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Structural investigations on coumarins leading to chromeno[4,3-c]pyrazol-4-ones and pyrano[4,3-c]pyrazol-4-ones: new scaffolds for the design of the tumor-associated carbonic anhydrase isoforms IX and XII

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Abstract

Human carbonic anhydrases (hCAs, EC 4.2.1.1) IX and XII are overexpressed in a wide variety of cancers and are considered available drug targets for anti-tumor therapy since their inhibition has been shown to reduce tumor growth and metastasis. A set of coumarin derivatives (**1-10**) and several 1-aryl and 2-aryl-substituted chromeno[4,3-c]pyrazol-4-ones (**11-37**) and pyrano[4,3-c]pyrazol-4-ones (**38-39**) were synthesized and tested against the tumor-associated hCAs IX and XII and the cytosolic isoforms hCAs I and II. Several compounds were potent (K_i < 41 nM) and selective inhibitors of the hCA IX (**13**, **14**, **19**, **21**, **25**, **31**, **33**, **37** and **39**), some derivatives (**6**, **11** and **17**) were active against both hCA IX and XII isoforms (K_i= 5.6-9.6 nM), while none were effective against the off-target cytosolic hCAs I and II. Some selected inhibitors (**6, 11**, **13**, **19**, **21**, **25**, **31** and **39**) showed activity as antiproliferative agents on HT-29 colon cancer cell lines both in normoxic and hypoxic conditions. This finding led us to hypothesize for these derivatives more than one mechanism of action, involving hCAs IX and XII inhibition in hypoxia and other not identified target(s) in normoxia.

Keywords

Carbonic anhydrase inhibitors, 2H-cromen-2-one, chromeno[4,3-c]pyrazol-4-one, pyrano[4,3-c]pyrazol-4-one, anticancer agents.

1. Introduction

Human carbonic anhydrases (hCAs, EC 4.2.1.1) are metallo-enzymes that regulate physiological functions connected to carbon dioxide/bicarbonate transport, pH, electrolyte secretion, and biosynthetic reactions, thus they influence many metabolic processes [1-2]. hCAs are divided into at least 15 isoforms differing in their molecular properties, subcellular localization and tissue distribution. hCA isoforms IX and XII are membrane-bound enzymes whose expression is strongly induced by hypoxia [3] that occurs in different types of solid tumors, such as glioma, breast cancer and colon carcinoma. Hypoxia shifts metabolism toward glycolysis and anaerobic fermentation thus affording a high production of lactic acid [3]. These conditions, together with an excessive formation of carbon dioxide, induce progressive acidification of the extracellular environment due to extrusion of protons from the cell and the entrance of bicarbonate ions [1-6]. Protons and bicarbonate ions are produced by carbon dioxide hydration catalyzed by the transmembrane hCAs IX and XII. Both these hCA isoforms contribute to tumor progression by maintaining a more alkaline resting intracellular pH and the acidic extracellular pH. These conditions up-regulate proteases, angiogenic and cell-growth factors, and impaired immune functions [5-9]. hCA IX activity has also been associated with chemoresistance which may be due to two mechanisms [10]. First, hCA IX inhibits apoptosis by maintaining a normal intracellular pH in response to chemotherapy-induced acidosis. Second, hCA IX-induced extracellular acidosis negatively impacts efficacy of weakly basic anticancer drugs by reducing their cellular uptake.

Acetazolamide (AZA) and other sulfonamide-containing hCA inhibitors were reported to reduce tumor growth and to delay tumor development. However, AZA and the classical aromatic sulfonamides are non-selective inhibitors, thus leading to side effects by blocking the physiological relevant cytosolic isozymes hCA I and II. Hence, several studies are being carried out to identify new selective inhibitors for the tumor-associated hCA isoforms which are considered promising targets for effective and safer agents in cancer therapy [8-13].

Up to now several classes of inhibitors have been disclosed [1, 11, 13, 15], for example

sulfonamides, metal-complexing anions, phenols and 2H-chromen-2-one derivatives, simply referred to as coumarins [16-20]. Within the latter class, the unsubstituted coumarin possesses potencies spanning the medium to high micromolar range. In particular, it showed K_i values of 3.1 and 9.2 μM at the highly abundant hCAs I and II, respectively, while lower potencies were found for membrane-bound hCA IX and XII isoforms (K_i> 500 μM) [16]. Insertion of substituents on the coumarin scaffold significantly enhanced inhibitory activity. In particular, the presence of ester functions or a carboxylic group at position 3, alone or combined with small substituents on the fused benzo ring (Fig. 1, 3-carboxy-substituted coumarin series), ameliorated the potency toward the tumor-associated hCA IX and XII, shiftinh the K_i values to the low micromolar range (0.047-9.9 μM) [16]. These data suggested that further decoration of coumarin might enhance potency and selectivity for the two hCA isoforms. Therefore, to continue our study in the field of hCA IX and XII inhibitors [21] we chose the coumarin scaffold for investigation. Thus, we synthesized compounds **1-10** (Fig. 1) which bear at position 3 the acetyl group, in place of the less stable ester function present in the previously reported 3-carboxycoumarins [16], and diverse substituents at the 4-position (R_4) and on the fused benzo ring (X).

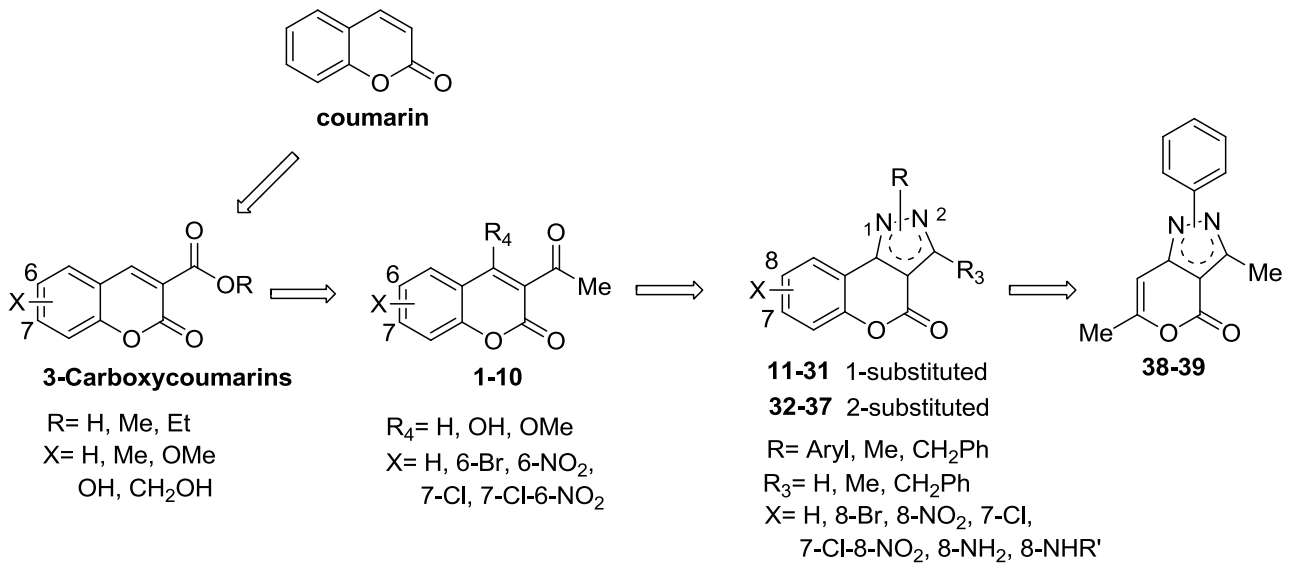


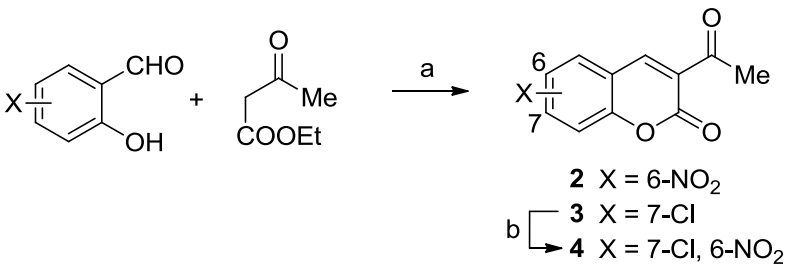
Fig. 1. Design of 2H-chromen-2-one derivatives **1-10** and their analogs chromeno[4,3-c]pyrazol-4-ones **11-37** and pyrano[4,3-c]pyrazoles **38-39** as inhibitors of the human carbonic anhydrases IX and XII.

Next, several chromeno[4,3-c]pyrazol-4-one derivatives (**11-37**) were synthesized and biologically tested. To explore the structural requirements of different areas around the tricyclic core, different substituents with diverse steric bulk and lipophilicity were placed at position 1 or 2 (R= Ar, CH_2Ph, Me) and 3 (R_3= H, Me, CH_2Ph), and on the fused benzo ring (X= H, Br, NO_2, Cl, NH-R') (Fig. 1). Finally, the pyrano[4,3-c]pyrazoles **38** and **39** were designed to evaluate how replacement of the fused benzo ring with the smaller methyl group affected complementarity with the hCAs.

2. Results and discussion

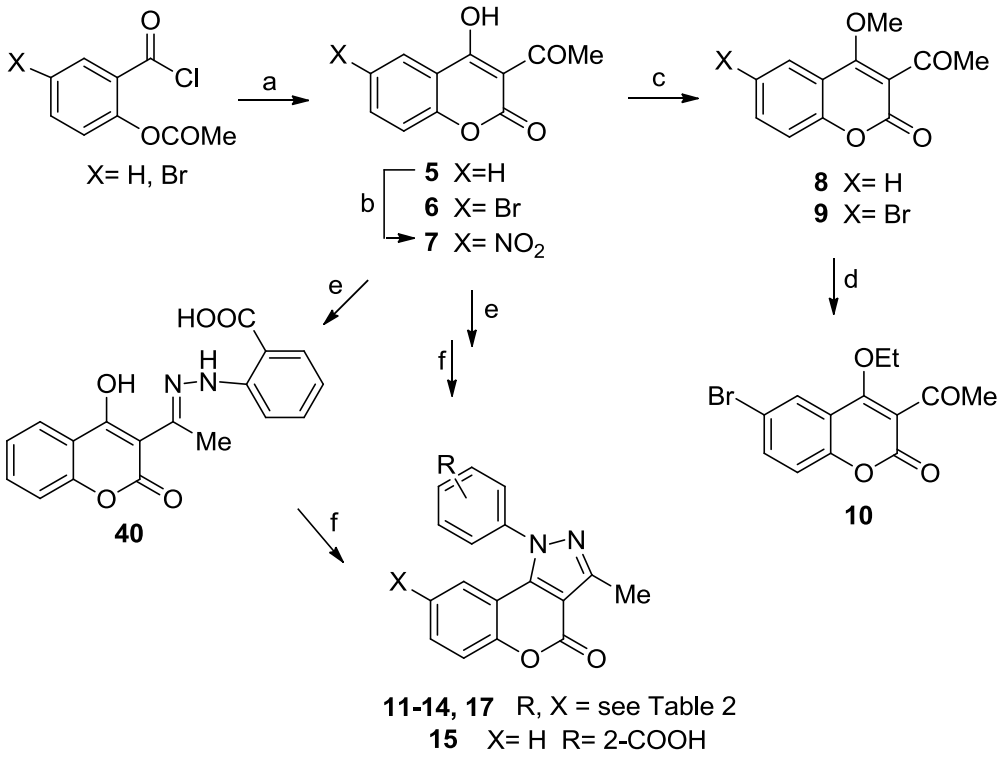
2.1.Chemistry

3-Acetylchromen-2-one (**1**) was purchased from Sigma-Aldrich while the other target compounds **2-39** were prepared as drawn in Schemes 1-6. The 3-acetylchromen-2-ones **2** [22] and **3** (Scheme 1) ensued from a Knoevenagel condensation between 5-substituted 2-hydroxybenzaldehydes and ethyl acetoacetate, carried out in the presence of piperidine and under microwave irradiation. Nitration of the 7-chloro-derivative **3** furnished the 7-chloro-6-nitro-substituted compound **4** in high yield.



Scheme 1. Reagents and conditions: a) piperidine, mw, 120 °C; b) 90% HNO_3, -10 °C.

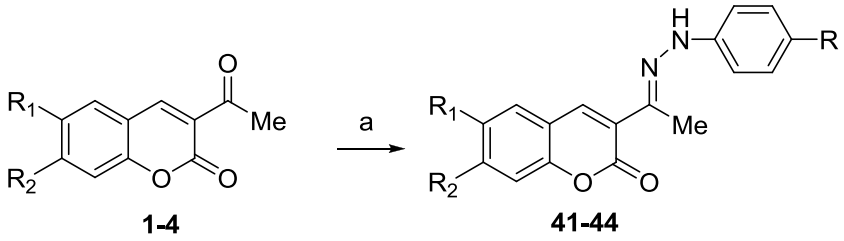
The synthesis of the 4-substituted 3-acetylchromen-2-one derivatives **5-10** and 3-methyl-1-aryl-chromeno[4,3-c]pyrazol-4-one derivatives **11-15** and **17** is shown in Scheme 2.



Scheme 2. Reagents and conditions: a) ethyl acetoacetate sodium salt, anhydrous Et_2O (X= H) or toluene (X= Br), reflux; b) KNO_3, 96% H_2SO_4, 0 °C; c) $(MeO)_2SO_2$, anhydrous acetone, K_2CO_3, mw, 125 °C; d) EtOH, reflux; e) Ar-$NHNH_2$.HCl, NEt_3, EtOH, reflux; f) p-TsOH, xylene, reflux.

The 3-acetyl-4-hydroxychromen-2-one **5** and its 6-bromo derivative **6** were synthesized as previously reported, i.e. by reacting the suitable acetylsalicyloyl choride with the sodium salt of ethyl acetoacetate [23, 24]. This method was not employed to prepare the 6-nitro-derivative **7** [24] which was obtained by nitration of derivative **5**. The 4-hydroxy group of **5** and **6** was then alkylated with dimethylsulfate in anhydrous acetone and in the presence of potassium carbonate, under microwave-assisted conditions, to yield the 4-methoxy derivatives **8** and **9**. The latter afforded the 4-ethoxy derivative **10** after heating in refluxing absolute ethanol. The 3-acetyl-4-hydroxy-chromen-2-ones **5-7** were used as starting material to prepare the 1-aryl-3-methylchromeno[4,3-c]pyrazol-4-one **11-14** [23] and **17** [24]. Briefly, compounds **5-7** were reacted with suitable arylhydrazines in boiling ethanol to give the corresponding arylhydrazones [23, 24] which were cyclized in refluxing xylene and in the presence of para-toluensulfonic acid to provide derivatives

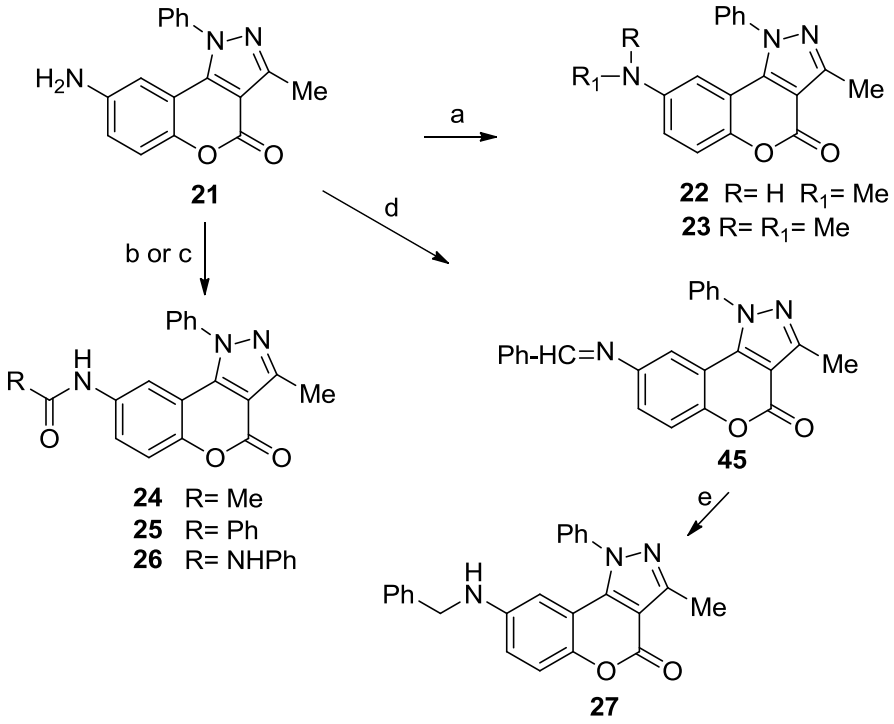
11-14, **17**. The same synthetic pathway was used to prepare the 1-(2-carboxyphenyl)-substituted compound **15** obtained starting from **5** and through the formation of the hydrazone derivative **40**.



	R_1	R_2	R
16, 41	H	H	COOH
18, 42	H	Cl	H
19, 43	NO_2	H	H
20, 44	NO_2	Cl	H
21	NH_2	H	H

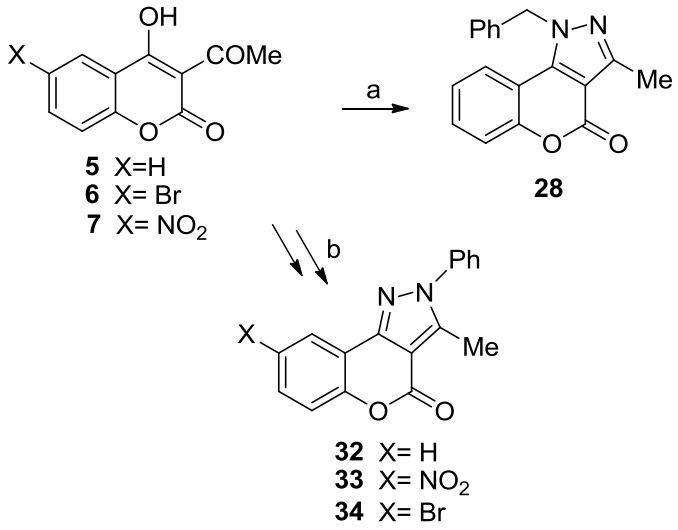
Scheme 3. Reagents and conditions: a) Ar-$NHNH_2$, EtOH, reflux; b) $Cu(AcO)_2$, $CuSO_4$, EtOH, reflux; c) from **19**, H_2, Pd/C, Parr apparatus 33 psi.

The tricyclic derivatives **16** and **18-20** (Scheme 3) were synthesized from the 3-acetylchromen-2-ones **1-4** which were reacted with arylhydrazines to afford the relative arylhydrazones **41-44**. The latter were cyclized by oxidation with a mixture of cupric sulfate and cupric acetate to give the chromeno[4,3-c]pyrazoles **16**, **18-20**. Catalytic reduction (Pd/C) of the 8-nitro derivative **19** provided the corresponding 8-amino derivative **21** which, besides being a target compound, was used as an intermediate for the preparation of the 1-phenylchromenopyrazoles **22-27** (Scheme 4).



Scheme 4. Reagents and conditions: a) excess of MeI, K_2CO_3, CH_3CN; b) RCOCl, NEt_3, anhydrous CH_2Cl_2, rt; c) PhNCO, anhydrous THF, reflux; d) PhCHO, anhydrous $ZnCl_2$, anhydrous THF, reflux, N_2; e) $NaBH_4$, MeOH, reflux, N_2.

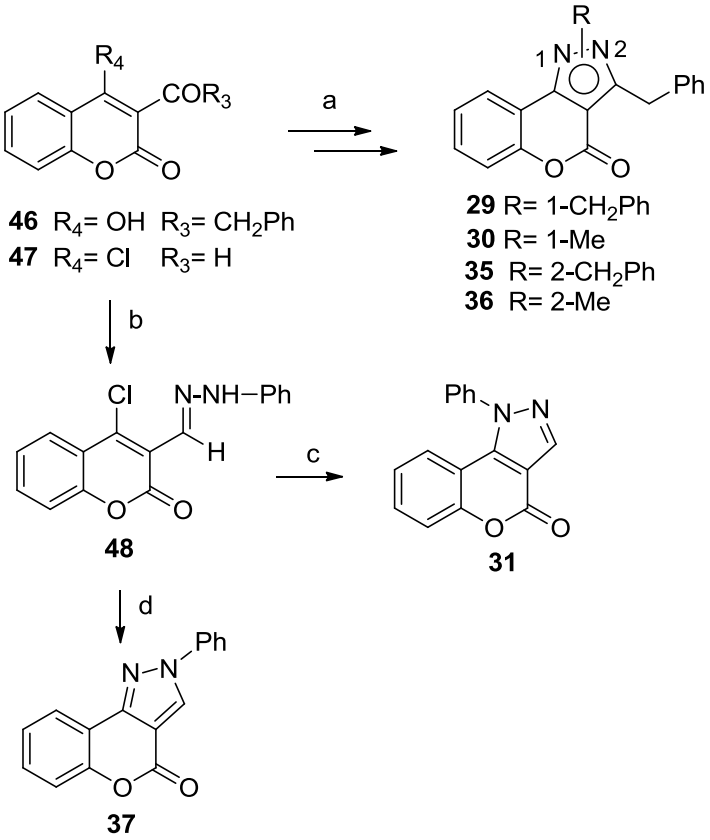
Reaction of **21** with an excess of methyl iodide furnished a mixture of the 8-methylamino- and 8-dimethylamino derivatives **22** and **23**, respectively, which were separated by column chromatography. When compound **21** was reacted with acyl chlorides or phenylisocyanate, the corresponding 8-amido- (**24**, **25**) or 8-phenylureido- (**26**) derivatives were obtained. The 8-benzylamino- substituted compound **27** was synthesized by treatment of **21** with benzaldehyde and subsequent reduction of the Schiff's base **45** with sodium borohydride. The 1-benzylchromenopyrazol-4-one derivative (**28**) (Scheme 5) was prepared by reacting 3-acetyl-4-hydroxychromen-2-one (**5**) with benzylhydrazine in refluxing ethanol. The 2-phenylchromeno[4,3-c]pyrazol-4-one derivatives **32-34** (Scheme 5) were obtained starting from the 3-acetyl-4-hydroxychromen-2-one derivatives **5-7**, as previously reported [23, 24].



Scheme 5. Reagents and conditions: a) Ph-CH_2NHNH_2 dihydrochloride, NEt_3, EtOH, reflux; b) see Ref. 23 and 24.

The synthesis of the tricyclic derivatives **29-31** and **35-37** is shown in Scheme 6. The chromeno[4,3-c]pyrazol-4-ones featuring a benzyl group at position 3, combined with a methyl and a benzyl residue at position 1 (**29** and **30**) [25] and 2 (**35** and **36**) [25], were obtained from 4-hydroxy-3-phenacetyl-chromen-2-one (**46**). The chromeno[4,3-c]pyrazol-4-one derivatives **31** and **37**, lacking a substituent at position 3 and bearing a phenyl group at position 1 or 2, were prepared starting from 4-chloro-2-oxo-2H-chromene-3-carbaldehyde **47** [26] which was transformed into the corresponding 3-phenylhydrazone **48** [26]. This compound was cyclized by heating in pyridine and a catalytic amount of piperidine to provide the 1-phenyl regioisomer **31** [26]. To obtain the 2-phenyl regioisomer **37** we employed different conditions from those described [27] as they afforded a mixture of the two isomer **31** and **37**. In our hands, the intermediate **47** was reacted with an equimolar amount of phenylhydrazine in refluxing glacial acetic acid and compound **37** was isolated as the sole isomer in good yields.

The synthesis of the 3,6-dimethylpyrano[4,3-c]pyrazole derivatives **38** [28] and **39** [29] was achieved, as previously reported, starting from the commercially available dehydroacetic acid.



Scheme 6. Reagents and conditions: a) see Ref. 25; b) $PhNHNH_2$.HCl, H_2O/AcOH, MeOH, 60 °C; c) piperidine, pyridine, 100 °C; d) $PhNHNH_2$, glacial AcOH reflux.

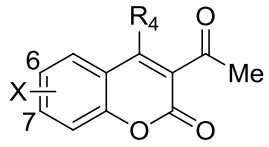
2.2.CA inhibitory activity

All the synthesized derivatives **1-39** were tested to determine their efficacy against the targeted hCAs IX and XII and also toward the hCAs I and II, which are physiologically relevant cytosolic isoforms, highly expressed throughout the human body, thus responsible for the side effects of nonselective hCA inhibitors.

The inhibition data of the synthesized compounds at hCAs are reported in Tables 1-3, together with those of acetazolamide (AZA) and coumarin, as reference inhibitors. The obtained results indicate that enhancement of molecular complexity of the coumarin scaffold ameliorated the potency and selectivity toward the targeted hCAs, thus confirming the concept that a more complex ligand can present better complementarity for a specific target. None of the tested derivatives was able to inhibit the off-target hCAs I and II, some compounds (**13**, **14**, **19**, **21**, **25**, **31**, **33**, **37** and **39**) were

potent (K_i < 41 nM) and selective against the hCA IX, and some (**6**, **11** and **17**) were dually potent hCAs IX and XII inhibitors (K_i= 5.6-9.6 nM).

Table 1. Inhibition data of the 3-acetyl-2H-chromen-2-one derivatives **1-10** and Acetazolamide (AZA) and coumarin, as reference compounds, against hCA I, II, IX and XII isoforms, as determined by a stopped-flow CO_2 hydrase assay.



1-10

			K_i (nM)[a]			
	R_4	X	hCAI	hCAII	hCAIX	hCAXII
1	H	H	>10000	>10000	151.8	7.8
2	H	6-NO_2	>10000	>10000	44.9	695.2
3	H	7-Cl	>10000	>10000	809.4	4529.4
4	H	6-NO_2-7-Cl	>10000	>10000	561.2	6400.0
5	OH	H	>10000	>10000	35.9	453.5
6	OH	6-Br	>10000	>10000	8.2	5.6
7	OH	6-NO_2	>10000	>10000	44.2	357.1
8	OMe	H	>10000	>10000	28.4	673.5
9	OMe	6-Br	>10000	>10000	90.7	706.1
10	OEt	6-Br	>10000	>10000	46.4	737.2
	Coumarin[b]		31000	92000	>500000	>500000
	AZA		250	12.1	25.4	5.6

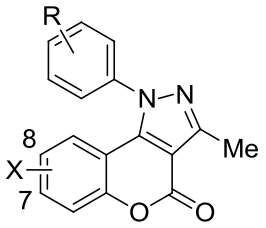
[a]Mean from three different assays, and errors were within 10% of the reported values. [b]Ref. 16.

Concerning the first set of inhibitors, i.e. the 3-acetylchromen-2-one derivatives **1-10** (Table 1), introduction of the acetyl group at the 3-position of coumarin resulted in a strong increase of potency at the hCAs IX and XII, compound **1** being active in the nanomolar range (K_i= 151.8 and 7.8 nM, respectively) against the two isoforms. Due to this encouraging result, further modifications were carried out on derivative **1**, such as introduction on the fused benzo ring of small substituents

(6-NO_2, 7-Cl or both) which, to the best of our knowledge, have been never probed on coumarin derivatives designed as hCA inhibitors. Derivatives obtained in this way (**2-4**) showed lower potencies, compared to the lead **1**, in particular against the hCA XII isoform. The only exception was the 6-nitro derivative **2** being about three-fold more active than **1** against the hCA IX. Introduction of a hydroxy group at position 4 of derivatives **1** and **2**, to give **5** and **7**, elicited different effects. Compound **5** was more effective than the parent **1** at the hCA IX (K_i= 35.9 nM) and significantly less potent at the hCA XII (K_i= 453.5 nM). In contrast, derivative **7** was as active as the parent compound **2**. However, compounds **5** and **7** are equally potent against each hCA of interest. Introduction of the lipophilic and bulky bromine atom at position 6 of compound **5** turned out to be profitable, compound **6** being one of the most potent dual inhibitors of hCAs IX and XII among the herein reported derivatives. The effect of 4-hydroxy alkylation carried out on compounds **5** and **6** is difficult to explain as it afforded opposite results (compounds **8-10**). Methylation of the 4-hydroxy group of **5** left the inhibition profile unchanged (see derivative **8**) while the same modification on derivative **6** worsened activity about 10-fold against the hCA IX and more drastically against the hCA XII (see derivative **9**). Transformation of the 4-methoxy derivative **9** into its superior homologue 4-ethoxy derivative **10** slightly enhanced the hCA IX potency.

In the second step of the work, we decided to merge a pyrazole nucleus with the bicyclic 3-acetylchromen-2-one **1** to verify whether the added ring, and other structural elements on it, could enhance affinity and selectivity for the targets. First, the 1-phenyl-3-methylchromeno[4,3-c]pyrazol-4-one **11** was synthesized and tested (Table 2). Compound **11** behaved as a balanced dual inhibitor of hCAs IX and XII, endowed with low nanomolar activity (K_i= 9.6 and 5.8 nM), and was more potent at the former isoform than the parent derivative **1**. These results indicate that compound **11** possesses a better structural complementarity for the hCA IX, thus suggesting that the fused pyrazole nucleus and the lipophilic area represented by the 1-phenyl ring reinforce the interaction with the enzyme.

Table 2. Inhibition data of derivatives **11-27** and Acetazolamide (AZA), as a reference compound, against hCA I, II, IX and XII isoforms, as determined by a stopped-flow CO_2 hydrase assay.



11-27

			K_i (nM)[a]			
	R	X	hCAI	hCAII	hCAIX	hCAXII
11	H	H	>10000	>10000	9.6	5.8
12	4-Cl	H	>10000	>10000	402.8	>10000
13	4-Me	H	>10000	>10000	22.2	>10000
14	4-OMe	H	>10000	>10000	40.2	>10000
15	2-COOH	H	>10000	>10000	76.8	27.3
16	4-COOH	H	>10000	>10000	1960.4	24.7
17	H	8-Br	>10000	>10000	8.5	7.1
18	H	7-Cl	>10000	>10000	57.9	818.7
19	H	8-NO_2	>10000	>10000	27.1	>10000
20	H	7-Cl, 8-NO_2	>10000	>10000	801.2	6062.5
21	H	8-NH_2	>10000	>10000	34.5	>10000
22	H	8-NHMe	>10000	>10000	51.3	737.2
23	H	8-N(Me)$_2$	>10000	>10000	59.4	359.8
24	H	8-NHCOMe	>10000	>10000	26.8	646.5
25	H	8-NHCOPh	>10000	>10000	15.3	>10000
26	H	8-NHCONHPh	>10000	>10000	77.9	583.2
27	H	8-$NHCH_2$Ph	>10000	>10000	37.8	359.8
	AZA		250	12.1	25.4	5.6

[a]Mean from three different assays, and errors were within 10% of the reported values.

To preliminarily investigate the structure-affinity relationships (SAR) in this new class of hCA inhibitors, small substituents with different lipophilic and electronic properties (Cl, OMe, Me, COOH) were introduced on the 1-phenyl ring (derivatives **12-16**). Only the carboxylic residue, both

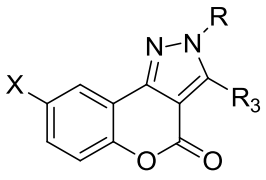
at the ortho (**15**) and para (**16**) position, afforded potent hCA XII inhibitors (K_i= 27.3 and 24.7 nM, respectively). The ortho-isomer **15** also showed quite good activity against the hCA IX. The other substituents yielded to hCA IX selective compounds (**12-14**) since they significantly reduced hCA XII inhibition. The 4-methyl- and 4-methoxy-substituted derivatives **13** and **14** are noteworthy, showing K_i values in the low nanomolar range (22.2 and 40.2 nM).

In the second set of tricyclic derivatives (**17-20**), structural modifications were carried out on the fused benzo ring where the same substituents probed on coumarins **1-10** were evaluated. The K_i values obtained for **17-20** are overall better than those of the relative bicyclic compounds and the observed SARs are similar. In detail, the 8-bromo derivative **17** stands out as one of the best dual hCAs IX and XII inhibitors (K_i= 8.5 and 7.1 nM) among those reported here. Also the 8-nitro derivative **19** is worth noting, being one of the most potent and selective inhibitors at the hCA IX isoform (K_i= 27.1 nM) of those investigated in this work. The 7-chlorine atom (compound **18**) resulted in a nanomolar potency at the hCA IX (K_i= 57 nM) and a significantly reduced inhibition of the hCA XII. The 7-chloro-8-nitro substitution made compound **20** scarcely active at both the targeted hCA isoforms. Reduction of the 8-nitro derivative **19** gave the 8-amino derivative **21** which, similarly to **19**, behaves as a selective hCA IX inhibitor endowed with nanomolar potency (K_i= 34.5 nM).

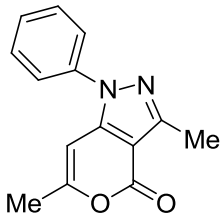
The 8-amino group of **21** was exploited for subsequent derivatizations. Its linkage to groups with different steric, lipophilic and electronic properties, such as methyl (**22** and **23**), acetyl (**24**), phenylcarbamoyl (**26**) or benzyl (**27**) showed little effect on the potency against the hCA IX, the obtained compounds being as active as **21** (K_i= 37.8-77.9 nM). These 8-amino-modified derivatives also showed some ability to inhibit the hCA XII (K_i= 359.9-737.2 nM). Instead, the presence of a benzoyl moiety on the 8-amino group (**25**) afforded the most potent (K_i= 15.3 nM) and selective hCA IX inhibitor of those reported.

Table 3. Inhibition data of derivatives **28-39** and Acetazolamide (AZA), as a reference compound, against hCA I, II, IX and XII isoforms, as determined by a stopped-flow CO_2 hydrase assay.

28-31



32-37



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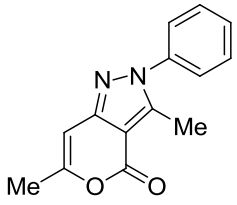

39

				K_i (nM)[a]			
	R	R_3	X	hCAI	hCAII	hCAIX	hCAXII
28	C_6H_4-CH_2	Me	-	>10000	>10000	371.1	731.9
29	C_6H_4-CH_2	C_6H_4-CH_2	-	>10000	>10000	31.5	818.7
30	Me	C_6H_4-CH_2	-	>10000	>10000	19.7	342.9
31	C_6H_5	H	-	>10000	>10000	30.1	>10000
32	C_6H_5	Me	H	>10000	>10000	38.1	593.2
33	C_6H_5	Me	Br	>10000	>10000	25.2	>10000
34	C_6H_5	Me	NO_2	>10000	>10000	35.8	359.8
35	C_6H_4-CH_2	C_6H_4-CH_2	H	>10000	>10000	289.7	671.9
36	Me	C_6H_4-CH_2	H	>10000	>10000	555.2	33.3
37	C_6H_5	H	H	>10000	>10000	37.6	>10000
38	-	-	-	>10000	>10000	26.6	517.7
39	-	-	-	>10000	>10000	41.4	>10000
	AZA			250	12.1	25.4	5.6

[a]Mean from three different assays, and errors were within 10% of the reported values.

Compounds **28**-**31** (Table 3) ensued from the chromeno[4,3-c]pyrazol-4-one **11** whose 1-phenyl and 3-methyl substituents were replaced by a benzyl, a methyl or a hydrogen atom to evaluate the steric and lipophilic requirements of the molecule in proximity of the two positions. None of these modifications ameliorated the inhibitory profile of the lead. In fact, the presence of a benzyl pendant at position 1 (**28**) caused a drop of potency at both hCAs of interest. Compound **29**, more

hindered than **28** since it features a benzyl group at both the 1- and 2-positions, showed a restored activity at the hCA IX, being active at low nanomolar concentration (K_i= 31.5 nM). A comparable inhibitory profile was obtained when the 1-benzyl moiety of **29** was replaced with the less lipophilic methyl group to give compound **30**. An interesting result, although difficult to explain, was found for compound **31**, where a hydrogen atom was present at position 3 in the place of the 3-methyl group of the potent dual hCA IX/XII inhibitor **11**. This small change made compound **31** inactive at the hCA XII isoform while it preserved activity against the hCA IX (K_i= 30.1 nM), thus suggesting that the methyl substituent of **11**, although being a small group, plays a crucial role for interaction with the hCA XII enzymatic site.

The set of derivatives **32-37** (Table 3) was synthesized to evaluate the effect of moving the lipophilic area (Ph, CH_2Ph, Me) from the 1- to the 2-position of the best inhibitors identified in the meanwhile, i.e. compounds **11**, **17**, **19**, **29-31**. This modification, although it significantly changed the shape of the molecules, gave rise to some potent hCA IX inhibitors (**32-34**, **37**). The compounds showed in fact nanomolar activities (K_i= 25.2-38.1 nM) even if they were in general less active than the 1-substituted counterparts (**11**, **17**, **19** and **31**) on both the isoforms. It is worth noting that the 8-bromo-derivative **33** was hCA IX selective while its regioisomer **17** was active on both the targeted enzymes. Derivatives **35** and **36**, featuring a benzyl moiety at position 3, displayed scarce potencies on the hCAs of interest, with the exception of the hCA XII inhibitor activity of **36** in the low nanomolar range.

Finally, the fused benzo ring of the 1-phenyl- and 2-phenyl- substituted chromenopyrazole derivatives **11** and **32** was replaced with a methyl residue to give the pyrano[4,3-c]pyrazol-4-one derivatives **38** and **39**. Both compounds maintained the capability of blocking the hCA IX with high potency (K_i= 26.6 and 41.4 nM) while they were less active against the hCA XII. As observed in the tricyclic series, the 1-phenyl derivative **38** showed a higher potency with respect to the 2-substituted regioisomer **39**.

2.3. Cell toxicity assays

Some selected compounds, belonging to the 3-acetylcoumarin set (**6**) and to the chromeno[4,3-c]pyrazol-4-one (**11**, **13**, **19**, **21**, **25**, **31**) and pyrano[4,3-c]pyrazol-4-one (**39**) series, were chosen among those possessing the best inhibitory profile and tested (30 – 300 μM) to evaluate their effects on viability of human colon cancer HT-29 cells. Tests were performed over time (16 and 48 h) under normoxic and hypoxic conditions. The efficacy of the compounds is summarized in Figures 2 (**6**, **11**, **13** and **19**) and 3 (**21**, **25**, **31** and **39**) in comparison to untreated control (100% viability).

As a general trend, after 16 h incubation, the tested derivatives significantly inhibited cell viability in normoxic conditions while most of them did not have any effect in hypoxia. A similar behavior was also observed after 48 h incubation, with the exception of compounds **19**, **31** and **39** which turned out to be more active under hypoxic conditions.

Analyzing the results in greater detail, in normoxic conditions, compounds **6**, **13**, **21** and **25** showed a concentration- and time-dependent efficacy in reducing cancer cell viability. It was not possible to test **6** and **13** at the higher concentration (300 μM) for their low solubility in the assay medium. Derivatives **6** and **25** (100 μM, 48 h) reduced the number of living cells up to about 35 and 50%, respectively. Compounds **11**, **19** and **39** showed the peculiarity of a dramatic concentration-dependent effect after 16 h incubation. The lower activity after 48 h could be related to a reduced stability of the solutions rather than an intrinsic variation of efficacy. In hypoxic conditions, the longer incubation time favors activity: after 48 h compounds **6**, **11**, **19**, **21**, **31** and **39** were significantly effective. In particular, the 2-phenylchromeno-pyrazol-4-one **31** (30 μM, 48 h, hypoxia) showed the best profile reducing cell viability up to 70%.

Finally, a long incubation time (72 h) was experimented. Only the activities of the most interesting derivatives (selected by solution stability and effectiveness) are reported in Figure 4. Compound **6** maintained efficacy as shown also for shorter times. With regard to compounds **21**, **25** and **39**, a longer time of incubation promoted activity in hypoxic conditions. In particular, compound **25** (100 μM) decreased cancer cell viability to 40%.

It is difficult to explain why some compounds showed cytotoxic effects not only in hypoxia (when hCA IX is overexpressed) but also in normoxia, when the enzyme is not present in these cells. Thus, it is possible that, besides interfering with the hCA IX/XII activity in hypoxia, they may exert their cytotoxic effects through other mechanisms of action, involving pathways in normoxia. A similar behavior was observed also for other hCA inhibitors, such as sulfocoumarins [13], 3-hydroxyquinazoline-2,4-diones [21] and selenoureido-benzensulfonamides [31].

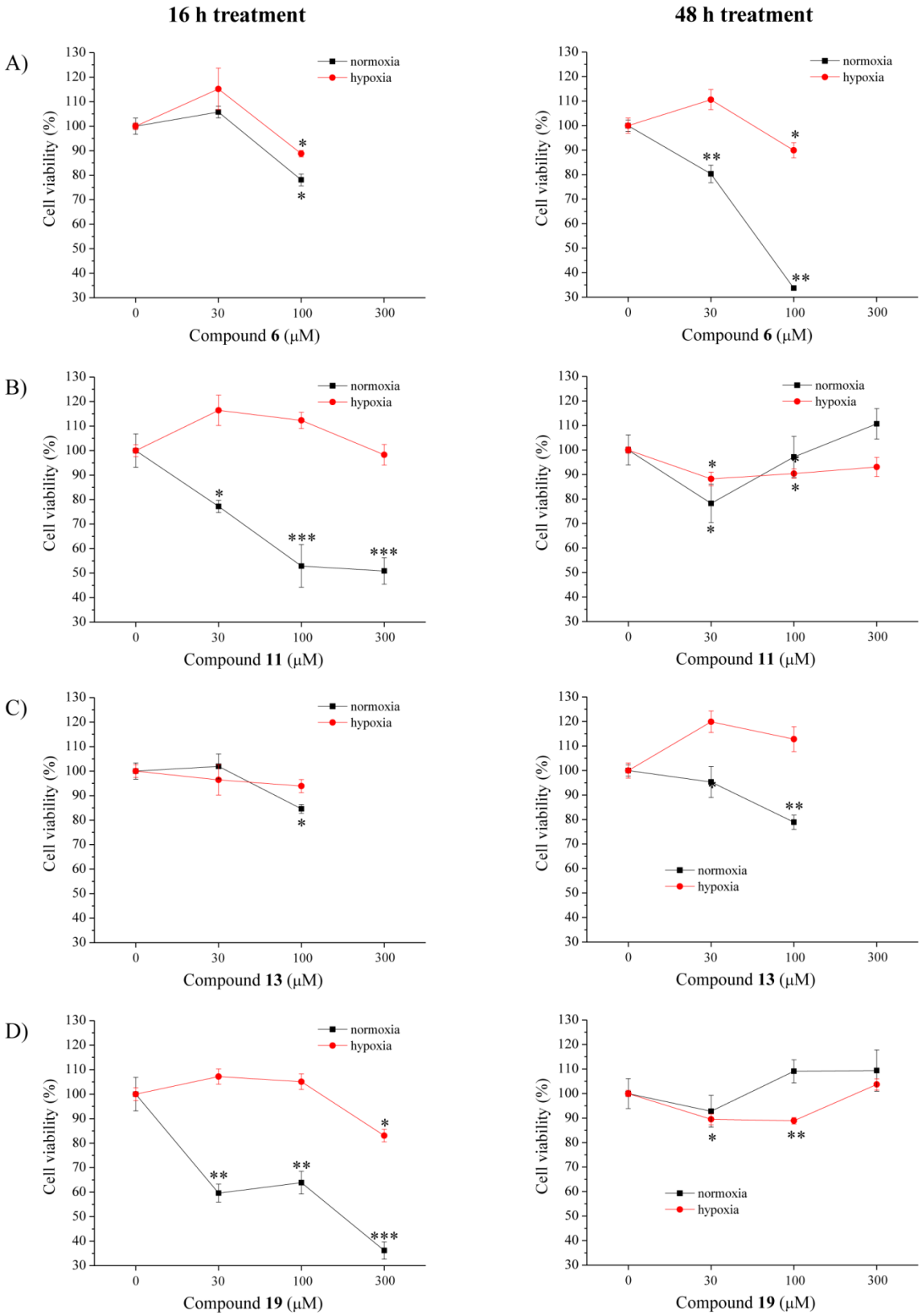

Fig. 2. HT-29 cells ($1 \cdot 10^4$/well) were treated with compounds **6** (A), **11** (B), **13** (C) and **19** (D) (0 - 300 μM). Incubation was allowed for 16 or 48 h in normoxic (20% O_2) and hypoxic conditions (0.1% O_2). Cell viability was measured by MTT assay. The control condition was arbitrarily set as 100% and values are expressed as the mean ± SEM of three experiments. *P<0.05, **P<0.01 and ***P<0.001 in comparison to control (0 μM).

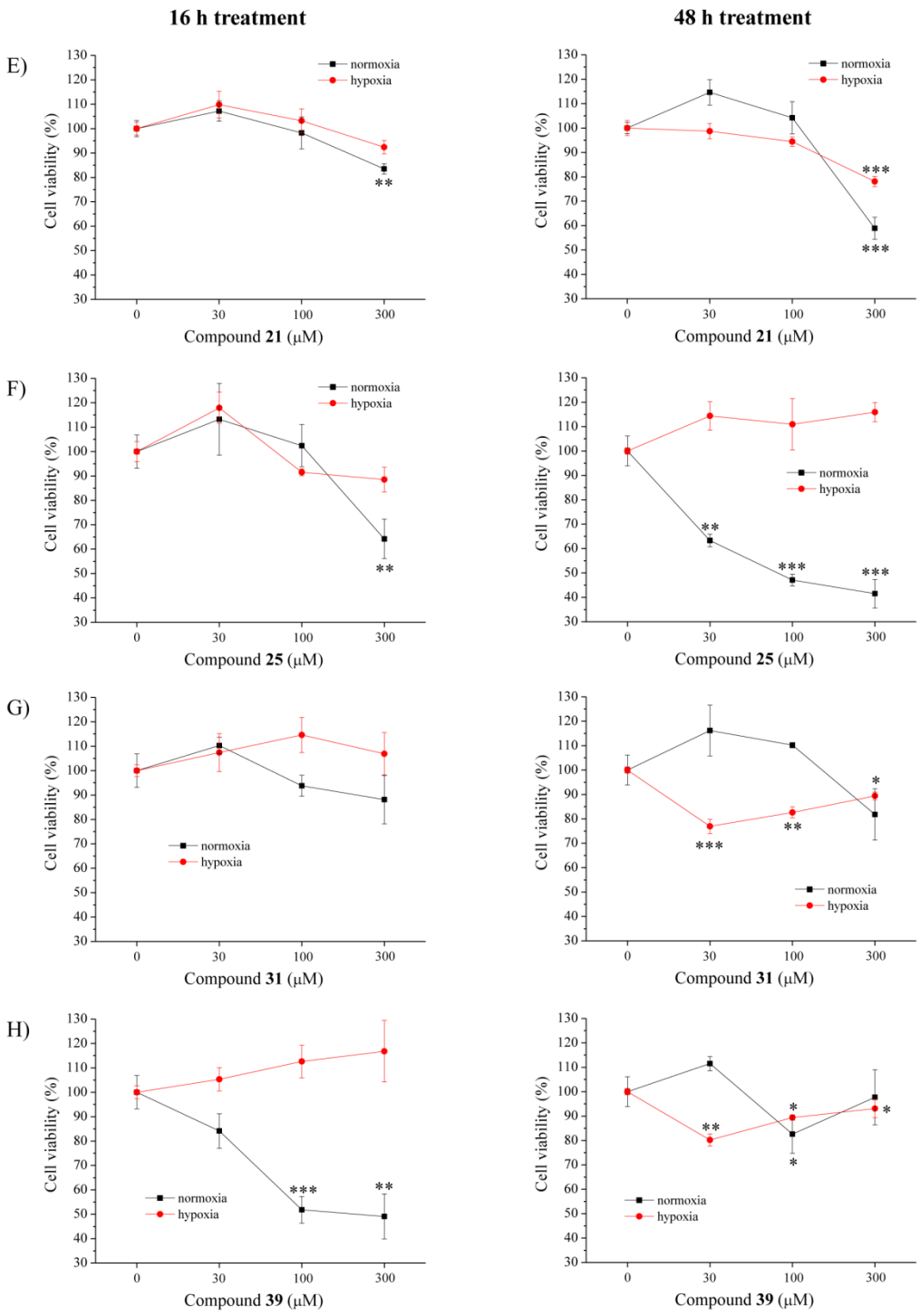

Fig. 3. HT-29 cells ($1 \cdot 10^4$/well) were treated with compounds **21** (E), **25** (F), **31** (G), and **39** (H) (0 - 300 μM). Incubation was allowed for 16 or 48 h in normoxic (20% O_2) and hypoxic conditions (0.1% O_2). Cell viability was measured by MTT assay. The control condition was arbitrarily set as 100% and values are expressed as the mean ± SEM of three experiments. *P<0.05, **P<0.01 and ***P<0.001 in comparison to control (0 μM).

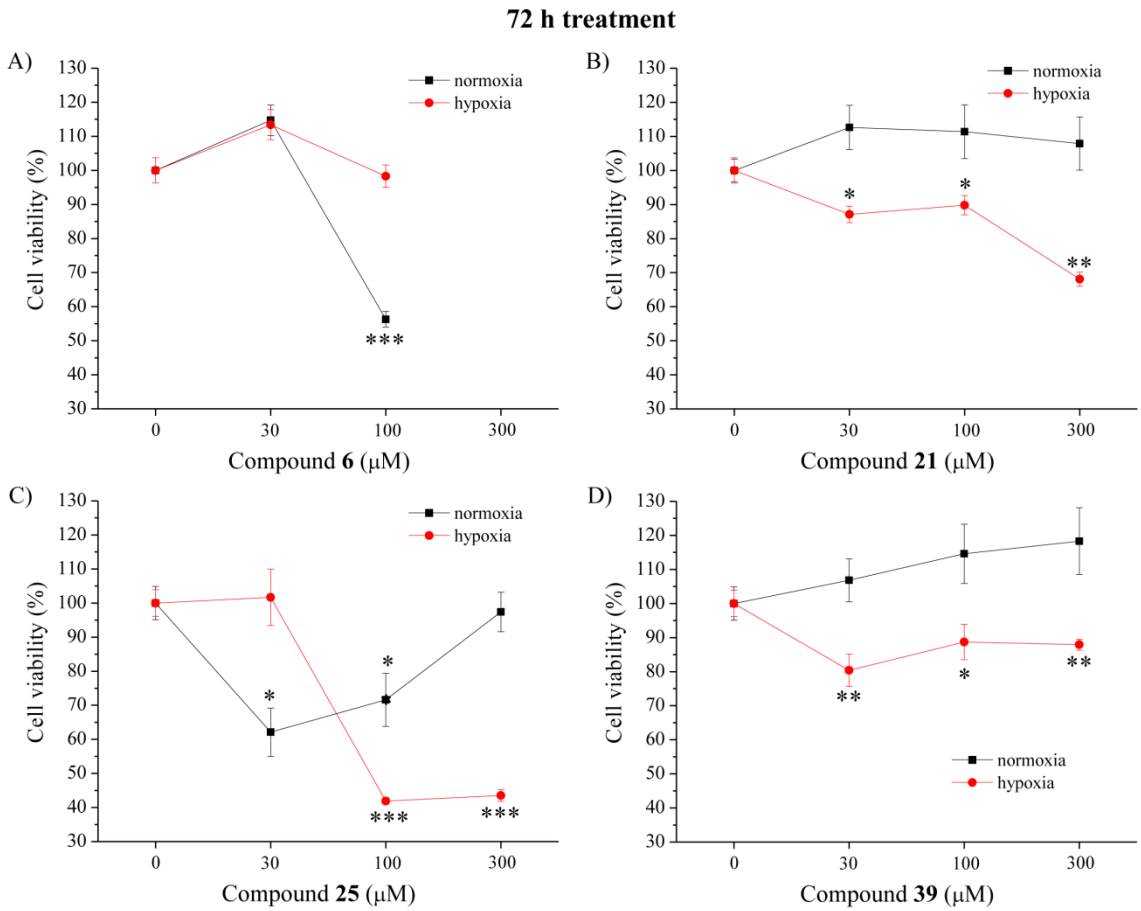

Fig. 4. HT-29 cells ($1 \cdot 10^4$/well) were treated with compounds **6** (A), **21** (B), **25** (C), and **39** (D) (0 - 300 μM). Incubation was allowed for 72 h in normoxic (20% O_2) and hypoxic conditions (0.1% O_2). Cell viability was measured by MTT assay. The control condition was arbitrarily set as 100% and values are expressed as the mean ± SEM of three experiments. *P<0.05, **P<0.01 and ***P<0.001 in comparison to control (0 μM).

3. Conclusions

In this work, different structural modifications were carried out on the coumarin scaffold leading to a set of 3-acetyl-coumarin derivatives and, more interestingly, to new classes of inhibitors of the tumor-associated hCAs IX and XII: the chromeno[4,3-c]pyrazol-4-ones and the pyrano[4,3-c]pyrazol-4-ones. Several synthesized derivatives showed K_i values in the low nanomolar range, being either selective against the hCA IX or dual inhibitors on both the hCA isoforms of interest. None of the compounds was effective in inhibiting the cytosolic enzymes hCAs I and II. Some inhibitors proved to be active as antiproliferative agents on HT-29 colon cancer cell lines, both in normoxic and hypoxic conditions. This finding led us to hypothesize for these derivatives more than one mechanism of action, involving hCAs IX and XII in hypoxia and other not identified target(s) in normoxia.

4. Experimental section

4.1 Chemistry.

All the commercially available reagents and solvents were used as purchased from Sigma-Aldrich and Alfa Aesar (Italy) without further purification. Analytical silica gel plates (Merck F254) and silica gel 60 (Merck, 70-230 mesh) were used for analytical TLC and for column chromatography, respectively. All melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Compounds were named following IUPAC rules as applied by ChemDrawUtra 9.0. Elemental analyses were performed with a Flash E1112 Thermofinnigan elemental analyzer for C, H, N and the results were within ± 0.4% of the theoretical values. All final compounds revealed purity not less than 95%. The IR spectra were recorded with a Perkin-Elmer Spectrum RX I spectrometer in Nujol mulls and are expressed in cm^{-1}. NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR). The chemical shifts are reported in δ (ppm) and are relative to the central peak of the solvent which was $CDCl_3$ or

$DMSOd_6$. The following abbreviations are used: s= singlet, d= doublet, t= triplet, q= quartet, m= multiplet, br= broad and ar= aromatic protons.

Compounds **2** [22], **5** [23], **6** [24], **11-14** [23], **17** [24], **29-30** [25], **32** [23], **33** and **34** [24], **35** and **36** [25], **38** [28], **39** [29] were prepared according previously reported procedures.

*4.1.1. 3-Acetyl-7-chloro-2H-chromen-2-one (**3**)*

A mixture of 4-chloro-2-hydroxybenzaldeyde (8.98 mmol), ethyl acetoacetate (22.4 mmol) and catalytic amount of piperidine (0.22 mmol) was stirred at room temperature for 10 min, then it was diluted with EtOH (10 mL). The solid which precipitated was collected by filtration and recrystallized. Yield 90%; mp 170-171 °C (2-Methoxyethanol); ^{1}H NMR ($CDCl_3$): δ 2.74 (s, 3H, CH_3), 8.34 (dd, 1H, H-6, J = 8.4, 1.9 Hz), 7.35 (d, 1H, H-8, J = 1.7 Hz), 8.01 (d, 1H, H-5, J = 8.3 Hz), 8.49 (s, 1H, H-4). ^{13}C NMR (DMSO-d_6): δ 30.92 (CH_3), 117.27, 118.18, 125.33, 126.26, 133.02, 139.74, 147.25, 155.92, 158.88, 195.80. IR: 1678, 1741. Anal. $C_{11}H_7ClO_3$ (C, H, N).

*4.1.2. 3-Acetyl-7-chloro-6-nitro-2H-chromen-2-one (**4**)*

3-Acetyl-7-chloro-2H-chromen-2-one **3** (2.25 mmol) was added portionwise to cooled HNO_3 (90 %, -10 °C). When the addition was complete, the mixture was stirred for about 2h at -10 °C, then the solution was poured onto ice (about 100 g) and the solid collected, washed with abundant water (about 100 mL), dried and recrystallized. Yield 88%; mp 210-212 °C (EtOH); ^{1}H NMR ($CDCl_3$): δ 2.75 (s, 3H, CH_3), 7.58 (s, 1H, H-8), 8.31 (s, 1H, ar), 8.49 (s, 1H, ar). ^{13}C NMR (DMSO-d_6): δ 30.36 (CH_3), 118.16, 120.01, 126.67, 128.61, 130.97, 143.92, 145.33, 156.66, 157.58, 194.95. IR: 1686, 1737. Anal. $C_{11}H_6ClNO_5$ (C, H, N).

*4.1.3. 3-Acetyl-4-hydroxy-6-nitro-2H-chromen-2-one (**7**)*

3-Acetyl-4-hydroxy-2H-chromen-2-one **5** (2.45 mmol) [23] was portionwise added to a cooled solution (0 °C) of KNO_3 (2.45 mmol) in H_2SO_4 (10 mL). When the addition was complete, the

mixture was stirred for about 1h at 0 °C, then the solution was poured onto ice (about 50 g) and the solid collected, washed with abundant water (about 50 mL), dried and recrystallized. Yield 98%; mp 230-231 °C (EtOH); ^{1}H NMR ($CDCl_3$): δ 2.83 (s, 3H, CH_3), 7.48 (d, 1H, H-8, J = 9.0 Hz), 8.55 (dd, 1H, H-7, J = 9.2, 2.5 Hz), 8.98 (d, 1H, H-5, J = 2.2 Hz).

*4.1.4. General procedure for the synthesis of 3-acetil-4-metossi-2H-chromen-2-one derivatives **8, 9***

A mixture of 3-acetil-4-hydroxy-2H-chromen-2-one derivatives **5** [23] or **6** [24] (0.98 mmol), K_2CO_3 (2.94 mmol) and dimethylsulfate (1.17 mmol) in anhydrous acetone (3 mL), was microwave irradiated at 125 °C for 6 min. The suspension was poured onto iced water (50 mL) and neutralized with 10% HCl solution. The mixture was extracted with Et_2O (30 mL x 3), then the organic phase was washed with a $NaHCO_3$ saturated solution (50 mL) and brine (50 mL). The dried (Na_2SO_4) organic phase was evaporated at reduced pressure to give an oil residue which solidified upon treatment with Et_2O (2-3 mL). The crude compound was purified by column chromatography (cyclohexane/EtOAc 6:4).

*4.1.4.1. 3-Acetyl-4-methoxy-2H-chromen-2-one (**8**).* Yield 74%; mp 81-83 °C (MeOH); ^{1}H NMR ($CDCl_3$): δ 2.72 (s, 3H, CH_3), 4.02 (s, 3H, OCH_3), 7.30-7.34 (m, 2H, ar), 7.61 (t, 1H, ar, J = 7.2 Hz), 7.91 (d, 1H, H-5, J = 7.9 Hz). IR 1699, 1713. Anal. $C_{12}H_{10}O_4$ (C, H, N).

*4.1.4.2. 3-Acetyl-6-bromo-4-methoxy-2H-chromen-2-one (**9**).* Yield 70%; mp 147-149 °C (MeOH); ^{1}H NMR ($CDCl_3$): δ 2.71 (s, 3H, CH_3), 4.01 (s, 3H, OCH_3), 7.22 (d, 1H, H-8, J = 7.8 Hz), 7.69 (dd, 1H, H-7, J = 8.4, 2.2 Hz), 8.03 (d, 1H, H-5, J =2.2 Hz). ^{13}C NMR (DMSO-d_6): δ 32.95 (CH_3), 62.67 (OCH_3), 110.63, 117.50, 118.96, 119.80, 127.40, 136.82, 151.93, 161.22, 162.02, 200.74. IR: 1693, 1710. Anal. $C_{12}H_9ClBrO_4$ (C, H, N).

4.1.5. *3-Acetyl-4-ethoxy-6-bromo-2H-chromen-2-one (**10**)*

A suspension of the 4-methoxy-2H-chromen-2-one derivative **9** (0.3 mmol) in absolute EtOH (25 mL) was refluxed for 6 h. The insoluble solid was collected by filtration and recrystallized. Yield 75%; mp 116-118 °C (EtOH); ^{1}H NMR ($CDCl_3$): δ 1.52 (t, 3H, CH_3, J = 6.9 Hz), 2.69 (s, 3H, CH_3), 4.15 (q, 2H, CH_2, J = 6.9 Hz), 7.22 (d, 1H, H-8, J = 8.8 Hz), 7.68 (dd, 1H, H-7, J = 8.4, 2.2 Hz), 8.01 (d, 1H, H-5, J = 2.2 Hz). ^{13}C NMR ($CDCl_3$): δ 15.36 (CH_3), 32.16 (CH_3), 70.97 (CH_2), 117.21, 118.37, 127.05, 135.95, 147.34, 149.04, 151.42, 153.07, 160.81, 161.63. IR: 1699, 1712. Anal. $C_{13}H_{11}ClBrO_4$ (C, H, N).

*4.1.6. 2-(2-(1-(2-Oxo-2H-chromen-3-yl)ethylidene)hydrazinyl)benzoic acid (**40**)*

A mixture of the suitable 2-hydrazinobenzoic acid hydrochloride [30] (2.58 mmol) and triethylamine (2.58 mmol) in EtOH (30 mL) was stirred at reflux for 5-10 min, then the 3-acetyl-4-hydroxy-2H-chromen-2-one **5** (1.29 mmol) was added and the mixture was heated for an additional 30 min. After cooling at room temperature, the solid was collected, washed with water (about 10-20 mL) and recrystallized. Yield 92%; mp 245-246 °C (EtOH); ^{1}H NMR ($DMSOd_6$): δ 2.69 (s, 3H, CH_3), 6.97 (t, 1H, ar, J = 7.6 Hz), 7.02 (d, 1H, ar, J = 8.5 Hz), 7.32–7.37 (m, 2H, ar), 7.57 (t, 1H, ar, J = 8.5 Hz), 7.66 (t, 1H, J = 7.8 Hz), 7.96 (d, 1H, ar, J = 7.8 Hz), 8.00 (d, 1H, ar, J = 7.6 Hz), 10.28 (s, 1H, NH), 13.37 (br s, 1H, OH) Anal. $C_{18}H_{14}N_2O_5$ (C, H, N).

*4.1.7. 2-(3-Methyl-4-oxochromeno[4,3-c]pyrazol-1(4H)yl)benzoic acid (**15**)*

A mixture of arylhydrazone **40** (2.96 mmol) and a catalytic amount of p-toluensulfonic acid in anhydrous xylene (50 mL) was refluxed for about 1h, while water was distilled off using a Dean-Stark apparatus. The mixture was concentrated by removing about half of the solvent, then cooled at rt and filtered to remove the tarry residue. After addition of ligroin to the filtrate, a solid was obtained which was collected by filtration and recrystallized. Yield 48%; mp > 300 °C (EtOH). ^{1}H NMR ($DMSOd_6$): δ 2.55 (s, 3H, CH_3), 6.79 (d, 1H, ar, J = 7.3 Hz), 7.17 (t, 1H, ar, J = 8.0 Hz), 7.52-

7.57 (m, 2H, ar), 7.77 (d, 1H, ar, J = 7.6 Hz), 7.83-7.89 (m, 2H, ar), 8.15 (d, 1H, ar, J = 7.4 Hz). ^{13}C NMR (DMSOd_6): δ 13.49 (CH_3), 105.93, 112.55, 118.64, 122.69, 125.38, 130.33, 130.57; 132.00, 132.31, 132.45, 134.56, 138.97, 143.31, 149.94, 153.48, 158.02, 166.50. IR 1713, 1747. Anal. $C_{18}H_{12}N_2O_4$ (C, H, N).

*4.1.8 General procedure for the synthesis of arylhydrazones of 3-acetyl-2H-chromen-2-ones **41-44***

A mixture of the suitable arylhydrazine (1.42 mmol) and 3-acetyl-derivatives **1-4** (1.29 mmol) in EtOH (5 mL) was heated at reflux for about 1-2 h. After cooling at room temperature, the solid was collected and recrystallized.

*4.1.8.1. 4-(2-(1-(2-Oxo-2H-chromen-3-yl)ethylidene)hydrazinyl)benzoic acid (**41**)* Yield 87%; mp 227-229 °C (2-methoxyethanol); ^{1}H NMR (DMSOd_6): δ 2.26 (s, 3H, CH_3), 7.29 (d, 2H, ar, J = 8.8 Hz), 7.42 (t, 1H, ar, J = 8.2 Hz), 7.62 (t, 1H, ar, J = 7.2 Hz), 7.82 (d, 2H, ar, J = 8.8 Hz), 7.86 (d, 1H, ar, J = 6.7 Hz), 8.25 (s, 1H, H-4), 9.89 (s, 1H, NH), 12.35 (br s, 1H, OH). IR 1682, 1720, 3290. Anal. $C_{18}H_{14}N_2O_4$ (C, H, N).

*4.1.8.2. 7-Cloro-3-(1-(2-phenyldrazono)ethyl)-2H-chromen-2-one (**42**).* Yield 58%; mp 153-155 °C (EtOH); ^{1}H NMR ($CDCl_3$): δ 2.31 (s, 3H, CH_3), 6.94 (t, 1H, ar, J = 7.2 Hz), 7.18 (d, 2H, ar, J = 7.6 Hz), 7.31 (m, 3H, ar), 7.38 (d, 1H, H-8, J = 1.6 Hz), 7.52 (d, 1H, H-5, J = 8.4 Hz), 7.58 (s, 1H, NH), 8.05 (s, 1H, H-4). IR: 1720, 3331. Anal. $C_{17}H_{13}ClN_2O_2$ (C, H, N).

*4.1.8.3. 3-(1-(2-Phenyldrazono)ethyl)-8-nitro-2H-chromen-2-one (**43**).* Yield 52%; mp 204-206 °C (EtOH); ^{1}H NMR ($CDCl_3$): δ 2.33 (s, 3H, CH_3), 6.97 (t, 1H, ar, J = 7.6 Hz), 7.20 (d, 2H, ar, J = 7.6 Hz), 7.34 (t, 2H, ar, J = 7.6 Hz), 7.48 (d, 1H, H-8, J = 9.2 Hz), 7.68 (s, 1H, NH), 8.16 (s, 1H, H-4), 8.39 (dd, 1H, H-7, J = 8.8, 2.8 Hz), 8.53 (d, 1H, H-5, J = 2.4 Hz). IR: 1340, 1516, 1726, 3328. Anal. $C_{17}H_{13}N_3O_4$ (C, H, N).

*4.1.8.4. 7-Chloro-3-(1-(2-phenyldrazono)ethyl)-8-nitro-2H-chromen-2-one (**44**).* Yield 70%; mp 220-221 °C (2-Methoxyethanol); ^{1}H NMR ($CDCl_3$): δ 2.32 (s, 3H, CH_3), 6.97 (t, 1H, ar, J = 7.6 Hz), 7.18 (d, 2H, ar, J = 8.0 Hz), 7.33 (t, 2H, ar, J = 8.0 Hz), 7.52 (s, 1H, H-8), 7.69 (s, 1H, NH), 8.08 (s, 1H, H-5), 8.23 (s, 1H, H-4). IR: 1355, 1525, 1741, 3342. Anal. $C_{17}H_{12}ClN_3O_4$ (C, H, N).

*4.1.9. General procedure for the synthesis of 1-aryl-3-methylchromeno[4,3-c]pyrazol-4-(1H)-one derivatives **16, 18-20***

A mixture of arylhydrazones (1.55 mmol), $Cu(AcO)_2$·monohydrate and $CuSO_4$·pentahydrate, respectively 20% and 10% w/w with respect to the hydrazone, in EtOH (8 mL) was heated at reflux for 6-24 h. After cooling, the insoluble solid was collected by filtration, washed with abundant water (about 20-30 mL), dried, and purified by column chromatography (CH_2Cl_2/MeCN 9.8:0.2). To obtain compound **16**, the crude solid was suspended in HCl 6N and the mixture was stirred at rt for about 30 min, then collected, washed with water, dried, and recrystallized.

*4.1.9.1. 4-(3-Methyl-4-oxochromeno[4,3-c]pyrazol-1(4H)yl)benzoic acid (**16**).* Yield 25%; mp >300 °C (2-Methoxyethanol) ^{1}H NMR ($DMSOd_6$): δ 2.57 (s, 3H, CH_3), 7.08 (d, 1H, ar, J = 7.9 Hz), 7.20 (t, 1H, ar, J = 7.5 Hz), 7.52 (d, 1H, ar, J = 8.1 Hz), 7.58 (t, 1H, ar, J = 7.8 Hz), 7.80 (d, 2H, ar, J = 8.0 Hz), 8.20 (d, 2H, ar, J = 8.0 Hz), 13.39 (br s, 1H, COOH). ^{13}C NMR ($DMSOd_6$): δ 13.43 (CH_3), 106.93, 112.22, 118.72, 123.21, 125.34, 128.05, 131.92, 132.55, 133.28, 142.37, 143.35, 150.77, 153.63, 157.93, 167.37. IR: 1690, 1742. Anal. $C_{18}H_{12}N_2O_4$ (C, H, N).

*4.1.9.2. 7-Chloro-3-methyl-1-phenylchromeno[4,3-c]pyrazol-4-(1H)-one (**18**).* Yield 48%; mp 193-195 °C (EtOH); ^{1}H NMR ($CDCl_3$): δ 2.71 (s, 3H, CH_3), 7.04 (br s, 2H, H-6 + H-8), 7.46 (s, 1H, H-9), 7.54-7.56 (m, 2H, ar), 7.63-7.65 (m, 3H, ar). ^{13}C NMR ($CDCl_3$): δ 12.84 (CH_3), 110.48, 118.31, 118.86, 123.28, 124.45, 126.80, 128.73, 130.06, 130.39, 136.91, 139.16, 141.08, 151.02, 153.61. IR: 1747. Anal. $C_{17}H_{11}ClN_2O_2$ (C, H, N).

*4.1.9.3. 3-Methyl-8-nitro-1-phenylchromeno[4,3-c]pyrazol-4-(1H)-one (**19**).* Yield 80%; mp 241-242 °C (EtOAc); ^{1}H NMR ($CDCl_3$): δ 2.75 (s, 3H, CH_3), 7.55-7.59 (m, 3H, ar), 7.60-7.80 (m, 3H, ar), 8.05 (d, 1H, H-9, J = 2.4 Hz), 8.33 (dd, 1H, H-7, J = 9.2, 2.4 Hz).

*4.1.9.4. 7-Chloro-3-methyl-8-nitro-1-phenylchromeno[4,3-c]pyrazol-4-(1H)-one (**20**).* Yield 77%; mp 265-267 °C (2-Methoxyethanol); ^{1}H NMR ($CDCl_3$): δ 2.72 (s, 3H, CH_3), 7.50-7.60 (m, 2H, ar), 7.61 (s, 1H, ar), 7.65-7.70 (m, 3H, ar), 7.74 (s, 1H, ar). IR: 1377, 1577, 1768. Anal. $C_{17}H_{10}ClN_3O_4$ (C, H, N).

*4.1.10. 8-Amino-3-methyl-1-phenylchromeno[4,3-c]pyrazol-4-(1H)-one (**21**).*

Pd/C 10% (10% w/w with respect to the nitro derivative) was added to a solution of the nitro derivative **19** (3.58 mmol) in DMF (5 mL). The mixture was hydrogenated in a Parr apparatus at 35 psi for 14 h. The catalyst was filtered off, and the clear solution was diluted with water (about 50 mL) and the obtained solid was collected by filtration, washed with water and purified by column chromatography (eluent cyclohexane/EtOAc 6:4). Yield 77%; mp 201-204 °C (EtOH); ^{1}H NMR ($CDCl_3$): δ 2.73 (s, 3H, CH_3), 3.52 (br s, 2H, NH_2), 6.33 (d, 1H, H-9, J = 2.4 Hz), 6.81 (dd, 1H, ar, J = 8.8, 2.4 Hz), 7.22 (d, 1H, ar, J = 8.8 Hz), 7.50-7.70 (m, 5H, ar). ^{13}C NMR ($CDCl_3$): δ 12.92 (CH_3), 106.69, 106.86, 112.18, 118.18, 118.88, 127.02, 129.76, 130.07, 139.48, 141.70, 142.38, 146.67, 150.79, 158.39. IR: 1726, 3228-3342. Anal. $C_{17}H_{13}N_3O_2$ (C, H, N).

*4.1.11. 3-Methyl-8-methylamino-1-phenylchromeno[4,3-c]pyrazol-4-(1H)-one (**22**) and 8-dimethylamino-3-methyl-1-phenylchromeno[4,3-c]pyrazol-4-(1H)-one (**23**)*

A mixture of the 8-amino derivative **21** (1.56 mmol), anhydrous K_2CO_3 (2.34 mmol) and methyl iodide (15.58 mmol) in anhydrous acetonitrile (10 mL), was stirred at rt for 22 h, then it was diluted with water (15 mL) and extracted with CH_2Cl_2 (33 mL x 3). The organic phase was anhydrified (Na_2SO_4) and the solvent was evaporated at reduced pressure to yield a solid which was treated with

a few Et_2O (3-5 mL) and collected by filtration. The crude solid was a mixture of compounds **22** and **23** (ratio 1:2 from ^{1}H-NMR spectrum) which were separated by column chromatography (cyclohexane/EtOAc 1:1).

*4.1.11.1. Compound **22***. Yield 10%; mp 220-222 °C (2-Propanol); ^{1}H NMR ($CDCl_3$): δ 2.51 (s, 3H, CH_3), 2.71 (s, 3H, NCH_3), 3.61 (br s, 1H, NH), 6.18 (d, 1H, H-9, J = 2.4 Hz), 6.74 (dd, 1H, H-7, J = 9.2, 2.4 Hz), 7.26 (s, 1H, H-6), 7.50-7.70 (m, 5H, ar). ^{13}C NMR ($CDCl_3$): δ 12.97 (CH_3), 31.05 (CH_3), 104.06, 112.49, 117.94, 118.91, 127.17, 129.75, 130.10, 139.57, 141.88, 146.60, 151.01, 156.19, 158.55, 177.25. IR: 1732, 3427. Anal. $C_{18}H_{15}N_3O_2$ (C, H, N).

*4.1.11.2. Compound **23***. Yield 20%; mp 189-191 °C (2-Propanol); ^{1}H NMR ($CDCl_3$): δ 2.68 (s, 6H, $N(CH_3)_2$), 2.71 (s, 3H, CH_3), 6.30 (d, 1H, H-9, J = 2.9 Hz), 6.87 (dd, 1H, H-7, J = 9.2, 2.9 Hz), 7.31 (d, 1H, H-6, J = 9.2 Hz), 7.50-7.70 (m, 5H, ar). ^{13}C NMR ($CDCl_3$): δ 13.03 (CH_3), 40.55 (CH_3), 104.62, 106.57, 111.72, 116.44, 118.38, 127.30, 129.60, 130.01, 139.55, 142.17, 145.44, 146.67, 150.60, 158.54. IR: 1735. Anal. $C_{19}H_{17}N_3O_2$ (C, H, N).

*4.1.12. General procedure for the synthesis of the 8-acylamino-3-methyl-1-phenylchromeno[4,3-c]pyrazol-4-(1H)-ones **24** and **25**.*

The suitable acyl chloride (0.56 mmol) was added to a solution of the 8-amino derivative **21** (0.47 mmol) and triethylamine (0.56 mmol) in anhydrous CH_2Cl_2 (10 mL). The mixture was stirred at rt for 3 h, then the solvent was evaporated at reduced pressure and the residue was treated with water (10 mL). The obtained solid was collected by filtration, washed with abundant water (50 mL) and dried. The 8-acetylamino derivative **24** was purified by column chromatography (eluent cyclohexane/EtOAc 2:8) while the 8-benzoylamino derivative **25** was recrystallized.

*4.1.12.1. 8-Acetylamino-3-methyl-1-phenylchromeno[4,3-c]pyrazol-4-(1H)-one (**24**).* Yield 65%; mp 282-285°C (2-Propanol); ^{1}H NMR ($CDCl_3$): δ 2.11 (s, 3H, $COCH_3$), 2.71 (s, 3H, CH_3), 6.99 (br s, 1H, NH), 7.40 (br s, 2H, ar), 7.59-7.62 (m, 3H, ar), 7.67-7.80 (m, 3H, ar). ^{13}C NMR (DMSO-d_6):

δ 13.44 (CH_3), 24.71 (CH_3), 106.62, 112.23, 112.99, 118.85, 123.51, 127.54, 130.88, 130.99, 136.37, 139.76, 142.17, 149.22, 150.23, 158.10, 169.01. IR: 1716, 1732, 3444. Anal. $C_{19}H_{15}N_3O_3$ (C, H, N).

*4.1.12.2. 8-Benzoylamino-3-methyl-1-phenylchromeno[4,3-c]pyrazol-4-(1H)-one (**25**).* Yield 76%; mp 284-286 °C (2-Propanol); ^{1}H NMR ($CDCl_3$): δ 2.73 (s, 3H, CH_3), 7.40-7.68 (m, 13H, 12 ar + NH), 7.78 (d, 1H, ar, J = 7.3 Hz). ^{13}C NMR (DMSO-d_6): δ 12.99 (CH_3), 106.15, 111.71, 114.13, 118.34, 124.61, 127.08, 128.06, 128.84, 130.4, 130.48, 132.09, 135.11, 135.63, 139.27, 141.68, 149.22, 149.79, 157.63, 165.89. IR: 1710, 1730, 3375. Anal. $C_{24}H_{17}N_3O_3$ (C, H, N).

*4.1.13. 1-Phenyl-3-(3-methyl-4-oxo-1-phenyl-1,4-dihydrochromeno[4,3-c]pyrazol-8-yl)urea (**26**).*

A solution of the 8-amino derivative **21** (0.62 mmol) and phenylisocyanate (0.99 mmol) in anhydrous THF anidro (10 mL), was heated at reflux for 12 h. The solvent was evaporated at reduced pressure and the solid residue was taken up with Et_2O (5 mL), collected and recrystallized. Yield 90%; mp < 300 °C (2-Methoxyetanol/H_2O); ^{1}H NMR (DMSO-d_6): δ 2.56 (s, 3H, CH_3), 6.96 (t, 1H, ar, J = 7.2 Hz), 7.20-7.35 (m, 4H, ar), 7.38 (d, 1H, ar, J = 7.6 Hz), 7.45 (d, 1H, ar, J = 8.8 Hz), 7.55-7.75 (m, 6H, ar), 8.59 (br s, 1H, NH), 8.64 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 12.97 (CH_3), 111.67, 111.89, 118.44, 118.64, 122.37, 122.61, 127.20, 129.24, 130.41, 130.53, 136.28, 139.35, 139.94, 141.65, 148.18, 149.76, 152.65, 157.67, 166.53. IR: 1689, 1710, 3356, 3394. Anal. $C_{24}H_{18}N_4O_3$ (C, H, N).

*4.1.14. 8-Benzylideneamino-3-methyl-1-phenylchromeno[4,3-c]pyrazol-4-(1H)-one (**45**).*

A mixture of the 8-amino derivative **21** (0.47 mmol), benzaldehyde (0.56 mmol) and anhydrous $ZnCl_2$ (0.56 mmol) in anhydrous THF (10 mL) was heated at reflux for 8 h, under nitrogen atmosphere. After cooling at rt, the mixture was diluted with iced water (25 mL) and extracted with CH_2Cl_2 (33 mL x 3). The organic phase was anhydrified (Na_2SO_4) and the solvent evaporated at

reduced pressure to give an oily residue which solidified upon treatment with Et_2O (about 2 mL). The solid was collected and directly used for the next step. Yield 68%; ^{1}H NMR ($CDCl_3$): δ 2.73 (s, 3H, CH_3), 6.95 (d, 1H, H-9, J = 2.0 Hz), 7.45-7.75 (m, 11 H, ar), 7.80 (d, 1H, H-6, J = 6.8 Hz), 8.19 (s, 1H, CH=).

*4.1.15. 8-Benzylamino-3-methyl-1-phenylchromeno[4,3-c]pyrazol-4-(1H)-one (**27**).*

$NaBH_4$ (0.59 mmol) was portionwise added to a boiling suspension of the 8-benzylideneamino derivative **45** (0.29 mmol) in anhydrous CH_3OH (10 mL). After the addition was complete (about 10 min), the suspension was heated at reflux for an additional 40 min. After cooling at rt, the mixture was poured onto ice (about 25 g) and the obtained solid was collected by filtration, washed with abundant water (100 mL), collected by filtration and dried. The crude compound was purified by column chromatography (eluent CH_2Cl_2/ acetonitrile 9.8: 0.2). Yield 65%; mp 200-202 °C (EtOH); ^{1}H NMR ($CDCl_3$): δ 2.69 (s, 3H, CH_3), 3.99 (br s, 3H, CH_2 + NH), 6.24 (d, 1H, H-9, J = 2.4 Hz), 6.78 (dd, 1H, H-7, J = 8.8, 2.4 Hz), 7.14 (d, 1H, H-6), 7.20-7.40 (br s, 5H, ar), 7.45-7.60 (m, 5H, ar). ^{13}C NMR ($CDCl_3$): δ 12.92 (CH_3), 47.84 (CH_2), 80.52, 100.00, 103.26, 112.13, 117.60, 118.82, 127.03, 127.38, 128.64, 129.56, 129.94, 138.37, 139.41, 142.00, 144.05, 146.14, 150.73. IR: 1720, 3340. Anal. $C_{24}H_{29}N_3O_2$ (C, H, N).

*4.1.16. 1-Benzyl-3-methylchromeno[4,3-c]pyrazol-4-(1H)-one (**28**).*

A mixture of 3-acethyl-4-hydroxychromen-4-one **5** (3.91 mmol), benzylhydrazine dihydrochloride (15.64 mmol) and triethylamine (31.28 mmol) in EtOH (30 mL) was heated at reflux for 6 h. Part of the solvent (about 20 mL) was evaporated at reduced pressure and the solid which precipitated was collected by filtration and purified by column chromatography (eluent $CHCl_3$/CH_3OH 9:1). Yield 45%, mp 169-171 °C (EtOH); ^{1}H NMR ($CDCl_3$): δ 2.69 (s, 3H, CH_3), 5.81 (s, 2H, CH_2), 7.16-7.49 (m, 8H, ar), 7.68 (d, 1H, ar, J= 7.9 Hz). Anal. $C_{18}H_{14}N_2O_2$ (C, H, N).

*4.1.17. 1-Phenylchromeno[4,3-c]pyrazol-4-(1H)-one (**31**).*

A solution of the 3-phenylhydrazone **48** [26] in pyridine (4 ml) and piperidine (0.02 mL) was heated at 100 °C for 2 h. After cooling at room temperature, the solution was diluted with H_2O (20 mL) and acidified (2 N HCl) until the formation of a solid which was collected, washed with H_2O (about 50 mL), dried and purified by column chromatography (eluent cyclohexane/EtOAc, 6:4) and recrystallized. Yield 66%, mp 196-197 °C (EtOH); ^{1}HNMR ($CDCl_3$): δ 7.09 (t, 1H, ar, J = 6.8 Hz), 7.16 (d, 1H, ar, J = 7.6 Hz), 7.45-7.55 (m, 2H, ar), 7.55-7.75 (m, 5H, ar), 8.39 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6): δ 13.53 (CH_3), 55.40 (CH_2), 106.47, 112.24, 118.58, 124.21, 125.49, 127.40, 128.65, 132.14, 137.04, 141.66, 149.53, 153.40, 157.92.

*4.1.18. 2-Phenylchromeno[4,3-c]pyrazol-4-(1H)-one (**37**).*

A solution of the hydrazone **48** [26] (0.67 mmol) and phenylhydrazine hydrochloride (0.67 mmol) in glacial acetic acid (10 mL) was heated under reflux for 10 min. After cooling at room temperature, a solid precipitated which was collected by filtration, washed with abundant water (20-30 mL), and recrystallized. Yield 64%, mp 210-211 °C (EtOH); ^{1}HNMR ($CDCl_3$): δ 7.35-7.65 (m, 6H, ar), 7.87 (d, 2H, ar, J =7.6 Hz), 7.87 (d, 1H, ar, J = 7.6 Hz), 8.71 (d, 1H, H-3).

4.2. CA inhibition

An applied photophysics stopped-flow instrument has been used for assaying the hCA catalyzed CO_2 hydration activity [32]. 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 7.4) as buffer, and 20 mM Na_2SO_4 for maintaining constant the ionic strength (this anion is not inhibitory and has a K_i> 200mM against these enzymes), 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 measurement three traces of the initial 5–10% of the reaction have been used for determining the initial velocity (which was the mean of the three traces),

working with 10-fold decreasing inhibitor concentrations ranging between 0.1nM and 10–100 mM (depending on the inhibitor potency, but at least five points at different inhibitor concentrations were employed for determining the inhibition constants). 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 0.1 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using the Cheng–Prusoff equation, and represent the mean from three independent experiments. All human isoforms were recombinant enzymes produced as described earlier in our laboratory [33-35].

4.3. Cytotoxicity Assay

4.3.1. Cell culture and treatments

Human colon cancer cell lines HT-29 were obtained from American Type Culture Collection (Rockville, MD). HT-29 were cultured in DMEM high glucose with 20% FBS in 5% CO_2 atmosphere at 37° C. Media contained 2 mM L-glutamine, 1% essential amino acid mix, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma, Milan, Italy). HT-29 cells were plated in 96-wells cell culture ($1 \cdot 10^4$/well) and, 24 h after, treated with the tested compounds (0-300 μM) for 16, 48 and 72 h. Low oxygen conditions were acquired in a hypoxic workstation (Concept 400 anaerobic incubator, Ruskinn Technology Ltd., Bridgend, UK). The atmosphere in the chamber consisted of 0.1% O_2 (hypoxia), 5% CO_2, and residual N_2. In parallel, normoxic (20% O_2) dishes were incubated in air with 5% CO_2.

4.3.2. Cell viability assay

HT-29 cell viability was evaluated by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an index of mitochondrial compartment functionality. Cells

were plated and treated as described. After the treatment and extensive washing, 1 mg/ml MTT was added into each well and incubated for 30 minutes at 37 °C. After washing, the formazan crystals produced by the reaction were dissolved in 150 μl DMSO and the absorbance was measured at 550 nm. Experiments were performed in quadruplicate on at least three different cell batches.

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