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Exploring heteroaryl-pyrazole carboxylic acids as human carbonic anhydrase XII inhibitors

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KEYWORDS: Carbonic anhydrase, hCA XII inhibitors, heteroaryl-pyrazole carboxylic acids, X-ray crystallography, computational docking, hypoxic tumours.

ABSTRACT: We report the synthesis, biological evaluation, and structural study of a series of substituted heteroaryl-pyrazole carboxylic acid derivatives. These compounds have been developed as inhibitors of specific isoforms of carbonic anhydrase (CA), with potential as prototypes of a new class of chemotherapeutics. Both X-ray crystallography and computational modelling provide insights into the CA inhibition mechanism. Results indicate that this chemotype produces an indirect interference with the zinc ion, thus behaving differently from other related non-classical inhibitors. Among the tested compounds, **2c** with $K_i = 0.21 \mu\text{M}$ towards hCA XII demonstrated significant antiproliferative activity against hypoxic tumour cell lines. Taken together, the results thus provide the basis of structural determinants for the development of novel anticancer agents.

Many currently used antitumor drugs demonstrate relatively poor selectivity for cancer cells, causing frequent toxicity and side effects. A current strategy to overcome this impasse relies on the identification of biological targets that are exclusive, or at least highly prevalent, in cancer cells. One of the most suited hallmarks of cancer is metabolic reprogramming, which offers new opportunities for the development of innovative chemotherapeutics.¹ In particular, hypoxia, a common condition in tumour microenvironment, promotes consistent metabolic adaptation, such as the switching of energetic metabolism from aerobic to anaerobic glycolysis.^{2,3} A critical consequence of this phenomenon is the overproduction of weak acid species, such as lactic acid. In order to preserve their acid-base homeostasis, cancer cells adopt a series of adaptive mechanisms through the activation of specific factors (such as the hypoxia-inducible factor 1, HIF-1), which promptly remove/rebalance the H^+ excess from the cells, thus lowering the extracellular pH.^{4,5}

As far as other putative biological pathways involved with hypoxic cancer are concerned, carbonic anhydrases (CAs), a family of metal enzymes that catalyze the biochemical reaction of carbon dioxide hydration to produce a bicarbonate ion and proton, are emerging as potential biological targets for cancer therapy.⁶ Previously, two tumour-associated extracellular transmembrane carbonic anhydrase isozymes (hCA IX and hCA XII) were identified, cloned and sequenced.⁶⁻⁸ These two CA isoforms have been shown to be expressed in a wide varie-

ty of malignancies and appear to be tightly regulated by micro-environmental hypoxia.^{7,8} This phenomenon is particularly evident in highly malignant solid tumours, and has been linked to poor blood perfusion that reduces the provision of oxygen and nutrients for cancer cells, thus inducing a series of adaptive responses (i.e. metabolic shift to anaerobiosis and increased amino acids catabolism) that lead to lower extracellular pH, a condition that is not favourable for cell survival.

In this scenario, the extracellular CA isoforms concur to rebalance the acid/base equilibrium of the extracellular medium.^{6,9,19} Specifically, hCA XII is minimally expressed in a variety of normal tissues including kidney, colon, prostate, pancreas, ovary, testis, lung, and brain, but its expression is up-regulated in cancer cells originating from these tissues.⁷ Several recent studies proposed hCA XII as a suitable and attractive target for both diagnostic and therapeutic intervention, particularly on the management of hypoxic tumours normally nonresponsive to classical chemo- and radiotherapies.⁹⁻¹²

Primary sulfonamides, the most clinically used CAs inhibitors (CAIs), have been shown to reverse the effect of the extracellular CAs, and inhibit the growth of cancer cells in the low nanomolar or micromolar range.¹³⁻¹⁵ However, one main drawback of these classical sulfonamide CAIs is the lack of selectivity for inhibiting transmembrane CAs over the other cytoplasmic CA isoforms present in humans and mouse model systems. For this reason, efforts are being initiated to find novel

CAIs, in order to explore molecular diversities and discover original pharmacophores and chemotypes.¹³⁻¹⁶

This study focuses on the structural optimization of a previously identified lead compound, the 3-(1-ethyl-1*H*-indol-3-yl)-1*H*-pyrazole-5-carboxylic acid (**1a**, Chart 1),¹⁷ in order to generate and evaluate a new series of indolylpyrazole-5-carboxylic acids/esters with improved inhibition profile toward *h*CA IX and *h*CA XII.

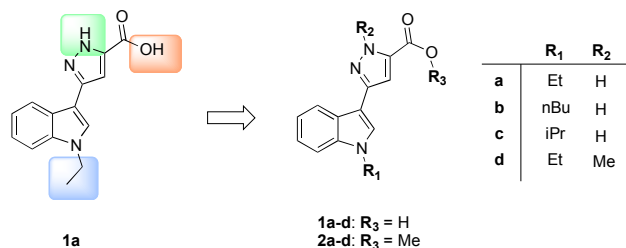
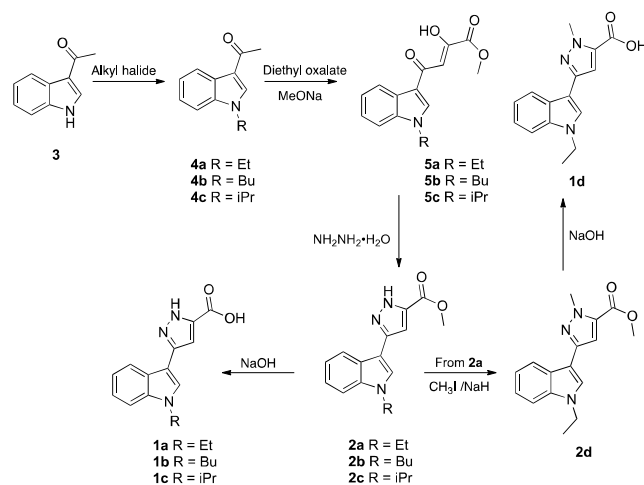


Chart 1. Design and general structure of title compounds.

Preliminary major modifications of **1a** have been made by varying the substituents at the nitrogen atoms both on the indole and pyrazole rings, and by esterification of the carboxylic functionality to generate the series **1a-d** and **2a-d** (Chart 1). Specifically, *N*-alkylation of the indole was first explored to modulate lipophilicity; second, *N*-methylation of the pyrazole was introduced to abolish N-H interaction; finally, on the basis of the assumption that the carboxylic moiety is a necessary feature for the inhibitory activity, we examined the effect of esterification on the activity.

Compounds **1a-d** and **2a-d** were synthesized starting from the 3-acetyl indole **3** by alkylation reaction with the appropriate alkyl bromide to obtain the *N*-alkyl-3-acetyl-indole derivatives **4a-c** (Scheme 1).



Scheme 1. Synthetic route for the preparation of compounds **1a-d** and **2a-d**.

These intermediates were reacted with diethyl oxalate in the presence of sodium methoxide in MeOH, to provide the respective β -diketoesters **5a-c**. The latter were found to exist in the keto-enol form in DMSO, since ¹H-NMR experiments showed a singlet centered at 6.87 ppm (for **5a,b**) and at 6.89

(for **5c**) attributable to the enolic CH of the hydroxy-keto motif. Next, the compounds **5a-c** were treated with hydrazine monohydrate to generate the pyrazole derivatives **2a-c** in appreciable yields (46-93%). Then, the acids **1a-c** were obtained by alkaline hydrolysis of the esters **2a-c**. Finally, the compound **2d**, containing a methyl group on the nitrogen of pyrazole ring, was synthesized by alkylation of intermediate **2a** with iodomethane in presence of NaH. The corresponding acid **1d** was obtained from **2d** by alkaline hydrolysis with NaOH in ethanol at reflux.

Compounds **1a-d** and **2a-d** were tested for their ability to inhibit a panel of representative isoforms of human CAs (I, II, IX, and XII, Table 1). Catalytic activities were measured by a stopped-flow technique, as previously described (see Supporting Information).

Table 1. Inhibition of *h*CA isoforms I, II, IX and XII with compounds **1a-d** and **2a-d** by a stopped-flow CO₂ hydrase assay. Data relative to the standard CAIs acetazolamide (AAZ), methazolamide (MZA), and dichlorophenamide are also shown for comparison.

Compound	K _i (μM) ^[a]			
	<i>h</i> CA I ^[b]	<i>h</i> CA II ^[b]	<i>h</i> CA IX ^[c]	<i>h</i> CA XII ^[c]
1a	0.042	1820	7.79	7.78
1b	5.33	4.70	4.51	0.34
1c	4.83	0.70	18.9	0.28
1d	6.61	0.78	2.91	0.44
2a	4.31	6.95	0.47	0.57
2b	5.93	0.53	7.90	0.35
2c	6.45	0.76	7.36	0.21
2d	0.62	0.41	3.02	0.31
AAZ	0.25	0.012	0.025	0.006
MZA	0.78	0.014	0.027	0.034
DCP	1.2	0.038	0.050	0.050

[a] Errors in the range of ± 5 –10% of the reported value from three different determinations. [b] Full length. [c] Catalytic domain.

Although structural modifications of the lead compound **1a** reduced the potency and selectivity against *h*CA I isoform (work is in progress to clarify the behaviour of this outlier compound), these new compounds showed an interesting selectivity toward *h*CA XII, with K_i values ranging from 0.21 to 0.57 μM (Table 1). Moreover, with the exception of **1b**, and **2a**, the tested compounds effectively interfere with *h*CA II catalytic activities in submicromolar/micromolar concentration range (K_i values of 0.41–6.95 μM, Table 1). On the contrary, no significant inhibition or slightly weaker inhibitory action toward *h*CA I and *h*CA IX (with the exception of **2d** and **2a**, with K_i values of 0.62 and 0.47 μM, for *h*CA I and *h*CA IX, respectively), were revealed.

Overall, the biological profile appeared to be substantially independent from the nature of substituents on both the indole and the pyrazole rings. However, although a proper structure–activity–relationship (SAR) for *h*CA XII inhibition is

not feasible at this stage, it is plausible that structural determinants of this chemotype (i.e. indole ring system, lipophilic substituents at the nitrogen indole, and carboxylic functionality) appear an optimal combination to effectively inhibit such transmembrane isoform, (with a certain mutual interference with cytosolic *hCA* II catalytic activity). Again, no relevant differences could be observed between acids and esters on the inhibition profile. Nevertheless, among the tested compounds, compound **2c** ($K_I = 0.21 \mu\text{M}$) proved to be the most active compound in inhibiting *hCA* XII, thus demonstrating to be one of the most effective nonsulfonamide-based inhibitor towards this CA isoform.

Since the indolylpyrazole-5-carboxylate backbone appeared to be an original chemotype for CA inhibition, the interaction of representative compound **1d** with *hCA* II was studied by X-ray crystallography and determined to 1.2 Å resolution (statistics summarized in Table S1). Structural analysis of the *hCA* II/**1d** complex indicates that the compound does not bind directly to the active site zinc(II) ion, making it dissimilar to other potent CA inhibitors.¹⁸ Instead, the compound utilizes a carboxylic acid moiety to substitute the typical location of a zinc-binding group (ZBG).^{19,20}

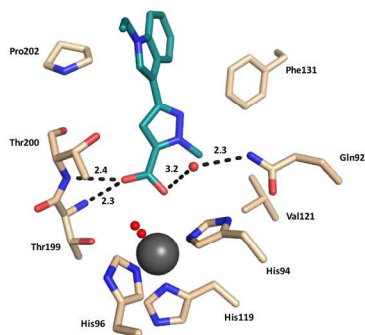


Figure 1. *hCA* II (wheat) in complex with compound **1d** (deep teal). Hydrogen bond interactions are shown with distances (Å) and amino acids labeled. Water molecules are depicted as red spheres. Zinc ion is represented as grey sphere. This chemotype binds with residues located in proximity of the catalytic site, which displaces the ordered water network, thus definitely producing an indirect interference with the zinc ion. Image was made in PyMol.

As such, the compound resides buried in the *hCA* II active site and interacts with adjacent residues to the catalytic zinc. This mode of binding results in the observation of a disordered water molecule coordinated to the active site zinc (Figure 1). However, compound **1d** displaces the ordered water network in the active site that has been shown to be important for catalysis. Key interactions are observed between the carbonyl oxygen of compound **1d** and the backbone NH of both Thr199 and Thr200 (2.3 and 2.4 Å respectively). The carbonyl hydroxyl also forms a hydrogen bond with a coordinated solvent molecule within the active site (bond distance = 3.2 Å), which is then stabilized by a hydrogen bond with the amide of Gln92 (bond distance = 2.3 Å). Compound **1d** also displays extensive interactions with the hydrophobic pocket of the *hCA* II active site. Specifically, van der Waals interactions with residues

Val121, Phe131, and Pro202 of the enzymes hydrophobic pocket are predicted to stabilize the “tail” region of compound **1d** and are therefore suggested to greatly stabilize the enzyme/inhibitor complex (Figure 2).

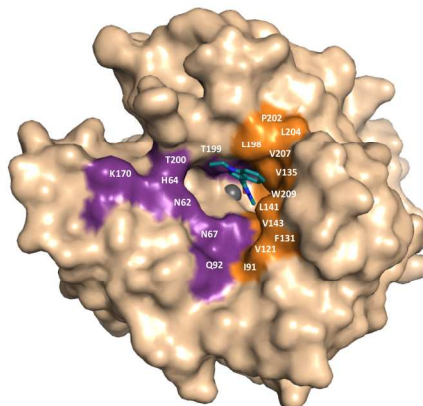


Figure 2. Surface representation showing the location of compound **1d** (deep teal) in the *hCA* II active site (wheat). Hydrophobic (orange) and hydrophilic (purple) residues are highlighted and relative locations labeled. Figure was produced using PyMol.

In order to investigate the interaction of the other compounds **1a-c** and **2a-d** within the catalytic sites of *hCA* II (new PDB code: 5CJL) and *hCA* XII (PDB code: IJCZ), a series of docking studies were performed. Preliminarily, we envisaged that in the assembly of ligand-target complex, water molecules could play a crucial role in careful calculations. To be able to accomplish the calculation with manageable computational complexity, the docking procedure recently proposed by Olson and Forli,²¹ was employed. This procedure allows the effect of water molecules located in proximity of ligands to be considered. This method entails the use of a monoatomic pseudoatom (W) as a surrogate of water molecule. Firstly, compound **1d** was docked into the X-ray crystal structure, obtaining the same binding mode as in the X-ray crystallography, within the experimental resolution limits (Figure S1A). This established the robustness of the method and then **1d** was also modelled with CA XII (Figure S1B).

After this, the docking settings were optimized, and docking runs were performed for all remaining ligands (i.e., **1a-c** and **2a-d**) in the active sites of *hCA* II and XII isoforms. Graphical representations of top-ranking binding modes obtained for these ligands are shown in Figures S2A, S2B, S3 and S4.

From the structural point of view, *hCA* II and XII exhibit considerable tertiary structure similarity, with an rmsd value of 1.2 Å; some discrepancies are present on the surface of proteins in concomitance of random coiled regions (Figure S5). Moreover, only three residues exhibited a rmsd greater than 3 Å. Focusing on the catalytic region, the primary sequence appears highly conserved, with the exception of nine residues (ie, A65S, N67K, E69D, I91T, P131A, G132S, V135S, Q136N, and L204N, indicated as Res_{CAII}-Number-Res_{CAXII}). As shown in Table S2, eighteen and twenty-two residues, for *hCA* II and XII, respectively, concur to stabilize the ligand-protein complex. Moreover, the residues at positions 94, 131, 135, 198, 200, and 202, were also involved in ligand interaction. Furthermore, the key residue Thr199 is predicted to es-

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establish interaction only for *hCA* II, whereas residues in positions 91, 92, 96, and 141 are predominantly involved in contacts with *hCA* XII active site. Although differences were found between the binding modes of ligands to the same CA isoform, a similar orientation pattern and disposition within the catalytic site were observed. In the case of the *hCA* II, all ligands accommodated almost perpendicularly to the plane of the three coordinating histidines with the carboxylate group directed towards the zinc-bound water. In the case of *hCA* XII, ligands are in a decentered position with the scaffold lying along the protein wall formed by residues Gln92 and His94. This particular arrangement implies that the carboxylate group makes contact with the deep-water from lateral side. In all cases (with the exception of the less active compound **1a**), it allows the formation of a hydrogen bond with the imidazole ring of His96. Regarding the most active compound **2c** against *hCA* XII, docking results showed that the most favourable binding conformation with *hCA* II is superimposable to that of compound **1d** obtained experimentally by X-ray crystallography (Figure S1A and 3A).

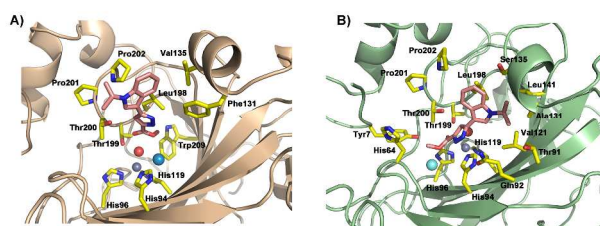


Figure 3. Predicted binding modes for the most promising compound **2c** within the catalytic pockets of *hCA* II (A) and XII (B) isoforms. *hCA* II and XII are coloured as wheat and pale green, respectively; side chain of significant residues are represented as yellow sticks and labelled. Ligand **2a** is in pink sticks. Zinc cofactor, zinc-bound water and pseudoatoms are depicted as spheres and coloured in gray, red, and pale blue or cyan, respectively.

Both compounds shared a common interacting residues pattern (Table S2), with slight differences for the absence of a hydrophobic contact with Val121 (for **2c**), due to the lacking N-methyl group in position 1 of pyrazole ring and the presence of a methoxy group receptor that is able to establish additional contact with Trp209. It is worth noting that, when docked in *hCA* XII catalytic site, compound **2c** appeared located inside a binding pocket formed by thirteen residues (Figure 3B and Table S2), with the presence of three strong hydrogen bonds with His96 and Thr200.

The inhibitory effect of the compounds **1a**, **2a**, **1c** and **2c** on CA activity (particularly of *hCA* IX and/or XII) was evaluated in terms of cell viability of three human cancer cell lines (hormone-independent prostate cells, PC-3; human embryonic kidney, HEK 293 cells; human neuroblastoma cells, SH-SY5Y). First, HEK 293 cells were treated for 24 hours with increasing concentrations of **1a**, **2a**, **1c**, and **2c** (Figure S6A). No significant antiproliferative effect was detected at the 1, 10, 30, or 100 μ M of compound treatment, with the exception of **2c**, which exhibited a dose dependent cell growth inhibition reaching roughly the 70% antiproliferative effect at the highest concentration. Better results were found when the compounds were tested on PC-3 cells at the same conditions, (Figure S6B)

as well on SH-SY5Y cell lines after 48 hours of treatment (\sim 85 growth inhibition, Figure S6C).

Since hypoxia-induced upregulation of *hCA* IX and XII are key components of the complex response of cancer cells to the evolving low oxygen environment, compound **2c** was further investigated for its antiproliferative potency on neuroblastoma SH-SY5Y cells in normal and in simulated hypoxic conditions using cobalt(II) chloride (Figure 4).²² As expected, the addition of cobalt(II) chloride, a known chemical inducer of hypoxia-inducible factors, significantly increased the susceptibility of the cells to the **2c** treatment, showing a 40% reduction of cell viability at an inhibitor concentration of 30 μ M ($p < 0.05$ vs untreated cells).

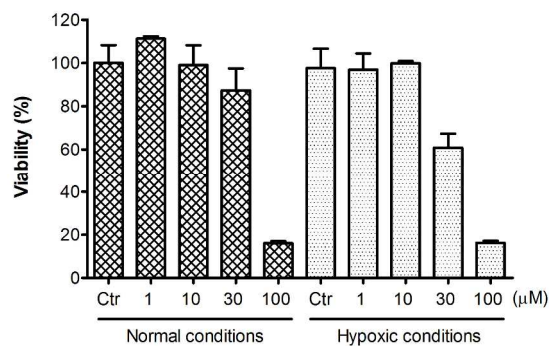


Figure 4. Dose-dependent antiproliferative activity of compound **2c** on human neuronal SH-SY5Y cells (measured by percentage of cell viability after 48 hours of treatment) assessed in normal and simulated hypoxic conditions.

A new series of compounds bearing a 3-(1*H*-indol-3-yl)pyrazole-5-carboxylic acid scaffold have been synthesised and evaluated for their selective inhibition of *hCA* XII. Among the tested compounds, **2c** ($K_i = 0.21$ μ M) resulted the most potent *hCA* XII inhibitor, and exhibited cytotoxicity towards three different cancer cell lines. Interestingly, this chemotype behaves differently from other related non-classical inhibitors. In fact, it does not directly bind to the metal ion or to the zinc-bound water/hydroxide ion. Otherwise, it seems to establish contacts with residues located in proximity of the catalytic site, which displaces the ordered water network, thus definitely producing an indirect interference with the zinc ion. However, **2c** displayed improved antiproliferative effects when tested in simulated hypoxia conditions, considering the putative expected involvement of *hCA* XII inhibition. These results could provide structural determinants for the discovery of novel anticancer agents with original mechanism of action, warranting further development.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Experimental details for chemistry, X-ray crystallography and molecular modeling. ¹H- NMR for title compounds **1b-d** and **2a-d**, and their intermediates **4a-c** and **5a-c**. Mass spectra for **1b-d**, **2a-d**, and **5a-c**. X-ray crystallography statistics for data processing and refinement of **4d** in complex with

hCA II (Table S1). Details of experimental biology procedure (CA inhibition assay, cell culture, assessment of cell viability). Molecular modelling additional data (Table S2 and Figures S1–S5). Antiproliferative activity of compounds **1a**, **2a**, **1c**, and **2c** (Figure S6). (Pdf).

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Author Contributions

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

Notes

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Authors will release the atomic coordinates and experimental data for PDB ID 5CJL (Table S1) upon article publication. The authors declare no competing financial interest.

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ABBREVIATIONS

hCA, human carbonic anhydrase; CAIs, carbonic anhydrase inhibitors; K_i , inhibition constant; ZBG, zinc-binding group; PC-3, Hormone-independent prostate cells; HEK 293, human embryonic kidney cells; SH-SY5Y, human neuroblastoma cells.

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1 SYNOPSIS TOC: X-ray crystallography of representative indolylpyrazole-5-carboxylate in complex with hCA II. Structural
2 analysis indicates that the compound does not bind directly to the active site zinc(II) ion, thus behaving differently from oth-
3 er related non-classical inhibitors. The most potent exhibited cytotoxicity towards three different cancer cell lines, and dis-
4 played improved antiproliferative effects when tested in simulated hypoxia conditions.
5

6 **Exploring heteroaryl-pyrazole carboxylic acids as human carbonic anhydrase XII inhibitors**

