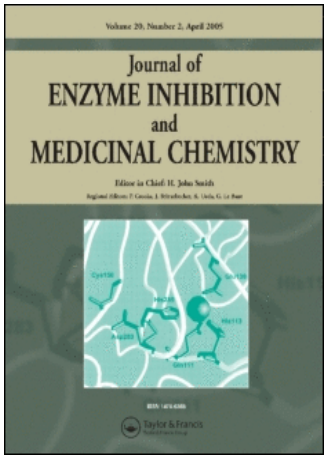

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In vitro inhibition of cytosolic carbonic anhydrases I and II by some new dihydroxycoumarin compounds

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In vitro inhibition of cytosolic carbonic anhydrases I and II by some new dihydroxycoumarin compounds

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Abstract

A new series of 6, 7-dihydroxy-3-(methylphenyl) chromenones, including three new derivatives, i.e. 6,7-dihydroxy-3-(2-methylphenyl)-2H-chromen-2-one (**OPC**); 6,7-dihydroxy-3-(3-methylphenyl)-2H-chromen-2-one (**MPC**); 6,7-dihydroxy-3-(4-methylphenyl)-2H-chromen-2-one (**PPC**) and one previously described, namely 6,7-dihydroxy-3-phenyl-2H-chromen-2-one (**DPC**), were synthesized. These compounds were investigated as inhibitors of human carbonic anhydrase I (hCA-I) and human carbonic anhydrase II (hCA-II) which had been purified from human erythrocytes on an affinity gel comprised of L-tyrosine-sulfonamide-Sepharose 4B.

Keywords: *Carbonic anhydrase, isozymes I and II, inhibitors, coumarin, phenols, cancer*

Introduction

The involvement of the carbonic anhydrase (CA) enzyme family, which catalyzes the physiological hydration of CO_2 to yield bicarbonate and a proton, in many physiological/pathological processes opens up widespread opportunities for the development of diverse, specific inhibitors for clinical application [1,2].

There is convincing evidence to suggest that the expression of CAs is increased in many tumours where their action in acidifying the extracellular milieu may give tumours a growth advantage over normal tissues [3,4]. Acetazolamide, a sulfonamide inhibitor of CA, has been shown to inhibit the *in vitro* invasion of renal cancer cells, which can provide additive delays in tumour growth when used in combination with other cytotoxic agents [5,6]. In view of the important role that CAs may have in supporting tumour growth there is currently considerable interest in developing CA inhibitors for use in cancer therapy [7]. Inhibition of CA in the ciliary processes of the eye decreases aqueous humor secretion, presumably by slowing the formation of bicarbonate ions with subsequent reduction in sodium and fluid transport, and is effective in the treatment of glaucoma [8,9]. Literature surveys reveal that sulfonamides and coumarin moieties are important pharmacophores and exhibit outstanding biological properties.

A wide spectrum of pharmacological activities are displayed by coumarins and their derivatives [10,11]. Coumarin derivatives are used widely as anticoagulants (such as warfarin, -OH group is attached at 4-position) for the treatment of disorders in which there is excessive or undesirable clotting, such as thrombophlebitis, pulmonary embolism, and certain cardiac conditions. They are also used as rodenticides due to their ability to cause fatal hemorrhaging [12].

In biosynthesis the coumarin nucleus (benzo-2-pyrone) is derived from cinnamic acid (phenylacrylic

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skeleton). Hydroxylation at the 7-position is common in biosynthesis yielding such derivatives as umbelliferone (7-hydroxy coumarin), esculetin (6,7-Dihydroxycoumarin), and scopoletin (7-hydroxy-6-methoxycoumarin) which are the most widespread coumarins in nature. Synthetic 7-hydroxy coumarins are used to absorb ultraviolet rays in sunscreen cosmetics and are also used in the synthesis of drugs especially against cancer [13].

In the present study we have synthesized derivatives of 6, 7-dihydroxy-3-(methylphenyl) chromenones for evaluation as potential inhibitors of cytosolic carbonic anhydrase isozymes that could be beneficial in the development of anti-glaucoma and anti-cancer therapies.

Materials and methods

All chemicals were from Sigma (USA) or Aldrich (Germany) and were used without further purification. 6, 7-Dihydroxy-3-phenyl-2H-chromen-2-one (DPC) was available from a previous study [14].

The structures of all synthesized compounds were identified from their IR (Perkin Elmer Spectrum BX II) and 400 MHz ^{1}H-NMR spectra (Bruker GmbH DPX-400). Melting points were measured on an Electrothermal 9200 instrument.

General procedure for the synthesis of 6, 7-dihydroxy-3-(methylphenyl) chromenones

Methyl-substituted coumarin compounds, i.e. the 6, 7-dihydroxy-3-(methylphenyl) chromenones, were synthesized by the procedure of Buu-Hoi et al. [15]. A solution of 2, 3, 4-trimethoxybenzaldehyde (**TMB**) (25.0 mmol) and methylphenylacetonitrile (37.5 mmol) in ethanol (100 mL) was heated to 70°C. Aqueous NaOH (20%) was then added dropwise to the stirred solution until the onset of turbidity. The acrylonitrile which precipitated when the solution was cooled was washed with water by filtration. It was then treated with HCl and then H_3O^+. The precipitates were collected by filtration, and the dried product was purified by recrystallisation from ethanol (Scheme 1).

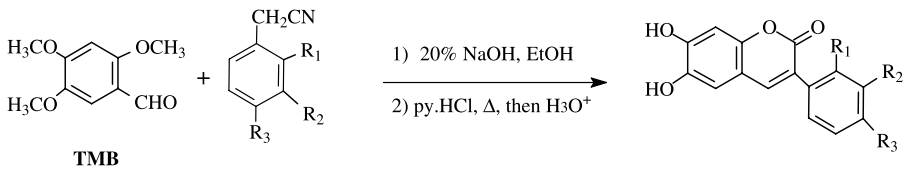
*6,7-dihydroxy-3-(2-methylphenyl)-2H-chromen-2-one (**OPC**).* 2-Methylphenylacetonitrile (**OMPA**) was used as the starting compound. Yield, 76.5%; mp, 242°C; IR (KBr), ν (cm^{-1}): 3464(OH), 1655(C═O), 1297(C—H), 1151(C—O). ^{1}H-NMR ($CDCl_3$ + DMSO), δ (ppm): 2.20 (s, 3H, CH_3), 6.83 (s, H, ArH), 6.98 (s, H, ArH), 7.32 (m, 4H, ArH), 7.50 (s, H, cumH), 7.86 (s, 2H, OH).

*6,7-dihydroxy-3-(3-methylphenyl)-2H-chromen-2-one (**MPC**).* 3-Methylphenylacetonitrile (**MMPA**) was used as the starting compound. Yield, 76.8%; mp, 211°C; IR (KBr), ν (cm^{-1}): 3172(OH), 1669(C═O), 1258(C—H), 1168(C—O). ^{1}H-NMR ($CDCl_3$ + DMSO), δ (ppm): 2.32 (s, 3H, CH_3), 6.75 (s, H, ArH), 6.91 (s, H, ArH), 7.08 (d, H, ArH), 7.21 (d, H, ArH), 7.37 (d, 2H, ArH), 7.70 (s, H, cumH), 7.75 (s, 2H, OH).

*6,7-dihydroxy-3-(4-methylphenyl)-2H-chromen-2-one (**PPC**).* 4-Methylphenylacetonitrile (**PMPA**) was used as the starting compound. Yield, 80.5%; mp, 257-258°C; IR (KBr), ν (cm^{-1}): 3151(OH), 1660(C═O), 1273(C—H), 1188(C—O). ^{1}H-NMR ($CDCl_3$ + DMSO), δ (ppm): 2.30 (s, 3H, CH_3), 6.75 (s, H, ArH), 6.90 (s, H, ArH), 7.13 (d, 2H, ArH), 7.48 (d, 2H, ArH), 7.67 (s, H, cumH), 7.73 (s, 2H, OH).

Preparation of hemolysates

Blood samples (25 mL) were taken from healthy human volunteers. They were anticoagulated with ACD (acid-citrate-dextrose), centrifuged at 1848 × g for 20 min at 4°C and the supernatant was removed. The packed erythrocytes were washed three times with 0.9% NaCI and then hemolysed in cold water. The ghosts and any intact cells were removed by centrifugation at 18924 × g for 25 min at 4°C, and the pH of the hemolysate was adjusted to pH 8.5 with solid Tris-base. The 25 mL hemolysate was applied to an affinity column containing L-tyrosine-sulfonamide-Sepharose-4B [16,17] equilibrated with 25 mM Tris–HCl / 0.1 M Na_2SO_4 (pH 8.5). The affinity gel was washed with



	R_1	R_2	R_3
OMPA	CH_3	H	H
MMPA	H	CH_3	H
PMPA	H	H	CH_3

	R_1	R_2	R_3
OPC	CH_3	H	H
MPC	H	CH_3	H
PPC	H	H	CH_3

Scheme 1. Synthesis of the various dihydroxy-coumarin derivatives.

50 mL of 25 mM Tris–HCl / 22 mM Na_2SO_4 (pH 8.5). The human CA (hCA) isozymes were then eluted with 0.1 M NaCl / 25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa / 0.5 M $NaClO_4$ (pH 5.6), which recovered hCA-I and hCA-II respectively. Fractions of 3 mL were collected and their absorbance measured at 280 nm.

Determination of protein content

Quantitative protein determination was performed on the pooled peaks of the eluted hCAs using the Coomassie Brilliant Blue G-250 method [18].

Enzyme assay and inhibition studies

CA enzyme activity was assayed both with respect to its hydratase activity (i.e. hydration of CO_2 [19]), and to its esterase activity (i.e. hydrolysis of an ester of *p*-nitrophenylacetate [20]).

The Maren method [19] is based on determination of the time required for the pH of a standard solution to decrease from pH 10.0 to 7.4 due to CO_2 hydration. The assay solution was 0.5 M Na_2CO_3/0.1M $NaHCO_3$ (pH 10.0) and Phenol Red was added as the pH indicator. One unit of CA activity is defined as that amount of the enzyme that reduces by 50% the time for CO_2 hydration measured in the absence of enzyme. The second hydrolytic method measures CA activity using *p*-nitrophenylacetate as a substrate followed by monitoring at 348 nm [20].

The inhibition of CA activity by the various coumarin derivatives (DPC, OPC, OPC and PPC) was studied by determining their effects on the CA-catalyzed CO_2 hydration rate at 1°C. CO_2 hydration rates were determined at five different inhibitor concentrations using an initial substrate concentration of 70 mM. Regression analysis was carried out on the graph of percent inhibition values as a function of inhibitor concentration.

Results and discussion

Carbonic anhydrase (CA) is a common enzyme that is present in key regulatory organs. By catalyzing the interconversion between carbon dioxide and bicarbonate, with generation of a proton, CAs operate on three very simple molecules/ions involved in a variety of critical life processes [1,2] Among them, the most important ones are pH regulation, respiration, secretion of electrolytes, biosynthesis of some important biomolecules such as urea, glucose, lipids, and pyrimidines, excretion of acid and salts, carcinogenesis, and cell signaling [20,21]. In the kidney, CA is involved in regulating acid-base equilibrium [22]. Its action in the renal tubules produces a mild metabolic acidosis by increasing bicarbonate excretion, which increases pulmonary ventilation. In the lungs, erythrocyte CA is responsible for the dehydration of H_2CO_3 and its hydration in the tissues. Without a catalyst, the interconversion between CO_2 and H_2CO_3 requires more than 1 minute for completion, while the capillary transit of erythrocytes takes 1 second. Inhibition of CA by acetazolamide is used as a prophylactic treatment for acute mountain sickness [23]. It is known that this substance produces an elevation of the arterial PO_2 during acute exposure to hypoxia. It is also an effective inhibitor in the treatment of glaucoma. Therapeutic inhibition of CA is thus feasible and effective, and new inhibitors with different properties and/or potencies are potentially valuable.

Coumarins are active components of herbs used for the treatment of various diseases [24]. In the literature, two coumarin-based sulfamate drugs (667 COUMATE and STX 118) have been reported to have IC_{50} values of 25–59 nM for the inhibition of hCA-II activity. These compounds therefore have similar CA-II inhibitory potencies to that of acetazolamide (IC_{50} 25 nM), the well-known hCA-II inhibitor [25]. In the literature, many sulfonamide derivatives have been described which inhibit members of the CA family [27–30]. Synthesis of coumarin 3-(N-aryl) sulfonamides has been accomplished either by Knoevenagel condensation of anilinosulfonylacetic acids with suitable salicylaldehydes, or by the reaction of methyl anilinosulfonylacetates with substituted salicylaldehydes in the presence of a catalytic amount of a base. All the compounds tested for antiproliferative activity in different cancer cell lines have shown GI_{50} values less than 100 μM [31]. Supuran and his group have reported that some of the coumarinyl-substituted benzenesulfonamide series are potent, low nanomolar CA-V inhibitors [26]. In another study, Zobel et al. demonstrated the enhanced anti-carcinogenic potential of combining coumarin at high concentrations (greater than 10 ppm) with the chemotherapy agent, Paclitaxel [32].

Recently, new synthetic routes to this class of compounds have been reported, also detailing their variable and interesting biological and pharmacological activities [10–13]. There have also been very interesting studies of the synthesis and selective biological properties of 7-hydroxy-4-methyl-2H-chromen-2-one (2), 7-hydroxy-4,5-dimethyl-2H-chromen-2-one (15) and some of their derivatives [10]. These stimulated us to synthesize a number of new dihydroxy coumarin compounds for testing against hCA-I and -II isozymes.

These new coumarin compounds were tested for their ability to modulate both the hydratase and esterase activities of cytosolic CA isozymes. Their *in vitro* inhibitory effects on hCA-I and hCA-II, purified by affinity chromatography from erythrocytes, are summarised in Table I. The hCA-II isozyme evolved to very efficiently catalyze the reversible hydration of CO_2, but it also exhibits promiscuous

Table I. IC_{50} values of cytosolic CA isozymes I and II with the phenold dscribed in the paper.

	IC_{50} (mM)			
	Esterase Activity		Hydratase Activity	
Coumarin compounds	hCA- I	hCA- II	hCA- I	hCA- II
DPC	0.160	0.246	0.124	0.0617
OPC	0.153	0.163	0.206	0.363
MPC	0.837	0.201	0.320	0.504
PPC	0.256	0.127	0.272	0.561

activity toward highly activated esters such as 4-nitrophenyl acetate. Among the compounds tested, MPC was the least effective inhibitor of hCA-I, exhibiting IC_{50} values of 0.837 mM and 0.320 mM against esterase and hydratase activities, respectively. For hCA-II, DPC was the weakest inhibitor of esterase activity (IC_{50} of 0.246 mM), but PPC was the weakest hydratase inhibitor (IC_{50} of 0.561 mM), closely followed by MPC (IC_{50} of 0.504 mM). The most effective inhibitor of hCA-I esterase activity was OPC (IC_{50} of 0.153 mM) and that of hCA-II esterase activity was PPC (IC50 of 0.127 mM). In contrast, the most potent inhibitor of hydratase activities was DPC with both hCA-I and –II isozymes (IC_{50} values of 0.124 mM and 0.0617 mM, respectively). Lindskog et al. [33] had previously found that the phenol is a weaker inhibitor for CA I and CA II and K_I values for CA-II with hydratase activity as 18 mM and for esterase activity was 30 mM.In the present study with the new compounds belonging to the diphenol class, we observed a better inhibition of the two CA isozymes with them, as compared to the simple phenol investigated earlier (Table I).

Our group had also previously synthesized an original sulfonamide derivative of coumarin, namely 2-(8-methoxycoumarine-3-carbamido)-1,3,4-thiadiazole-5-sulfonamide (MCTS). This compound showed IC_{50} values of 0.0047, 0.0096 and 0.0253 mM against the hydratase activities of hCA-I, hCA-II and dog carbonic anhydrase (dCA), respectively [9,26].

Inhibitors of CA play an important role in ophthalmology, where they are used to reduce elevated intraocular pressures. Apart from transient myopia and blurred vision, no adverse reactions from the eye have been described in spite of long-term treatment of patients over many years. The dihydroxycoumarin derivatives described here do not exhibit the inhibitory potencies of acetazolamide, or even that of the coumarin-sulfonamide MCTS. However, they do demonstrate that this synthetic route has some potential that is worth further investigation in the search for more therapeutically-effective CA inhibitors for potential use in the treatment of glaucoma, or other pathologies, where acidification by carbonic anhydrases plays a role.

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