm⁻¹) 1700.8, 1671.5, 1609.1, 1556.8; δ_H (400 MHz, DMSO-d₆) 2.95 (2H, t, J = 6.4 Hz, 2'-H), 3.55 (2H, q, J = 6.4 Hz, 1'-H), 4.56 (2H, s, OCH₂CO), 6.54 (1H, d, J = 9.6 Hz, 3-H), 7.21–7.30 (4H, m, 7, 5, 6', 8'-H), 7.39 (1H, d, J = 8.8 Hz, 8-H), 7.70 (1H, t, J = 7.8 Hz, 7'-H), 8.04 (1H, d, J = 9.6 Hz, 4-H), 8.27 (1H, t, J = 6.4 Hz, exchange with D₂O, NH), 8.51 (1H, m, 5'-H); δ_C (100 MHz, DMSO-d₆) 36.4, 54.1, 67.0, 112.9, 113.4, 113.9, 117.6, 118.3, 120.1, 120.9, 136.9, 143.7, 144.9, 149.1, 154.9, 158.7, 160.9, 168.1; m/z (ESI positive) (C₁₈H₁₆N₂O₄) 325.3 [M + H]⁺.

Molecules 2018, 23, 153

2-((2-Oxo-2H-chromen-7-yl)oxy)-N-(2-(pyridin-2-yl)ethyl)acetamide (11a). Using 3a and 11 as starting materials and the general procedure described above compound 11a was obtained as a white solid in 62% yield; m.p. 158–159 °C; silica gel TLC Rf = 0.43 (MeOH/DCM 10% v/v); IR (KBr, cm⁻¹) 1699.7, 1670.3, 1605.2, 1555.1; δ_H (400 MHz, DMSO-d₆) 4.42 (2H, t, J = 6.1 Hz, 1'-H), 4.70 (2H, s, OCH₂CO), 6.55 (1H, d, J = 9.6 Hz, 3-H), 7.27–7.29 (2H, 6, 8-H), 7.33–7.36 (2H, m, 3'-H), 7.42 (1H, d, J = 8.9 Hz, 5-H), 8.05 (1H, d, J = 9.6 Hz, 4-H), 8.50–8.52 (2H, m, 4'-H), 8.85 (1H, t, J = 6.1 Hz, exchange with D₂O, NH); δ_C (100 MHz, DMSO-d₆) 41.8, 68.1, 112.9, 117.6, 118.3, 120.0, 121.0, 123.0, 144.8, 149.1, 149.2, 150.3, 154.9, 160.9, 168.8; m/z (ESI positive) (C₁₇H₁₄N₂O₄) 311.2 [M + H]⁺.

2-((2-Oxo-2H-chromen-7-yl)oxy)-N-(pyridin-4-ylmethyl)acetamide (11b). Using 3b and 11 as starting materials and the general procedure described above compound 11b was obtained as a white solid in 60% yield; m.p. 160–161 °C; silica gel TLC Rf = 0.40 (MeOH/DCM 10% v/v); IR (KBr, cm⁻¹) 1710.3, 1671.6, 1606.5, 1555.0; δ_H (400 MHz, DMSO-d₆) 4.36 (1H, d, J = 6.1 Hz, 1'-H), 4.78 (2H, s, OCH₂CO), 6.36 (1H, d, J = 9.6 Hz, 3-H), 7.05–7.07 (2H, m, 6, 8-H), 7.27–7.30 (2H, m, 3'-H), 7.70 (1H, d, J = 8.6 Hz, 8-H), 8.06 (1H, d, J = 9.6 Hz, 4-H), 8.51–8.53 (2H, m, 4'-H), 8.85 (1H, t, J = 6.1 Hz, exchange with D₂O, NH); δ_C (100 MHz, DMSO-d₆) 41.8, 68.1, 102.7, 113.7, 113.8, 113.9, 123.0, 130.4, 145.1, 149.1, 150.4, 156.1, 157.3, 161.6, 168.4; m/z (ESI positive) (C₁₇H₁₄N₂O₄) 311.2 [M + H]⁺.
N-(2-Morpholinoethyl)-2-((2-oxo-2H-chromen-6-yl)oxy)acetamide (12b). Using 3b and 12 as starting materials and the general procedure described above compound 12b was obtained as a white solid in 60% yield; m.p. 141–142 °C; silica gel TLC Rf = 0.60 (MeOH/DCM 10% v/v); IR (KBr, cm⁻¹) 1700.2, 1669.8, 1670.7, 1605.8, 1558.4; δH (400 MHz, DMSO-d₆) 1.37–1.41 (2H, m, 6'-H), 1.45–1.50 (4H, m, 5'-H), 2.33–2.38 (6H, m, 2', 4'-H), 3.27 (2H, q, J = 6.4 Hz, 1'-H), 4.65 (2H, s, OCH₂CO), 6.34 (1H, d, J = 9.6 Hz, 3'-H), 7.00–7.04 (2H, m, 6, 8-H), 7.33 (1H, d, J = 9.6 Hz, 3-H), 7.30 (1H, d, J = 8.9 Hz, 7-H), 7.42 (1H, d, J = 9.6 Hz, 7'-H), 8.04 (1H, d, J = 9.6 Hz, 4-H), 8.51–8.53 (2H, m, 4'-H).

2-((2-Oxo-2H-chromen-7-yl)oxy)-N-(2-(piperidin-1-yl)ethyl)acetamide (13a). Using 3a and 13 as starting materials and the general procedure described above compound 13a was obtained as a white solid in 45% yield; m.p. 116–117 °C; silica gel TLC Rf = 0.24 (MeOH/DCM 10% v/v); IR (KBr, cm⁻¹) 1669.8, 1670.7, 1605.8, 1558.4; δH (400 MHz, DMSO-d₆) 1.37–1.41 (2H, m, 6'-H), 1.45–1.50 (4H, m, 5'-H), 2.33–2.38 (6H, m, 2', 4'-H), 3.27 (2H, q, J = 6.4 Hz, 1'-H), 4.65 (2H, s, OCH₂CO), 6.34 (1H, d, J = 9.6 Hz, 3'-H), 7.00–7.04 (2H, m, 6, 8-H), 7.69 (1H, d, J = 8.4 Hz, 5-H), 8.03 (1H, d, J = 9.6 Hz, 4-H); δC (100 MHz, DMSO-d₆) 24.9, 26.4, 36.9, 54.9, 58.3, 68.2, 102.7, 113.7, 113.9, 130.4, 145.1, 115.1, 161.1, 161.6, 167.7; m/z (ESI positive) (C₁₈H₂₆N₂O₄) 331.3 [M + H]+.
were preincubated together for 6 h at 4 °C. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor CA-catalyzed CO$_2$ hydration activity [36]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM HEPES (pH 7.5) as buffer, and conclusions.

3.3. Soybean Lipoxygenase Inhibition Studies

A DMSO solution of the tested compound was incubated with sodium linoleate (0.1 mM) and 0.2 mL of soybean LOX solution (1/9 × 10$^{-4}$ w/v in saline) in buffer pH 9 (tris) and at room temperature. The conversion of sodium linoleate to 13-hydroperoxylinoleic acid was recorded at 234 nm and compared with the standard inhibitor NDGA (IC$_{50}$ = 0.45 µM). The results are given in Table 2 expressed as IC$_{50}$ values or % inhibition at 100 µM [34].

3.4. CA Inhibition Assay

An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA catalysed CO$_2$ hydration activity [36]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM HEPES (pH 7.5) as buffer, and 20 mM Na$_2$SO$_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO$_2$ hydration reaction for a period of 10–100 s. The CO$_2$ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 mM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 6 h at 4 °C prior to assay, in order to allow for the formation of the E-I complex and for the active site mediated hydrolysis of the inhibitor [18,19]. Data reported in Table 1 show the inhibition after 6 h incubation, which led to the completion of the in situ hydrolysis of the coumarin and formation of the hydroxycinnamic acid [18,19]. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier [42–50] and represent the mean from at least three different determinations. The four CA isoforms were recombinant proteins obtained as reported earlier in our laboratory [42–50].

4. Conclusions

We report here a series of carboxamide derivatives of 6- and 7-substituted coumarins. They have been prepared by an original procedure starting from the corresponding 6- or 7-hydroxycoumarins which were alkylated with ethyl iodoacetate, then the obtained ester was converted to the corresponding carboxylic acid which was thereafter reacted with a series of aromatic/aliphatic/heterocyclic amines leading to the desired amides. The present study shows that these compounds represent a promising class of multi-targeting derivatives which can interact with several biological targets, in this case,
lipoxygenase and carbonic anhydrases. Compounds 4a and 4b were potent LOX inhibitors, whereas many effective hCA IX inhibitors (K_{I5} in the range of 30.2–30.5 nM) were detected in this study. Two compounds 4b and 5b showed the phenomenon of dual inhibition. Furthermore, these coumarins did not significantly inhibit the widespread cytosolic isoforms hCA I and II, whereas they were weak hCA IV inhibitors, making them hCA IX-selective inhibitors. As hCA IX and LOX are validated antitumor targets, these results are promising for the investigation of novel drug targets involved in tumorigenesis.

**Acknowledgments:** Hadjipavlou-Litina and Peperidou (PhD student) are thankful to Biobyte and A. Leo for the free use of C-QSAR software. Peperidou had EU financial support as an Erasmus student.

**Author Contributions:** A.P. and M.B. prepared the compounds, S.B. tested them, D.H.-L. and C.T.S. supervised all the project, designed the experiments and wrote the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Supuran, C.T. Advances in structure-based drug discovery of carbonic anhydrase inhibitors. *Expert Opin. Drug Discov.* 2017, 12, 61–88. [CrossRef] [PubMed]
2. Supuran, C.T. Structure and function of carbonic anhydrases. *Biochem. J.* 2016, 473, 2023–2032. [PubMed]
3. Supuran, C.T. Carbonic anhydrases: Novel therapeutic applications for inhibitors and activators. *Nat. Rev. Drug Discov.* 2008, 7, 168–181. [PubMed]
4. Neri, D.; Supuran, C.T. Interfering with pH regulation in tumours as a therapeutic strategy. *Nat. Rev. Drug Discov.* 2011, 10, 767–777. [CrossRef] [PubMed]
5. Capasso, C.; Supuran, C.T. An overview of the alpha-, beta-and gamma-carbonic anhydrases from Bacteria: Can bacterial carbonic anhydrases shed new light on evolution of bacteria? *J. Enzym. Inhib. Med. Chem.* 2015, 30, 325–332. [CrossRef] [PubMed]
6. Masini, E.; Carta, F.; Scozzafava, A.; Supuran, C.T. Antiglaucoma carbonic anhydrase inhibitors: A patent review. *Expert Opin. Ther. Pat.* 2013, 23, 705–716. [CrossRef] [PubMed]
7. Supuran, C.T. Structure-based drug discovery of carbonic anhydrase inhibitors. *J. Enzym. Inhib. Med. Chem.* 2012, 27, 759–772. [CrossRef] [PubMed]
8. Monti, S.M.; Supuran, C.T.; De Simone, G. Anticancer carbonic anhydrase inhibitors: A patent review (2008–2013). *Expert Opin. Ther. Pat.* 2013, 23, 737–749. [CrossRef] [PubMed]
9. Supuran, C.T. Carbonic Anhydrase Inhibition and the Management of Hypoxic Tumors. *Metabolites* 2017, 7, 48. [CrossRef] [PubMed]
10. Scozzafava, A.; Supuran, C.T.; Carta, F. Antiobesity carbonic anhydrase inhibitors: A literature and patent review. *Expert Opin. Ther. Pat.* 2013, 23, 725–735. [CrossRef] [PubMed]
11. Carta, F.; Supuran, C.T. Diuretics with carbonic anhydrase inhibitory action: A patent and literature review (2005–2013). *Expert Opin. Ther. Pat.* 2013, 23, 681–691. [CrossRef] [PubMed]
12. Carta, F.; Di Cesare Mannelli, L.; Pinard, M.; Ghelardini, C.; Scozzafava, A.; McKenna, R.; Supuran, C.T. A class of sulfonamide carbonic anhydrase inhibitors with neuropathic pain modulating effects. *Bioorg. Med. Chem.* 2015, 23, 1828–1840. [CrossRef] [PubMed]
13. Scozzafava, A.; Menabuoni, L.; Mincione, F.; Supuran, C.T. Carbonic Anhydrase Inhibitors. A General Approach for the Preparation of Water-Soluble Sulfonamides Incorporating Polyamino—Polycarboxylate Tails and of Their Metal Complexes Possessing Long-Lasting, Topical Intraocular Pressure-Lowering Properties. *J. Med. Chem.* 2002, 45, 1466–1476. [CrossRef] [PubMed]
14. Supuran, C.T. How many carbonic anhydrase inhibition mechanisms exist? *J. Enzym. Inhib. Med. Chem.* 2016, 31, 345–360. [CrossRef] [PubMed]
15. Vullo, D.; Del Prete, S.; Di Fonzo, P.; Carginale, V.; Donald, W.A.; Supuran, C.T.; Capasso, C. Comparison of the Sulfonamide Inhibition Profiles of the β- and γ-Carbonic Anhydrases from the Pathogenic Bacterium *Burkholderia pseudomallei*. *Molecules* 2017, 22, 421. [CrossRef] [PubMed]
16. Berrino, E.; Bua, S.; Mori, M.; Botta, M.; Murthy, V.S.; Vijayakumar, V.; Tamboli, Y.; Bartolucci, G.; Mugelli, A.; Cerbai, E.; et al. Novel Sulfamide-Containing Compounds as Selective Carbonic Anhydrase I Inhibitors. *Molecules* 2017, 22, 1049. [CrossRef] [PubMed]
17. Carta, F.; Supuran, C.T.; Scozzafava, A. Sulfonamides and their isosters as carbonic anhydrase inhibitors. *Future Med. Chem.* 2014, 6, 1149–1165. [CrossRef] [PubMed]

18. Maresca, A.; Temperini, C.; Vu, H.; Pham, N.B.; Poulsen, S.A.; Scozzafava, A.; Quinn, R.J.; Supuran, C.T. Non-zinc mediated inhibition of carbonic anhydrases: Coumarins are a new class of suicide inhibitors. *J. Am. Chem. Soc.* 2009, 131, 3057–3062. [CrossRef] [PubMed]

19. Maresca, A.; Temperini, C.; Pochet, L.; Masereel, B.; Scozzafava, A.; Supuran, C.T. Deciphering the mechanism of carbonic anhydrase inhibition with coumarins and thiocoumarins. *J. Med. Chem.* 2010, 53, 335–344. [CrossRef] [PubMed]

20. Touisni, N.; Maresca, A.; McDonald, P.C.; Lou, Y.; Scozzafava, A.; Dedhar, S.; Winum, J.Y.; Supuran, C.T. Glycosyl coumarin carbonic anhydrase IX and XII inhibitors strongly attenuate the growth of primary breast tumors. *J. Med. Chem.* 2011, 54, 8271–8277. [CrossRef] [PubMed]

21. Bozdag, M.; Ferraroni, M.; Carta, F.; Vullo, D.; Lucarini, L.; Orlandini, E.; Rossello, A.; Nuti, E.; Scozzafava, A.; Masini, E.; et al. Structural insights on carbonic anhydrase inhibitory action, isoform selectivity, and potency of sulfonamides and coumarins incorporating arylsulfonylureido groups. *J. Med. Chem.* 2014, 57, 9152–9167. [CrossRef] [PubMed]

22. Carta, F.; Maresca, A.; Scozzafava, A.; Supuran, C.T. Novel coumarins and 2-thioxo-coumarins as inhibitors of the tumor-associated carbonic anhydrases IX and XII. *Bioorg. Med. Chem.* 2012, 20, 2266–2273. [CrossRef] [PubMed]

23. Davis, R.A.; Vullo, D.; Maresca, A.; Supuran, C.T.; Poulsen, S.A. Natural product coumarins that inhibit human carbonic anhydrases. *Bioorg. Med. Chem.* 2013, 21, 1539–1543. [CrossRef] [PubMed]

24. Ferraroni, M.; Carta, F.; Scozzafava, A.; Supuran, C.T. Thioxocoumarins Show an Alternative Carbonic Anhydrase Inhibition Mechanism Compared to Coumarins. *J. Med. Chem.* 2016, 59, 462–473. [CrossRef] [PubMed]

25. Kucukbay, F.Z.; Kucukbay, H.; Tanc, M.; Supuran, C.T. Synthesis and carbonic anhydrase inhibitory properties of amino acid—Coumarin/quinolinone conjugates incorporating glycine, alanine and phenylalanine moieties. *J. Enzym. Inhib. Med. Chem.* 2016, 31, 1198–1202. [CrossRef] [PubMed]

26. Ding, X.Z.; Kuszynski, C.A.; El-Metwally, T.H.; Adrian, T.E. Lipoxygenase Inhibition Induced Apoptosis, Morphological Changes, and Carbonic Anhydrase Expression in Human Pancreatic Cancer Cells. *Biochem. Biophys. Res. Commun.* 1999, 266, 392–399. [CrossRef] [PubMed]

27. Kostova, I.; Bhatia, S.; Grigorov, P.; Balkansky, S.; Parmar, V.S.; Prasad, A.K.; Saso, L. Coumarins as antioxidants. *Curr. Med. Chem.* 2011, 18, 3929–3951. [CrossRef] [PubMed]

28. Detsi, A.; Kontogiorgis, C.; Hadjipavlou-Litina, D. Coumarin derivatives: An updated patent review (2015–2016). *Expert Opin. Ther. Pat.* 2017, 27, 1201–1226. [CrossRef] [PubMed]

29. Roussaki, M.; Zelianaios, K.; Kavetsou, E.; Hamilakis, S.; Hadjipavlou-Litina, D.; Kontogiorgis, C.; Liargkova, T.; Detsi, A. Structural modifications of coumarin derivatives: Determination of antioxidant and lipoxygenase (LOX) inhibitory activity. *Bioorg. Med. Chem.* 2014, 22, 6586–6594. [CrossRef] [PubMed]

30. Morphy, R.; Kay, C.; Rankovic, Z. From magic bullets to designed multiple ligands. *Drug Discov. Today* 2004, 9, 641–651. [CrossRef]

31. Morphy, R.; Rankovic, Z. Designed multiple ligands: An emerging drug discovery paradigm. *J. Med. Chem.* 2005, 48, 6523–6543. [CrossRef] [PubMed]

32. Crooks, S.W.; Stockley, R.A. Leukotriene B4. *Int. J. Biochem. Cell. Biol.* 1998, 30, 173–178. [CrossRef]

33. Peperidou, A.; Pontiki, E.; Hadjipavlou-Litina, D.; Voulgaris, E.; Avgoustakis, K. Multifunctional Cinnamic Acid Derivatives. *Molecules* 2017, 22, 1247. [CrossRef] [PubMed]

34. Müller, K. 5-Lipoxygenase and 12-lipoxygenase: Attractive targets for the development of novel antipsoriatic drugs. *Arch. Pharm.* 1994, 327, 3–19. [CrossRef]

35. Pontiki, E.; Hadjipavlou-Litina, D. Lipoxygenase inhibitors: A comparative QSAR study review and evaluation of new QSARs. *Med. Res. Rev.* 2008, 28, 39–117. [CrossRef] [PubMed]

36. Khalifah, R.G. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* 1971, 246, 2561–2573. [PubMed]

37. BioByte Home Page. Available online: http://www.biobyte.com/ (accessed on 1 June 2012).
38. Kavetsou, E.; Gkionis, L.; Galani, G.; Gkolfinopoulou, C.; Argyri, L.; Pontiki, E.; Chroni, A.; Hadjipavlou-Litina, D.; Detsi, A. Synthesis of prenyloxy coumarin analogues and evaluation of their antioxidant, lipoxygenase (LOX) inhibitory and cytotoxic activity. *Med. Chem. Res.* 2017, 26, 856–866. [CrossRef]

39. Chang, K.M.; Chen, H.H.; Wang, T.C.; Chen, I.L.; Chen, Y.T.; Yang, S.C.; Chen, Y.L.; Chang, H.H.; Huang, C.H.; Chang, J.Y.; et al. Novel oxime-bearing coumarin derivatives act as potent Nrf2/ARE activators in vitro and in mouse model. *Eur. J. Med. Chem.* 2015, 106, 60–74. [CrossRef] [PubMed]

40. Gao, Z.; Maloney, D.J.; Dedkova, L.M.; Hecht, S.M. Inhibitors of DNA polymerase beta: Activity and mechanism. *Bioorg. Med. Chem.* 2008, 16, 4331–4340. [CrossRef] [PubMed]

41. Mujahid, M.; Trendafilova, N.; Arfa-Kia, A.F.; Rosair, G.; Kavanagh, K.; Devereux, M.; Walsh, M.; McClean, S.; Creaven, B.S.; Georgieva, I. Novel silver(I) complexes of coumarin oxyacetate ligands and their phenanthroline adducts: Biological activity, structural and spectroscopic characterisation. *J. Inorg. Biochem.* 2016, 163, 53–67. [CrossRef] [PubMed]

42. Supuran, C.T.; Nicolae, A.; Popescu, A. Carbonic anhydrase inhibitors. Part 35. Synthesis of Schiff bases derived from sulfanilamide and aromatic aldehydes: The first inhibitors with equally high affinity towards cytosolic and membrane-bound isozymes. *Eur. J. Med. Chem.* 1996, 31, 431–438. [CrossRef]

43. Pacchiano, F.; Aggarwal, M.; Avvaru, B.S.; Robbins, A.H.; Scozzafava, A.; McKenna, R.; Supuran, C.T. Selective hydrophobic pocket binding observed within the carbonic anhydrase II active site accommodate different 4-substituted-ureido-benzenesulfonamides and correlate to inhibitor potency. *Chem. Commun.* 2010, 46, 8371–8373. [CrossRef] [PubMed]

44. Garaj, V.; Puccetti, L.; Fasolis, G.; Winum, J.Y.; Montero, J.L.; Scozzafava, A.; Vullo, D.; Innocenti, A.; Supuran, C.T. Carbonic anhydrase inhibitors: Synthesis and inhibition of cytosolic/tumor-associated carbonic anhydrase isozymes I, II, and IX with sulfonamides incorporating 1,2,4-triazine moieties. *Bioorg. Med. Chem. Lett.* 2004, 14, 5427–5433. [CrossRef] [PubMed]

45. Garaj, V.; Puccetti, L.; Fasolis, G.; Winum, J.Y.; Montero, J.L.; Scozzafava, A.; Vullo, D.; Innocenti, A.; Supuran, C.T. Carbonic anhydrase inhibitors: Novel sulfonamides incorporating 1,3,5-triazine moieties as inhibitors of the cytosolic and tumour-associated carbonic anhydrase isozymes I, II and IX. *Bioorg. Med. Chem. Lett.* 2005, 15, 3102–3108. [CrossRef] [PubMed]

46. Carta, F.; Garaj, V.; Maresca, A.; Wagner, J.; Avvaru, B.S.; Robbins, A.H.; Scozzafava, A.; McKenna, R.; Supuran, C.T. Sulfonamides incorporating 1,3,5-triazine moieties selectively and potently inhibit carbonic anhydrase transmembrane isoforms IX, XII and XIV over cytosolic isoforms I and II: Solution and X-ray crystallographic studies. *Bioorg. Med. Chem.* 2011, 19, 3105–3119. [CrossRef] [PubMed]

47. Sentürk, M.; Gülçin, I.; Beydemir, S.; Küfrevio˘glu, O.; Supuran, C.T. In Vitro inhibition of human carbonic anhydrase I and II isozymes with natural phenolic compounds. *Chem. Biol. Drug Des.* 2011, 77, 494–499. [CrossRef] [PubMed]

48. Fabrizi, F.; Mincione, F.; Somma, T.; Scozzafava, G.; Galassi, F.; Masini, E.; Impagnatiello, F.; Supuran, C.T. A new approach to antiglaucoma drugs: Carbonic anhydrase inhibitors with or without NO donating moieties. Mechanism of action and preliminary pharmacology. *J. Enzym. Inhib. Med. Chem.* 2012, 27, 138–147. [CrossRef] [PubMed]

49. Krall, N.; Pretto, F.; Decurtins, W.; Bernardes, G.J.; Supuran, C.T.; Neri, D. A small-molecule drug conjugate for the treatment of carbonic anhydrase IX expressing tumors. *Angew. Chem. Int. Ed.* 2014, 53, 4231–4235. [CrossRef] [PubMed]

50. Köhler, K.; Hillebrecht, A.; Schulze Wischelser, J.; Innocenti, A.; Heine, A.; Supuran, C.T.; Klebe, G. Saccharin inhibts carbonic anhydrases: Possible explanation for its unpleasant metallic aftertaste. *Angew. Chem. Int. Ed.* 2007, 46, 7697–7699. [CrossRef] [PubMed]

Sample Availability: Samples of the compounds 2–13b are available from the authors.