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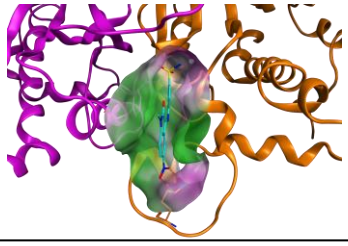

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Isatin analogs as novel inhibitors of *Candida spp.* β-carbonic anhydrase enzymes

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ABSTRACT

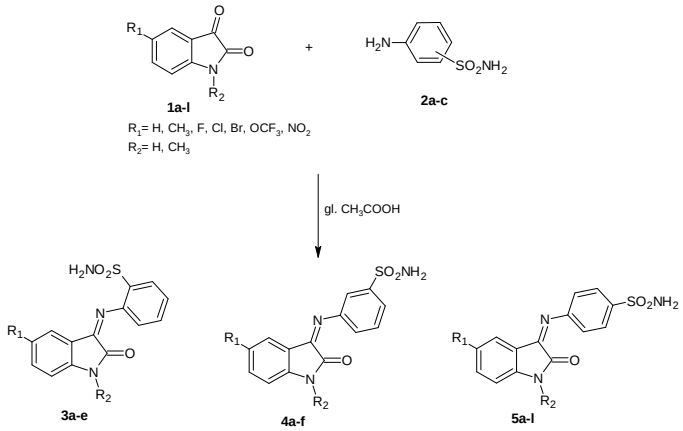
Enzyme inhibition data of structurally novel isatin-containing sulfonamides were determined for two carbonic anhydrases (CAs, EC 4.2.1.1) from pathogenic *Candida* species (CaNce103 from *C. albicans* and CgNce103 from *C. glabrata*). The compounds show K_I values in the low nanomolar range for the fungal CAs, while they have significantly higher K_I values for the human CAs. Homology models were constructed for the CaNce103 and CgNce103 and subsequently the ligands were docked into these models to rationalize their enzyme inhibitory properties.

1. Introduction

The reversible hydroxylation of carbon dioxide to form bicarbonate is a simple but very important chemical reaction that can be performed by various classes of carbonic anhydrases (CAs, EC 4.2.1.1).[1] The reaction not only provides means to control physiological pH values, but it also supplies bicarbonate ions for various physiological processes or metabolic, biosynthetic pathways.[2-5] As such, carbonic anhydrase inhibitors (CAIs) have a role as pharmaceuticals in various pathophysiological conditions.[6-10]

The CA enzymes are found in many living organisms and they belong to distinct families, i.e. α-CAs (humans and other mammals), β-CAs (most procaryotes and plant chloroplasts), γ-CAs (methanogens), δ-CAs (diatoms), ζ-CAs (some chemolitotrophs) and the recently identified η-CAs (plasmodium).[2-5] The α-CAs such as the human CA IX and XII isozymes (hCA IX and XII) are putative targets in cancer treatment,[11, 12] while hCA V inhibitors may form a class of novel antiobesity drugs.[6, 8, 13] The β-CAs are found in various infection causing organisms such as protozoa (*Trypanosome cruzi*) and fungi (*Candida albicans* and *Candida glabrata*) and selective CAIs targeting these organisms have pharmaceutical potential as antiprotozoal and antifungal drugs.[6, 14-16]

In a recent study, we synthesized 23 structurally novel 2/3/4-[(2-oxo-1,2-dihydro-3*H*-indol-3-ylidene)amino]benzenesulfonamides (compounds **3a-e**, **4a-f** and **5a-l**), obtained from substituted 1*H*-indole-2,3-diones and 2-, 3- or 4-aminobenzenesulfonamide (scheme 1).[17] These compounds showed selectivity against the tumor-associated hCA IX and XII enzymes over the widely distributed hCA I and II enzymes.



Scheme 1. Preparation of 2/3/4-[(2-oxo-1,2-dihydro-3*H*-indol-3-ylidene)amino]benzene-sulfonamides.

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In this new study, we tested these compounds for their inhibition against CAs from *Candida* species, i.e. *Candida albicans* Nce103 (CaNce103) and *Candida glabrata* Nce103 (CgNce103). Molecular modeling studies were conducted to rationalize the obtained inhibition values.

2. Results and discussion

2.1. Carbonic Anhydrase inhibition assays

Compounds **3a-e**, **4a-f** and **5a-l** were tested in CaNce103 and CgNce103 enzyme inhibition assays (Table 1). The compounds show K_I values in the lower nanomolar range for CaNce103 (21.7 – 295 nM) and 16 of 23 compounds have K_I values lower than 100 nM. The compounds show lower K_I values for CgNce103 (16.3 – 154 nM) compared to CaNce103 in all cases. All compounds show selectivity for the tested Candida CAs over the widely distributed human off-targets hCA I and II (Table 1).[17]

Interestingly, compounds with a nitro group on the R1 position of the indole ring showed the lowest K_I values for compound series **4** and **5** (compounds **4f**, **5k** and **5l**; Table 1). The nitro group is electronegative and could form hydrogen bonds with the enzyme active sites.

For both enzymes, the lowest and highest measured K_I values were displayed by compounds **5l** and **3e**, respectively (Table 1). However, it should be noted that the K_I values are close to each other. This makes it difficult to construct clear structure-activity relationships for this set of compounds. Still the possible binding pose was investigated with docking studies.

2.2. Homology model building for CaNce103 and CgNce103

No crystal structures of the CA enzymes from *Candida glabrata* (CgNce103) and *Candida albicans* (CaNce103) are available. Therefore, homology models have been constructed for both enzymes. To this end, the sequences for CaNce103 and CgNce103 have been obtained and a BLAST search was performed to identify crystal structures in the PDB server with high sequence similarities. Two crystal structures have been identified, namely the crystal structure of the yeast *Saccharomyces cerevisiae* CA Nce103 (ScNce103; 3eyx; 2.04 Å) and the crystal structure of the green alga *Coccomyxa* CA in complex with acetazolamide (CmCA; 3ucj; 1.85 Å).

Figure 1. Sequence alignment of ScNce103 (3eyx), CmCA (3ucj), CaNce103 and CgNce103. The residues in gray are not solved in the crystal structure, the residues in blue are within 4.5 Å of acetazolamide (3ucj), and the residues in yellow have a different backbone fold.

The ScNce103 structure shows the highest sequence identity to our target structures (33.3% to CaNce103 and 52.3 5 to CgNce103), while the CmCA structure shows lower but still significant sequence identity (28.2% to CaNce103 and 24.7% to CgNce103)(Figure 1).

The CmCA structure reveals the location of the active site, located at the interface of two subunits, and the binding interactions between the enzyme and acetazolamide (Figure 2). Acetazolamide forms an interaction with the Zn^{2+}-ion via its sulfonamide nitrogen atom. One of the sulfonamide oxygen groups forms a hydrogen bond to the Gln38 sidechain.

The CmCA crystal structure was superposed on the Cα-atoms of the ScNce103 structure (RMSD: 2.402 Å, 382 residues) to compare the general fold of the enzymes and the active site architecture (Figure 3). Gln38 and the loop surrounding this amino acid is not visible in the ScNce103 crystal structure. In addition, Phe is present instead of Tyr88. Finally, the backbone near Trp115 of the CmCA structure and the corresponding backbone of the ScNce103 structure have a different regional fold. However, the overall sequence identity with the Candida CAs is higher compared to the CmCA structure. Therefore, two different homology model building strategies were applied, namely i) using CmCA as a template and ii) using ScNce103 as a template but with the coordinates of Gln38 and its 5 neighboring residues from CmCA.

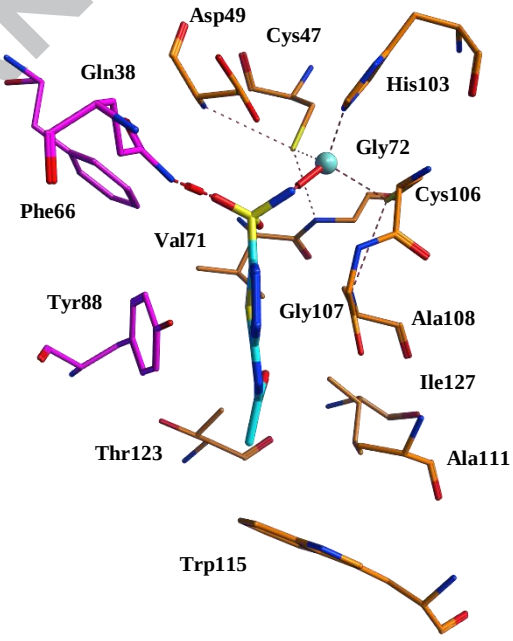

Figure 2. The binding interactions between the well-known CA inhibitor acetazolamide with CmCA (3ucj). The residues of the two protein chains that form the active site are depicted in different colors (carbon atoms in magenta and light brown). The ligand carbon atoms are depicted in turquoise. The hydrogen bond between the ligand and Gln38 and the interaction between the ligand and Zn^{2+} are depicted in thick red dashed lines. The interactions between the protein atoms and between the protein atoms and the Zn^{2+}-ion are depicted in thin dashed lines.

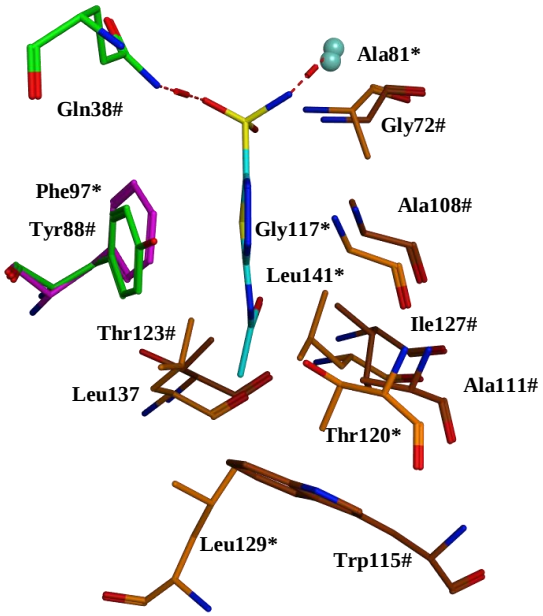

Figure 3. Differences in the backbone fold of the two templates near the ligand acetazolamide. Only the residues that are different in both structures are shown for clarity. The ScNce103 carbon atoms are depicted in magenta and light brown and the corresponding residues numbers are indicated with *. The CmCA carbon atoms are depicted in green and brown and the corresponding residue numbers are indicated with #. The ligand carbon atoms are depicted in turquoise.

2.3. Docking studies of isatin analogs

All compounds have been docked into each of the two binding pockets for all four generated homology models (CaNce103[3eyx], CaNce103[3ucj], CgNce103[3eyx] and CgNce103[3ucj]) and similar docked poses have been obtained.

The compounds are most likely able to form hydrophobic contacts between their phenyl groups and the sidechain of Phe146 (π-π stackings) in the CaNce103 active site. In addition, the ligands indole moiety can form hydrophobic interactions with the sidechains of Val150, Trp168 and Ala169.

Potential residues in the CaNce103 structure that are able to form hydrogen bonds to the ligands nitro group are Gln149 and Lys175. For example, the docked pose of compound **5l** can most likely form hydrogen bonds with the sidechain of Gln149 in the CaNce103[3eyx] model and with the sidechain of Lys175 in the CaNce103[3ucj] model (Figure 4). This can be explained by differences in the backbone fold, and as a consequence, a difference in the position and orientation of some active site residues (such as Gln141, Gln149, Trp168, Lys175).

The docked poses observed for the CgNce103 models are in general similar to the ones observed for the CaNce103 models (Figure 5). Hydrophobic interactions are most likely formed between the ligands phenyl group and the sidechain of Phe93 (located at similar position as Phe146 of CaNce103; see Figure 3). Hydrogen bonds can be formed between the ligands nitro group and the side chain of Asn97 in the CgNce103[3eyx] model (located at the Val150 position of CaNce103; see Figure 4).

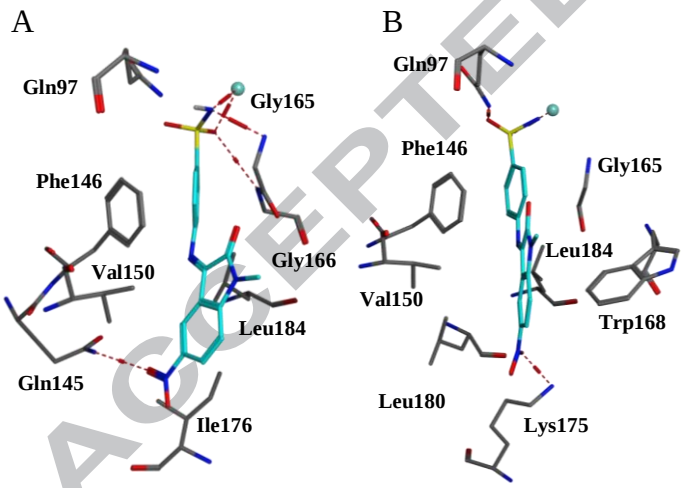

Figure 4. The docked poses of compound **5l** in the homology models of CaNce103 based on 3eyx (**A**) and 3ucj (**B**).

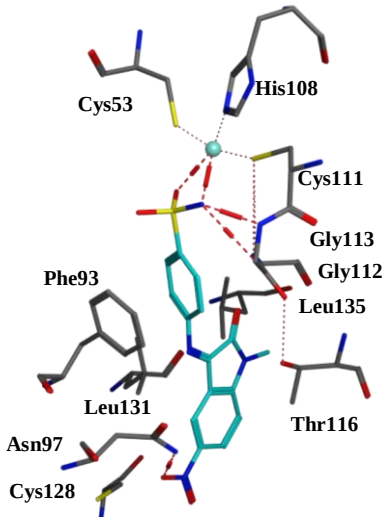

Figure 5. The docked pose of compound **5l** in the homology model of CgNce103 based on 3eyx.

Table 1. The enzyme inhibition values (K_I) of compounds **3**, **4** and **5**.

Compounds			K_I (nM)				Selectivity ratio		
#	R1	R2	hCA I[17]	hCA II[17]	CaNce103	CgNce103	hCA I / CgNce103	hCA II / CgNce103	CaNce103/ CgNce103
3a	H	H	816	728	136	66,5	12,27	10,95	2,05
3b	CH_3	H	600	711	139	62,3	9,63	11,41	2,23
3c	F	H	652	428	91,5	55,4	11,77	7,73	1,65
3d	Cl	H	778	652	65,7	58,9	13,21	11,07	1,12
3e	OCF_3	H	742	683	295	154	4,82	4,44	1,92
4a	H	H	579	618	98,1	85,1	6,80	7,26	1,15
4b	CH_3	H	510	565	157	45,9	11,11	12,31	3,42
4c	F	H	426	264	61,3	45,1	9,45	5,85	1,36
4d	Cl	H	490	547	54,8	50,1	9,78	10,92	1,09
4e	OCF_3	H	539	484	88	53,5	10,07	9,05	1,64
4f	NO_2	H	378	250	35,7	23,1	16,36	10,82	1,55
5a	H	H	422	523	86,5	48,4	8,72	10,81	1,79
5b	H	CH_3	586	549	75,9	39,7	14,76	13,83	1,91
5c	CH_3	H	414	454	128	29,7	13,94	15,29	4,31
5d	CH_3	CH_3	536	491	84,1	35,3	15,18	13,91	2,38
5e	F	H	375	468	85,2	21,6	17,36	21,67	3,94
5f	F	CH_3	259	432	79,3	32,4	7,99	13,33	2,45
5g	Cl	H	249	309	83,3	38,2	6,52	8,09	2,18
5h	Cl	CH_3	368	462	178	36,5	10,08	12,66	4,88
5i	Br	CH_3	457	514	156	37,1	12,32	13,85	4,20
5j	OCF_3	H	295	236	53,6	45,1	6,54	5,23	1,19
5k	NO_2	H	229	164	34,1	22,8	10,04	7,19	1,50
5l	NO_2	CH_3	146	101	21,7	16,3	8,96	6,20	1,33
AZ	---	---	250	12	132	11	22,73	1,09	12,00

The backbone fold of the homology models shows marked differences near the active site, especially in the region where most likely the nitro group is located. As such, it is difficult to identify the identity of the hydrogen bonding partner of the ligands nitro group. Nevertheless, docking studies suggest candidates for such hydrogen bond forming residues, namely Gln145 and Lys175 for CaNce103 and Asn97 for CgNce103.

3. Conclusions

The enzyme inhibition values of structurally novel isatin-containing sulfonamides were determined for two Candida CAs, namely CaNce103 (*Candida albicans* CA) and CgNce103 (*Candida glabrata* CA). The compounds show K_I values in the low nanomolar range for the Candida CAs, while they have significantly higher K_I values for the human CA I and II isozymes. Homology models were constructed for the Candida CAs and subsequently the ligands were docked into these models to rationalize their enzyme inhibition values.

4. Experimental protocols

4.1. Carbonic Anhydrase inhibition assay

A stopped-flow instrument (SX.18MV-R Applied Photophysics model) was used for assaying the CA-catalyzed CO_2 hydration activity. Inhibitor and enzyme were preincubated for 15 min for allowing the complete formation of the enzyme-inhibitor adduct. IC_{50} values were obtained from dose response curves working at seven different concentrations of test compound (from 0.1 nM to 50 μM), by fitting the curves using PRISM (www.graphpad.com) and non-linear least squares methods, the obtained values representing the mean of at least three different determinations. The inhibition constants (K_I) were derived from the IC_{50} values by using the Cheng-Prusoff equation, as follows: $K_I = IC_{50}/(1 + [S]/K_m)$ where [S] represents the CO_2 concentration at which the measurement was carried out, and K_m the concentration of substrate at which the enzyme activity is at half maximal. All enzymes used were recombinant, produced in *E. coli* as reported earlier. The concentrations of the CaNce103 and CgNce103 enzymes used in the assay were 16.5 nM and 14.8 nM, respectively.

4.2. Homology model building of CaNce103 and CgNce103

Sequences of CaNce103 (UniProtKB/Swiss-Prot: Q5AJ71.1; 281 amino acids) and CgNce103 (GenBank: CAG59355.1; 219 amino acids) were obtained from the National Center for Biotechnology Information (NCBI). A BLAST search was performed (default settings) to identify crystal structures β-CAs with sequence similarity from the Research Collaboratory for Structural Bioinformatics (RCSB) PDB server. Two templates have been identified for the construction of CaNce103 and CgNce103 homology models, namely yeast *Saccharomyces cerevisiae* CA Nce103 (ScNce103; 3eyx; 2.04 Å) and green alga *Coccomyxa* CA in complex with acetazolamide (CmCA; 3ucj; 1.85 Å). These templates were superposed on the Cα-atoms of the ScNce103 structure (RMSD: 2.402 Å, 382 residues) prior to the construction of homology models.

Homology models were constructed for CaNce103 and CgNce103 using both templates (50 models per template per protein) with the MOE software package (v2014.09, Chemical Computing Group Inc., Montreal). During this procedure, the backbone was fixed. The homology model with the highest contact score was selected per template for both CaNce103 and CgNce103 (2 homology models derived from both crystal structures per enzyme). Subsequently, steepest-descent energy minimization protocols were applied using the AMBER12:EHT force field (MOE software package). To this end, all heavy atoms of acetazolamide, the active site residues (all residues within 4.5 Å of acetazolamide), the zinc ion, the zinc-binding residues, and the protein backbone were fixed and the other parts were minimized using a controlled release of position restraints. The minimized structures were used in the docking studies.

4.3. Preparation of isatin structures for docking studies

Three-dimensional molecule structures of isatin analogs **3**, **4** and **5** were prepared with the MOE software package (v2014.09, chemical computing group inc., montreal, Canada). Subsequently, the ligands were energy minimized using a steepest-descent protocol (MMFF94x force field).

4.4. Docking studies of the isatin analogs into the CaNce103 and CgNce103 homology models

The GOLD Suite software package (v5.2, CCDC, Cambridge, UK) and the ChemScore scoring function were used to dock the compounds into the homology models (50 dockings per ligand). In our previous docking studies, the ChemScore scoring function proved to be faster and more efficient in identifying hydrogen bonds between ligand and binding pocket of CAs.[17-19] The binding pocket for all homology models was defined as all residues within 12 Å of the N3 atom of acetazolamide. All ligands were forced to place their sulfur and nitrogen atoms at similar positions as observed for acetazolamide (in complex to CmCA) by applying position restraints in the GOLD Suite software package (default settings).

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