The 11th International Conference On Carbonic Anhydrases

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11th International Conference on Carbonic Anhydrases

ICCA 2018

June 27-30, 2018, Bucharest, Romania

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ICCA 2018 PROGRAMME

27.06.2018		28.06.2018		29.06.2018		30.06.2018	
		SESSION I: Comparative aspects of new CA families, isozymes and their structural investigations *Chairman:* C.T. Supuran		**SESSION III: CAs as drug and diagnostic targets: anti-tumor agents** *Chairman:* C. Capasso		**SESSION VI: Various applications of the CAs** *Chairman:* G. Costantino	
		8.30	**R. Mckenna**	**8.30**	**S. Dedhar**		
				8.45	**H. Konig**		
		9.00	**G. De Simone**	**9.00**	**A. Hjelmeland**	**9.00**	**T. Eysteinsson**
		9.30	**M. Ferraroni**	**9.30**	**H. Becker**	**9.30**	**T. Tuccinardi**
		10.00	**Y. Gao**	**10.00**	**M. Mboge**	**10.00**	**Coffee Break**
		10.30	**Coffee Break**	**10.30**	**Coffee Break**	**SESSION VI: Young researchers** *Chairman:* W.A. Donald	
		SESSION II: CA inhibitors and activators drug design, Part 1 ***Chairman.*** G. De Simone		**SESSION IV: CAs as drug and diagnostic targets: anti-infective agents** *Chairman:* R. McKenna		**10.30**	**Murat Bozdag**
						10.45	**A. Aspatawar**
		11.00	**M. Barboiu**	**11.00**	**G. Costantino**	**11.00**	**A. Angeli**
						11.15	**B. Harlan**
		11.30	**M. Krasavin**	**11.30**	**P. Skelly**	**11.30**	**A. Nocentini**
						11.45	**E. Berrino**
		12.00	**M. Ilies**	**12.00**	**M. Patrauchan**	**12.00**	**N. Chiaramonte**
						12.15	**P. Zuvela**
		12.30	**Lunch**	**12.30**	**C. Capasso**	**12.30**	**Concluding remarks by the organizers**
				13.00	**Lunch**		
		SESSION II: CA inhibitors and activators drug design, Part 2 *Chairman:* M. Barboiu		**SESSION V: CA inhibitors/activators in human diseases (non-cancer) Part 1** *Chairman:* S. Carradori			
		14.30	**J.Y. Winum**	**14.30**	**S.M. Monti**		
		15.00	**S. Carradori**	**15.00**	**D. Vullo**		
		15.30	**F. Carta**	**15.30**	**G. Provensi**		
		16.00	**Coffee Break**	**16.00**	**Coffee Break**		
		SESSION II: CA inhibitors and activators drug design, Part 3 *Chairman:* S. Dedhar		**SESSION V: CA inhibitors/activators in human diseases (non-cancer) Part 2** *Chairman:* M. Ilies			
***Chairman*: Acad. Marius Andruh**		**16.30**	**R. Zalubovskis**	**16.30**	**L. Micheli**		
17.00	**Welcome from the organizers**	**17.00**	**S. Thibaudeau**	**17.00**	**E. Swenson**		
17.15	**C.T. Supuran**						
		17.30	**B. Iorga**	**17.30**	**W.A. Donald**		
18.00	**Welcome Cocktail**	**18.00**	**S. Akocak**	**18.00 - 19.30**	**Poster sesion and Editorial Board meeting for *J. Enzyme Inhib. Med. Chem.* and *Expert Opin. Ther. Pat.***		
		18.30 - 19.30	**Poster session**				
				19.30	**Departure to Social Dinner**		

Summary

PLENARY INTRDUCTORY LECTURE	Supuran C.T.	*Update on carbonic anhydrase research, 2015-2018*	6
ORAL COMMUNICATIONS			
O1	Mckenna R.	*"Seeing" CA in action: Towards generating a molecular movie of its catalytic mechanism*	7
O2	De Simone G.	*Binding of carbon dioxide in the active site of zeta-CAs*	8
O3	Ferraroni M.	*Dioxygen, an unexpected carbonic anhydrase ligand*	9
O4	Gao Y.	*The LCIB protein family: a new group of β-carbonic anhydrases in CO_2- concentrating mechanism*	10
O5	Barboiu M.	*Dynamic constitutional screening of ca inhibitors and activators*	11
O6	Krasavin K.	*Latest in CAI developments from Saint Peterburg*	12
O7	Ilies M.	*CA IX inhibitors in the design of theranostic nanosystems for hypoxic tumor detection and treatment*	13
O8	Winum J.Y.	*Multivalent carbonic anhydrase inhibitors*	14
O9	Carradori S.	*New chemical scaffolds for the selective inhibition of CA Anhydrase*	15
O10	Carta F.	*Recent advances in rheumatoid arthritis treatment*	16
O11	Žalubovskis R.	*Towards novel inhibitors of cancer associated enzymes*	17
O12	Thibaudeau S.	*Molecular diversity: superacid chemistry and carbonic anhydrases inhibitors*	18
O13	Iorga B.	*Activation mechanism of carbonic anhydrases explored by molecular dynamics simulations*	19
O14	Akocak S.	*Design and synthesize of novel, potent and selective carbonic anhydrase inhibitors by using different linkers and tail approach*	21
O15	Dedhar S.	*Co-Targeting Carbonic Anhydrase IX with Immune check-point Inhibitors*	22
O16	Hjelmeland A.	*Targeting carbonic anhydrase IX in brain tumor Initiating cells*	23
O17	Becker H.	*Carbonic anhydrase II supports lactate transport in cancer cells by non-catalytic function*	24
O18	Mboge M.	*Sulfonamide based targeting of UFH-001 a novel triple negative, CAIX-positive breast cancer cell line*	25
O19	Costantino G.	*Hit to Lead optimization of selective CA inhibitors as enhancers of antifungal therapy*	27
O20	Skelly P.	*Carbonic anhydrase at the host-interactive surface of the human blood fluke Schistosoma mansoni*	28
O21	Patrauchan M.	*The Role of β-carbonic anhydrases in calcium deposition by Pseudomonas aeruginosa*	29
O22	Capasso C.	*An overview of the bacterial carbonic anhydrases*	30
O23	Monti S.M.	*Are carbonic anhydrases involved in the protection from oxidative damage?*	32
O24	Vullo D.	*Carbonic Anhydrase Activators: mechanism of action, drug design and pharmacologic applications*	33

O25	Provensi G.	*Unravelling the role of carbonic anhydrases on fear memory extinction*	34
O26	Micheli L.	*Carbonic Anhydrase Inhibitors as strategy for chronic pain treatment: effects of new synthetic inhibitors*	36
O27	Swenson E.R.	*Carbonic Anhydrase Activity, Inhibition and Activation: How do measurements in vitro and in vivo inform our understanding?*	37
O28	Donald W.A.	*Nanoscale ion emitters in native mass spectrometry: Rapid and accurate determination of ligand-protein binding affinities*	38
O29	Eysteinsson T.	*Membrane permeant but not membrane impermeant carbonic anhydrase inhibitors dilate pre-contracted porcine retinal arteries*	39
O30	Tuccinardi T.	*Development of a fingerprint scoring function for the prediction of the binding mode of Carbonic Anhydrase inhibitors*	41
YOUNG RESEARCHERS			
Y1	Bozdag M.	*Investigations of new analogs of SLC-0111*	43
Y2	Aspatawar A.	*Zebrafish: an animal model for testing of novel carbonic anhydrase inhibitors against multidrug resistant tuberculosis*	44
Y3	Angeli A.	*First activation studies with amines and amino acids of β-, γ-, δ-, ζ- and η-carbonic anhydrases*	46
Y4	Harlan B.	*Computational analysis of transcription factor binding sites in cancer related carbonic anhydrases*	47
Y5	Nocentini A.	*Deciphering the mechanism of human carbonic anhydrases inhibition with sulfocoumarins: synthesis, inhibition and computational studies of novel 6- and 7-substituted benzo[e][1,2]oxathiine 2,2-dioxide*	48
Y6	Berrino E.	*Synthesis and Evaluation of Carbonic Anhydrase Inhibitors with Carbon Monoxide Releasing Properties*	49
Y7	Chiaramonte N.	*2-Benzylpiperazine: a new scaffold for potent human Carbonic Anhydrase inhibitors. Synthesis, enzyme inhibition, enantioselectivity, computational and crystallographic studies and in vivo activity for a new class of intraocular pressure lowering agents*	50
Y8	Zuvela P.	*The role of non-covalent interactions in design of potent and selective inhibitors of carbonic anhydrases*	51
Y9	Konig H.	*Targeting Carbonic Anhydrase-IX as a novel approach to overcome hypoxia-mediated drug resistance in FLT3/ITD mutated AML*	52
POSTER COMMUNICATIONS			
P1	Abdul Malik A.-T.	*New Perspectives in Rheumatoid Arthritis Treatment*	55
P2	Alghamdi, Amira	*Targeted Drug Delivery of Carbonic Anhydrase IX liposomes Mediated by a Novel Peptide for Pancreatic Cancer Therapy*	56
P3	Akocak S.	*Synthesize of new Histamine Schiff base derivatives as an isoform-selective and potent carbonic anhydrase VII activators*	57

P4	Andring J.	*Increasing Lipophilicity of Inhibitors Induces Conformational Changes in Human Carbonic Anhydrase II and IX*	58
P5	Angeli A.	*Organochalcogenide as new modulator of Carbonic Anhydrase activity*	59
P6	Bonardi A.	*Is three better than two? Structural development of the tail approach by incorporation of three tails on benzenesulfonamide scaffolds.*	60
P7	Bua S.	*Design, synthesis and stability study in plasma of New Nonsteroidal Anti-Inflammatory Drugs and Carbonic Anhydrase Inhibitors Hybrids*	61
P8	D'Ambrosio K.	*The crystallographic structure of human ca vii provides insights into the complicated puzzle of carbonic anhydrase catalytic mechanism*	62
P9	Diudea M.V.	*Personalized therapies in cancer: Carbonic anhydrase peptide- based motifs as templates for biomarkers*	63
P10	Eldehna W.M.	*New SLC-0111 enaminone analogs as selective subnanomolar inhibitors of the tumor-associated carbonic anhydrase isoform IX*	64
P11	Grandane A.	*Heterocyclic analogues of sulfocoumarin as a potent inhibitors of carbonic anhydrases*	65
P12	Gheorghiu M.	*Specific carbonic anhydrase inhibitors effect on hypoxic cells – a quantitative assay*	66
P13	Guglielmi P.	*A novel library of saccharin and acesulfame derivatives as potent and selective inhibitors of carbonic anhydrase IX and XII isoforms*	68
P14	Karioti A.	*Salvianolic Acids From theRoots of Salvia miltiorrhizza as Effective Carbonic Anhydrase Inhibitors*	70
P16	Kazokaitė J.	*Novel benzenesulfonamides as selective carbonic anhydrase IX inhibitors exhibit functional effects to reduce hypoxia-induced acidification and clonogenicity in cancer cell lines*	72
P17	Lomelino C.L.	*Sweetener-Based Inhibition of Carbonic Anhydrase*	74
P18	Lucarini L.	*Carbonic anhydrase inhibition and anti-inflammatory effects of a new class of NSAIDs in the airways remodeling of a murine model of bleomycin-induced lung fibrosis*	75
P19	Murray A.B.	*Acesulfame K Displays Alternative Binding Modes Dependent on Carbonic Anhydrase Isoform*	77
P20	Petrikaitė V.	*Carbonic anhydrase IX: a promising therapeutic target for cancer treatment*	78
P21	Swenson E.R.	*Benzolamide is protective in cardiac ischemia: Role of endothelial nitric oxide (NO) synthase-mediated NO production*	79
P22	Tanc M.	*Novel Atg4B-inhibitors and dual [Atg4B-Carbonic Anhydrase] inhibitors for interfering with cytoprotective mechanisms of cancer cells in the acidic tumor micro-environment*	80
P23	Urbański L.	*The production and characterization of novel beta-carbonic anhydrase of Trichomonas vaginalis*	81
Alfabetical Index of Authors			83

Oral Presentations

Update on carbonic anhydrase research, 2015-2018

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There are now seven CA genetic families with the discovery of the θ-CA class in 2016, in the diatom *Phaeodactylum tricornutum* [1]. No further characterization of the δ- or η-CA classes (for which X-ray crystal structures are not available) were performed, except the activation profile with amines and amino acids [2]. X-ray crystallography was extensively performed on the human isoforms already characterized with various classes of inhibitors [3-6], but no other new structures (e.g., CA VB, X or XI) were reported. The sulfonamide, sulfamide and sulfamate inhibitors continued to be the most investigated ones [3-5], and the benzoxaboroles were discovered as a new class of inhibitors [6]. The tumor-associated isoform CA IX remains the most investigated one, with its PG domain [7], interactome [8,9] and involvement in many processes connected to tumorigenesis thoroughly investigated [9-12]. The sulfonamide inhibitor SLC-0111, now programmed to enter Phase II clinical trials for the treatment of solid metastatic tumors [10,12] is the most advanced new therapeutic agent in this class of drugs. The use of CA inhibitors for novel therapeutic applications, such as neuropathic pain [13], arthritis [14] and cerebral ischemia [15] has been explored in several proof-of-concept studies. In the anti-infectives field, interesting developments were registered for inhibiting the growth of bacteria such as *Mycobacterium tuberculosis* [16], *Helicobacter pylori* [17], *Burkholderia pseudomallei* [18] and fungi such as *Malassezia globosa* [19] with various classes of inhibitors. The immobilization of thermostable CAs from extremophiles on the surface of *Escherichia coli* was achieved by Capasso's group [20], leading to the potential of significant biotechnologic applications in the field of biomimetic CO_2 capture. Relevant advances were done in the field of CA activators [21], with the report from Blandina's group [22] that treatment of rats with CA activators leads to an enhanced cognition in spatial learning, whereas CA inhibitors of the sulfonamide type show the opposite effect, with impairments of fear memory consolidation. The mechanism by which these agents exert their effects involves the extracellular signal-regulated kinase (ERK) pathways. This opens the way for innovative therapeutic strategies for the management of psychiatric disorders, such as phobia, obsessive-compulsive disorder, generalized anxiety and post-traumatic stress. Parkkila's group demonstrated the role of CA IV in skin wound healing [23], paving the way for understanding fundamental biological processes as well as new therapeutic applications for modulators of this enzyme.

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“Seeing” Carbonic Anhydrase in Action: Towards Generating a Molecular Movie of its Catalytic Mechanism

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Introduction

Carbonic Anhydrase II (CA II, Figure 1A), catalyzes the reversible hydration of CO_2, with a kcat of 10^6 sec^{-1}. In the hydration direction, a zinc-bound OH^- performs a nucleophilic attack on the substrate CO_2, resulting in a zinc-bound HCO_3^- product that is subsequently displaced by a water molecule. The zinc-bound solvent is regenerated through a proton transfer mechanism facilitated by a His residue at the entrance of the active site (Figure 1B).

Results and discussion

In an attempt to visualize the steps of this reaction: medium resolution room temperature neutron crystallography, is used to study the proton transfer mechanism [1]; ultra-high resolution cryo-cooled CO_2 X-ray crystallographic is used to study substrate binding and active site solvent replenishment [2]; and preliminary X-ray free electron laser studies in combination with serial femtosecond crystallography, are discussed towards generating a “molecular movie” of CA II catalytic mechanisms.

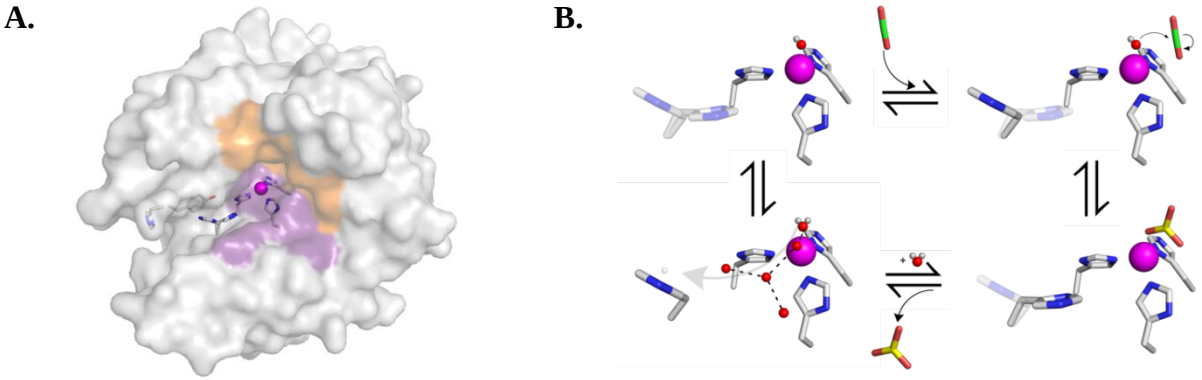

Fig. 1. **A**. Surface representation of CA II. **B**. Ping-pong catalytic mechanism of CA II.

Conclusions

These studies combined provide a “physical” glimpse of how CO2, protons, and water flow into/and out of the CA II active site during its catalytic cycle.

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Binding of carbon dioxide in the active site of zeta-carbonic anhydrases

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CDCA1 is a Carbonic Anhydrase (CA) isolated from the marine diatom *Thalassiosira weissflogii*, which based on its sequence and structural features has been used to define a new CA class, namely ζ-class. This enzyme, which consists of three repeats (R1, R2 and R3), is a cambialistic CA that can spontaneously exchange Zn or Cd in its active site. Even if structural studies are available on the three repeats and on the full-length protein [1,2], information on the substrate binding site are completely missing. To fill this gap, a structural study of CDCA1-R3 in complex with CO_2 will be here presented. The comparison of obtained data with those already available on α- and β-classes, will provide detailed information on the catalytic mechanism of ζ-class.

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Dioxygen, an unexpected Carbonic anhydrase ligand

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One of the most abundant zinc enzymes in the blood is carbonic anhydrase (CA, EC 4.2.1.1), which catalyzes a simple but essential reaction in all life kingdoms, CO_2 hydration to bicarbonate and protons. [1]

The crystallographic structure of human Zn,Cu(II)-CA II has been solved from data collected on a crystal of the native enzyme soaked in a solution containing 2 mM $CuSO_4$. In the structure a copper ion is bound at the hCA II N-terminal, coordinated to His4 and His64. Unexpectedly, a dioxygen molecule was found coordinated to the zinc ion in the active site. Since dioxygen is a rather unexpected CA inhibitor (up until now, molecular oxygen was never evidenced as a possible ligand of zinc in the CAs, except for one case [2] which has been poorly understood and discussed), molecular dynamics simulations were performed which suggested a superoxide character of the zinc bound O_2.

In native hCA II loaded with Cu(II) ions at the N-terminal region, the copper ion probably forms a redox center within the active site, which leads to the transfer of one electron to an oxygen molecule which thereafter replaces the water coordinated to the zinc ion deep within the CA active site, becoming a zinc ligand.

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The LCIB protein family: a new group of β-carbonic anhydrases in CO_2-concentrating mechanism

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Abstract

In many aquatic photosynthetic microorganisms, CO_2-concentration mechanisms (CCMs) have evolved to boost photosynthesis hindered by the slow diffusion rate of CO_2 in aquatic environment and the intrinsically low catalytic efficiency of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The limiting-CO_2 inducible B protein (LCIB) is indispensable to the CCMs in green algae *Chlamydomonas reinhardtii* (*C. Reinhardtii)*, however its molecular mechanism is poorly understood. Here we characterized a number of LCIB homologues from diverse organisms, of which constitutively carbonic anhydrase (CA)-active proteins were found in diatom *P. tricornutum*. Furthermore, we determined crystal structures of LCIB and LCIC in *C. reinhardtii*, as well as the CA-active homologue PtLCIB4 in diatom. All three structures harbor the motifs bearing close resemblance to the active site of canonical β-CAs, i.e. a zinc ion coordinated by a conserved trio of Cys-His-Cys, and a water molecule which interacts with an aspartate (a proton acceptor). The binding of the substrate analogue acetate to PtLCIB4 stabilizes the structure, leading to a complete model of the active site. Structural analysis together with biochemical data revealed a key residue, Arg194/193 in LCIB/LCIC and Ser47 in PtLCIB, appears to conduct the integrity of the active site and the functional activity of carbonic anhydrase within the LCIB family. Our results identify the LCIB family as a previously unidentified group of β-CAs, and provide a biochemical foundation for their function in the microalgal CCMs.

Furthermore, we recently also detemined the structure of PtLCIB3, which is much more active compared with PtLCIB4. I also will prosent our recent data to demonstrate how PtLCIB3 can be more active.

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Dynamic constitutional screening of ca inhibitors and activators

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Constitutional dynamic chemistry (CDC) and its application Dynamic combinatorial chemistry (DCC) are new evolutional approaches to produce *chemical diversity* [1]. In contrast to the stepwise methodology of classic combinatorial techniques, DCC allows for the generation of large molecular libraries from small sets of building blocks based on reversible interconversion between the library species. Dynamic combinatorial chemistry (DCC) has been extensively implemented during the last decade as a powerful approach in drug discovery that gives access to rapid and attractive identification of active ligands for biological targets [2].

The high selectivity and specificity of different bioreceptors may be used to describe the complex constitutional behaviors through component selection from a constitutional library. The examples presented in this talk, will shed light on the most major advantage with reversible dynamic combinatorial libraries (DCLs) and Carbonic Anhydrase. It will show the DCLs potential adaptability to express the sorting behaviours in response to an selection pressure, based on constitutional dynamics within a confined enzymatic pocket or specifically binding on the surface of the CA. Recent studies showed that a fine analysis can be performed to identify enzyme inhibitors [3] and to evaluate their relative affinities toward the human hCA I, II [4]. Moreover, synergetic screening via Dynamic Deconvolution method of CDLs of inhibitors (CAIs) and activators (CAAs) show that the inhibitory effects dominate over the activating ones, while the CAAs may be identified in the absence of CAIs [5]. Finally a straight-forward carbonic anhydrase activation strategy via dynamic encapsulation has been achieved by direct addition of multivalent amide dynamers into the enzyme reaction solutions [6,7].

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Latest in CAI development from Saint Petersburg

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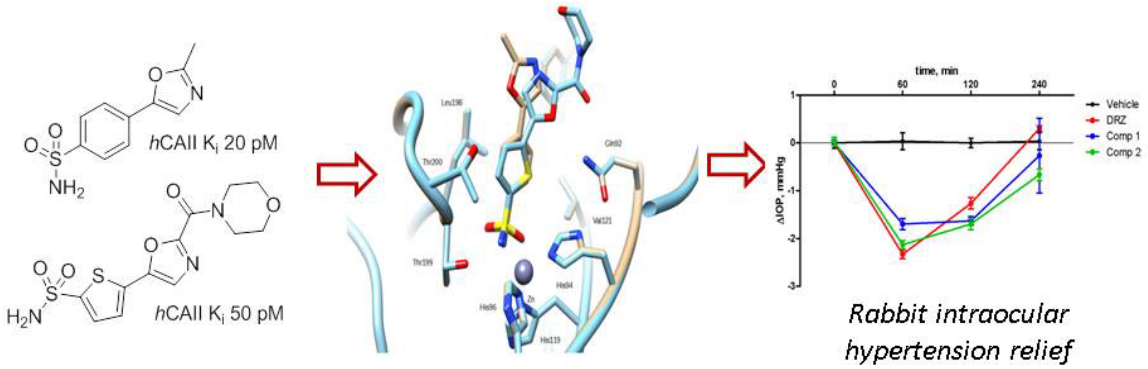
Since 2015, our Laboratory has been engaged in the development of isoform-selective inhibitors of carbonic anhydrases, in collaboration with the University of Florence (Prof. Claudiu Supuran's lab). Our initial approach was to apply direct sulfochlorination of various aromatic and heteroaromatic moieties in order to introduce primary sulfonamide as a zinc-binding group [1]. This has led to potent, *h*CAII-selective leads [2] that showed efficacy in a rabbit model of intraocular hypertension [3].



However, our synthetic methodology pursuits lie mostly in the area of multicomponent chemistry. It came to our attention that, unfortunately, multicomponent reactions (MCR) remain largely underutilized as a synthetic tool to construct carbonic anhydrase inhibitors (CAIs) [4]. Thus, we have set off to demonstrate the power of MCR in the design and synthesis of CAIs. This talk will provide some latest news in this regard.

Additionally, we have applied thermal shift assay (TSA) also known as differential scanning fluorimetry (DSF) to screen a library of compounds from Enamine, Ltd. (Ukraine). This has yielded an unusual class of CAIs, namely, Strecker-type alpha-aminonitriles [5]. A mechanism for their action has been proposed which is currently being verified by co-crystallization experiments.

Finally, we have screened some imidazoline-based sulphonamides for CA inhibition [6] and detected an interesting switch in their isoform selectivity profile on moving the aromatic groups around the imidazoline core [7].

Acknowledgements
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CA IX inhibitors in the design of theranostic nanosystems for hypoxic tumor detection and treatment

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Cancer remains a leading cause of mortality worldwide and its incidence is expected to increase dramatically in the next decades due to epigenetic factors, increased lifespan of population and better management of cardiovascular diseases. Many cancers are very hard to treat because in early stages they are asymptomatic, and relatively hard to detect under routine investigations. Finding new methods for early cancer detection and efficient treatment is thus essential for better management of many malignancies.

In this context, it was recognized that microtumors generated from early malignant cells that divide rapidly become quickly hypoxic due to lack of vascularization. Hypoxia triggers the expression of HIF-1, which in turn triggers the expression of more than 500 genes that are translated into pumps, transporters, proteins involved in angiogenesis and in different metabolic pathways that become overexpressed in hypoxic tumors such as glycolysis, anabolic processes, etc. Carbonic anhydrase IX is highly overexpressed in hypoxic tumors, which makes CA IX inhibitors potential theranostic agents for the detection and treatment of the corresponding cancers.

The latest developments in our lab towards the use of CA IX inhibitors in the design of various nanosystems for tumor detection and treatment, either alone, or in combination with chemotherapeutic drugs, will be presented. The efficacy of the new nanoplatforms will be supported by biological data acquired in 2D and 3D cell cultures and in animal models of cancer.

Multivalent Carbonic Anhydrase Inhibitors

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Carbonic anhydrases (CAs, EC 4.2.1.1) are well known zinc metalloproteins which catalyze a simple but essential physiological reaction: carbon dioxide hydration to bicarbonate and proton. These enzymes are of clinical relevance in cancer therapy as, among the 15 isoforms known in humans, some catalytically active isozymes are critical in various fundamental physiological processes and have been clinically exploited for the treatment or prevention of various pathologies such as glaucoma and neurological disorders. The lack of selectivity of conventional inhibitors against the different CA isozymes have led researchers to investigate new approaches for achieving such a goal.

Beside the rational drug design of more selective enzyme inhibitors, the multivalent approach was applied by our group to carbonic anhydrases. Multivalent enzyme inhibitors that combine multiple copies of an inhibitor conjugated to a single scaffold may lead to an overall increase of the binding affinity, avidity, and/or specificity. Exploration of new multivalent structures as potential enzyme inhibitors using straightforward and versatile method that enables the construction of multivalent systems for the inhibition of carbonic anhydrases (CA) will be presented.

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New chemical scaffolds for the selective inhibition of Carbonic Anhydrase

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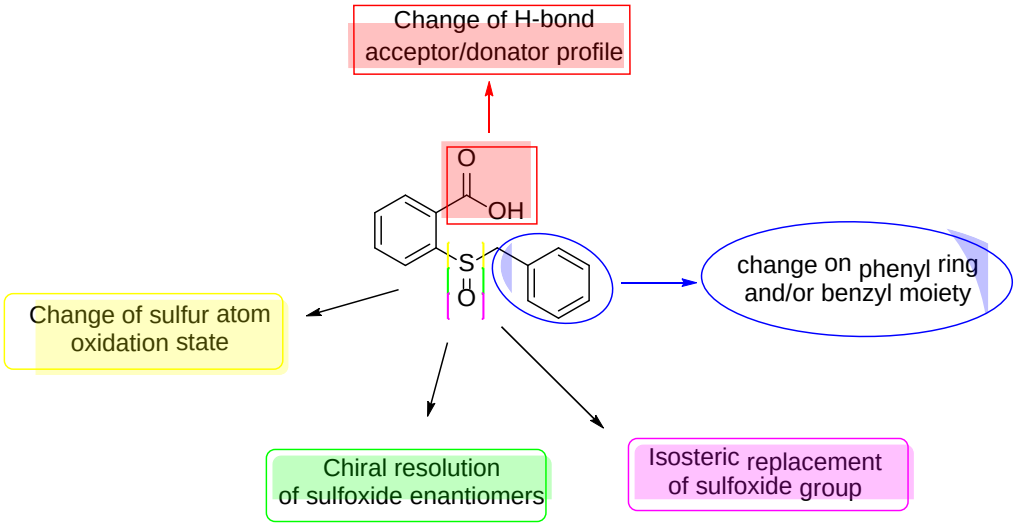
Starting from the structural study of the adduct which 2-benzylsulfinylbenzoic acid forms with human carbonic anhydrase II, showing a binding mode completely different from any other class of carbonic anhydrase inhibitors investigated so far, in a pocket situated out of the enzyme active site, we explored the chemical space around the lead compound to better understand this innovative mechanism of action (**Scheme 1**) [1]. All the compounds were fully characterized and tested against four different isoforms of human carbonic anhydrase (hCA I, II, IX and XII). Keeping also in mind that sulfoxide derivatives can exist as a mixture of two enantiomers, we separated them by means of chiral HPLC to evaluate the influence of the stereochemistry on the enzyme-inhibitor recognition.



Scheme 1. General overview of newly synthesized CA inhibitors.

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Recent Advances in Rheumatoid Arthritis Treatment

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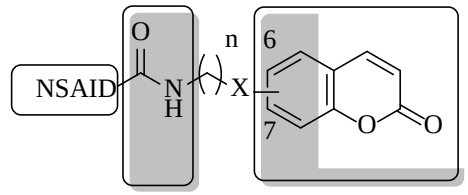
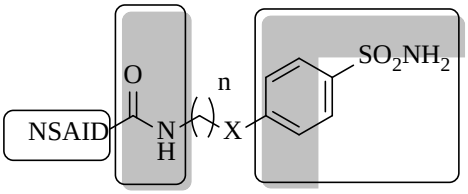
We firstly reported, as *proof-of-concept*, the use of small molecule hybrids composed of Nonsteroidal-Anti-Inflammatory-Drugs and Carbonic-Anhydrase-Inhibitors of the coumarin type (NSAIDs−CAIs) for the management of ache symptoms associated to inflammatory diseases such as rheumatoid arthritis (RA).[1]

As follow up study here we report next generation of hybrids bearing the sulfonamide CAI head instead. [2] The compounds tested in an *in vivo* model of the RA disease resulted highly effective, in terms of potency and time efficacy when compared to the reference NSAID alone. A peculiar feature of new hybrids is represented by their high affinity on the ubiquitous and erythrocyte expressed hCAs I and II which can be of particular advantage both for the systemic distribution of the drugs into the organism as well as for enhancement of their half-life. [2]



First Generation NSAID-CAI

n=2; X=O



Second Generation NSAID-CAI

n=0-4; X=none or O

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Towards novel inhibitors of cancer associated enzymes

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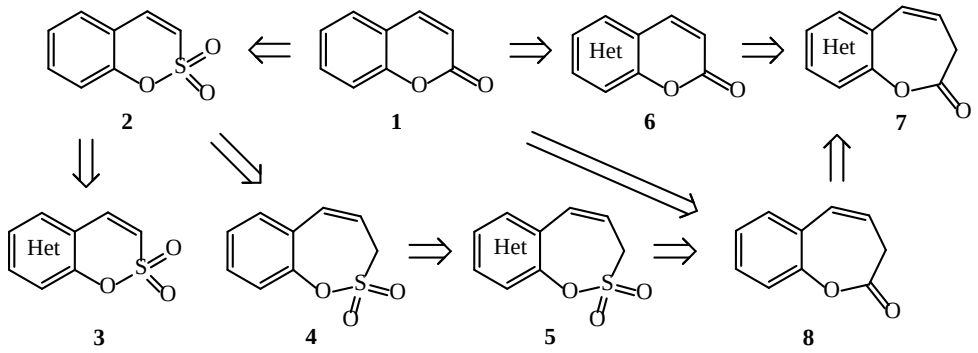
The efforts of our group are mainly focused on the design and synthesis of inhibitors of two enzymes groups - carbonic anhydrases (CA) and thioredoxin reductases (TrxR).

Carbonic anhydrases are zinc containing enzymes which catalyze reversible hydration and transport of carbon dioxide and, along with other functions, provide pH regulation in cells. Among 15 isoforms of human CA special attention is dedicated to inhibition of tumor associated CA IX and CA XII [1], where good inhibitory activities and selectivities for series of bioisosteres of coumarin have been recently demonstrated [2].

Thioredoxin reductases are selenoenzymes which are the only known enzymes to reduce Trx from its oxidized form. Since TrxR plays an important role in cellular redox balance and cancer cells are vulnerable at elevated ROS levels TrxR is an attractive drug target [3]. In our recent studies we have found, that some of bioisosteres of coumarin effectively inhibit cancer associated TrxR1.



Design and synthesis of bioisosteres of coumarin and corresponding derivatives will be discussed. Overview of they inhibition of CA, TrxR and cytotoxicity on tumor cell lines will be presented.

Acknowledgements
The authors would like to acknowledge ERA.Net RUS Plus project THIOREDIN.

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Molecular diversity: superacid chemistry and carbonic anhydrases inhibitors

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The strategic and practical role of organic synthesis is critical to the success of discovering and developing new drugs. The challenge now is for scientists to attack major diseases with fresh ingenuity [1]. A general consensus has emerged that library size is not everything; library diversity, in terms of molecular structure and thus function, is crucial [2]. Diversity-oriented synthesis aims to generate such structural diversity in an efficient manner [3,4].

In this context, superelectrophilic activation [5] of organic molecules under superacid conditions [6] has already been demonstrated to be a method of choice to generate molecular diversity and bioactive molecules in a straightforward way [7,8]. This strategy is now applied to the discovery of carbonic anhydrase inhibitors [9,10].

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Activation mechanism of carbonic anhydrases explored by molecular dynamics simulations

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Introduction

The research in the field of carbonic anhydrases (CAs) has known a tremendous development during the recent years, especially in the design of new inhibitors [1]. Meanwhile, the CA activators were studied to a lesser extent, in part due to lacking details in the molecular mechanism of the activation process. In this work, we explored the different aspects of CA activation mechanism using molecular dynamics simulations.

Experimental

CA structures were downloaded from the Protein Data Bank [2]. Docking calculations were carried out using GOLD [3] and molecular dynamics simulations using GROMACS [4] and the OPLS-AA force field [5]. Ligand parameters were obtained using the MOL2FF software.

Results and discussion

An initial attempt of OPLS-AA parametrization of CA binding site residues was published in 2013 [6]. We developed and validated recently a dataset of generalized OPLS-AA force field parameters for Zn metallo-enzymes. These parameters were used for molecular dynamics simulations of several CA isozymes in the apo form and in complex with different activators. The positions of stabilized water molecules and hydrogen-bonded networks were identified using HOP [7]. The influence of different structural and functional parameters evidenced by these simulations in the context of the enzyme activation mechanism will be discussed.

Conclusion

These results will provide a better understanding of the CA activation mechanism and will open opportunities for the design of new CA activators.

Acknowledgements

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Design and synthesize of novel, potent and selective carbonic anhydrase inhibitors by using different linkers and tail approach

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Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc enzyme responsible for the reversible hydration of carbon dioxide to bicarbonate, being involved in respiration and CO_2 transport between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion, aqueous humor secretion, biosynthetic reactions, etc. In mammals 16 isozyme or CA-related proteins have been described to date, with different catalytic activity, sub-cellular localization, and tissue distribution [1-3].

Carbonic anhydrase inhibitors were exploited for more than five decades in the treatment of edema, glaucoma, obesity, cancer, epilepsy and osteoporosis. Of recent interest is the development of selective inhibitors against membrane-bound isozymes, which will leave untouched the cytosolic ones, thus reducing the side effects associated with existing drugs on the market [1-5].

Isosteric replacement is an effective way to decrease side effects and improve pharmacological and pharmacokinetic properties of drugs for a specific biological target. In the current work, I will present our most recent studies towards design and synthesize of potent and selective carbonic anhydrase inhibitors by using different linkers such as cyano guanidine, 1,3-triazen, sulfamit..etc. Our bis-inhibitors will also be discussed in detail.

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Co-Targeting Carbonic Anhydrase IX with Immune check-point Inhibitors

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Carbonic Anhydrase IX (CAIX), a hypoxia induced pH and metabolic regulator is a promising cancer therapeutic target. We have developed a highly selective CAIX small molecule inhibitor, SLC-0111, which has recently completed a Phase 1 clinical trial and is now entering multiple Phase 1b/II clinical trials.

Immune check-point inhibitors, such as anti-PD1 and anti-CTLA4 antibodies have been approved for the treatment of malignant melanoma with dramatic results. However, only ~30% of the patients benefit fully from these therapeutics. Tumour hypoxia is a major hurdle in the response to immunotherapy, due to the hypoxia-mediated acidification of the tumour microenvironment, and high expression of CAIX contributes to this acidification. We therefore wanted to determine whether co-targeting CAIX with the immune check-point inhibitors resulted in increased efficacy relative to the check-point inhibitors alone.

We utilized the syngeneic B16F10 melanoma model in vivo in c57bl mice. We found that daily oral administration of SLC-0111 together with anti-PD1, anti-CTLA4, or combination of anti-PD1 and anti-CTLA4, resulted in significantly increased efficacy in terms of both tumour growth and survival, relative to the check-point inhibitors alone. Importantly, we found complete cures in 3 of 10 mice in the combination treated mice, compared to 0 of 10 in the check-point antibody alone treated mice.

Mechanistically we found a decrease in the extracellular pH, as well as in PD-1 expression on effector T-cells and a switch to a more favourable ratio of T-regs versus T-effector cells in the SLC-0111 treated mice.

These data suggest that reducing CAIX-mediated acidification of the hypoxic tumour microenvironment, results in a more favourable tumour microenvironent for T-cell mediated tumour cell killing promoted by the check-point inhibitors.

Combining CAIX inhibitors with immunotherapy thus represents a promising therapeutic strategy.

Targeting Carbonic Anhydrase IX in Brain Tumor Initiating Cells

A.B. Hjelmeland

Modeling of physiologic tumor environments and analysis of brain tumor sections demonstrates conditions such as hypoxia and acidic stress can enrich for a subset of thriving neoplastic cells, identified as brain tumor initiating cells (BTICs). BTICs are resistant to chemo- and radiotherapy, providing a reservoir for tumor recurrence and a desirable target for brain tumor treatments. Surgical resection, radiation therapy, and the chemotherapeutic agent temozolomide, which prolongs median life expectancy by mere months, constitutes current standard of care for the grade IV astrocytoma, glioblastoma (GBM). Prior studies suggested the efficacy of chemotherapies including temozolomide was increased by reducing expression of carbonic anhydrase 9 (CA9). After confirming basal and hypoxia-induced expression of CA9 in GBM BTICs, we targeted CA9 activity with the small molecule inhibitor SLC-0111 alone or in combination with temozolomide. In multiple GBM BTIC lines sensitive to temozolomide, addition of SLC-0111 to temozolomide reduced cell growth in vitro and in vivo. Mechanistically, SLC-0111 in combination with temozolomide altered cellular metabolism and pH in chemotherapy sensitive GBMs. While loss of p53 did not completely mitigate the benefit of SLC-0111 and temozolomide in GBM cells in vitro, the growth inhibitory effects of the combinatorial therapy were lost in temozolomide resistant cells. More recent kinomic and transcriptomic analysis of SLC-0111 and temozolomide treated GBMs reveals cell signaling changes that may offer new opportunities for pathway interventions to further improve therapy. Together, our data suggest that SLC-0111 can sensitize BTICs to temozolomide and extend animal survival in chemotherapy sensitive GBMs.

Carbonic anhydrase II supports lactate transport in cancer cells by non-catalytic function

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Tumor cells, especially those that reside in a hypoxic environment, produce vast amounts of lactate and acid, which have to be quickly removed from the cell to avoid intracellular lactacidosis and suffocation of metabolism. In the present study, we demonstrate that proton-coupled lactate flux is facilitated by the intracellular carbonic anhydrase CAII in MCF-7 breast cancer cells. In these cells CAII is colocalized with the monocarboxylate transporter MCT1, as shown by *in situ* proximity ligation assay. Coexpression of MCT1 and MCT4 with various CAII mutants in *Xenopus* oocytes demonstrated that CAII facilitates MCT transport activity via CAII-Glu69 and CAII-Asp72, which have been suggested to function as surface proton antennae for the enzyme [1]. While CAII-Glu69 and CAII-Asp72 seem to mediate proton transfer between enzyme and transporter, CAII-His64, the central residue of the enzyme's intramolecular proton shuttle, is not involved in proton transfer between the two proteins, but mediates binding of CAII to the transporter's C-terminal tail. Taken together, the results suggest that CAII features a moiety that exclusively mediates proton exchange with the transporter, while the central residue for the intramolecular proton shuttle, His64, mediates binding to MCT (Fig. 1).

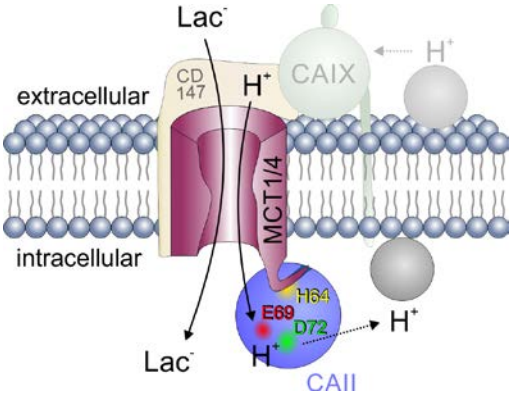

Fig. 1. CAII functions as a proton antenna for MCTs.

Intracellular CAII is anchored to the C-terminal tail of MCT1/4 via CAII-His64. This binding brings CAII close enough to the transporter pore to shuttle protons between transporter and surrounding protonatable residues (gray circles). Proton shuttling is mediated by CAII-Glu69 and CAII-Asp72. Under hypoxic conditions CAIX could bind to MCT1/4 via their chaperon CD147 to facilitate the exchange of protons between transporter and extracellular protonatable residues in a similar fashion as CAII [2]. By this non-catalytic mechanism intracellular and extracellular carbonic anhydrases could cooperate non-enzymatically to facilitate proton-driven lactate flux across the cell membrane of cancer cells.

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Sulfonamide Based Targeting of UFH-001 a Novel Triple Negative, CAIX-positive Breast Cancer Cell Line

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Introduction

Membrane-bound carbonic anhydrase IX (CAIX) has been shown to play an important role in tumor progression in xenograft models and is considered a potential therapeutic target for the treatment of several aggressive types of cancers [1,2]. In breast cancer, CAIX expression is a marker for hypoxia and associated with the triple negative phenotype (TNBC). Therefore, targeting CAIX activity, using small molecule inhibitors, in this highly aggressive and metastatic setting may serve to improve overall disease free survival and clinical outcomes [3,4]. Our goal for this study was to 1) Identify and/or develop a cell line that expresses CAIX in an hypoxia-independent manner and is representative of the TNBC phenotype and 2) To target CAIX activity in this cell line and test the effects of CAIX inhibition/ablation on its growth and metastasis. Our hypothesis is that CAIX inhibition, in the context of an hypoxic and/or acidic microenvironment, will prevent its ability to accelerate growth, migration and invasion, which we infer is from deregulation of the tumor microenvironment.

Experimental

To achieve our aims, we developed and characterized a new CAIX-expressing cell line named UFH-001. We used both biophysical and biochemical methods as well as cell-based assays to evaluate the effects of sulfonamide-mediated inhibition of CAIX on UFH-001 cell growth, migration, and invasion.

Results and Discussion

Our results show that CAIX expression and activity in UFH-001 cells, is increased under hypoxic conditions, although substantial levels were also noted under normoxic conditions. This cell line is also representative of the TNBC phenotype (ER/PR and HER2 negative) but with a more epithelial-like morphology relative to basal-like cells. UFH-001 cells grow aggressively and have the ability to migrate/invade and form tumors *in vivo*. In these studies, we have also shown how inhibition of CAIX activity, using sulfonamide-based inhibitors, affects UFH-001 growth in an apoptosis-independent manner and decrease the migratory/inhibitory capacity of these cells.

Conclusions

Taken together, our observations support the hypothesis that CAIX can serve as a selective target for the treatment of patients with TNBC.

Acknowledgements

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Hit to Lead optimization of selective CA inhibitors as enhancers of antimicrobial resistance therapy

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Introduction

A growing number of pathogens are becoming resistant to the available antibiotic treatments. Infections caused by these resistant strains fail to respond to classical antibiotic therapies, therefore leading to prolonged illness, higher healthcare costs, and a greater risk of death. In this scenario, the discovery and development of novel approaches to counteract the emergence of resistant pathogens is of great demand. Recently, it has been demonstrated that in many pathogens, carbonic anhydrases (CAs) are essential for their life cycle and the inhibition of such enzymes leads to growth impairment or growth defects. However, the principal drawback in using CA inhibitors (CAIs) as antimicrobial agents is the side effects due to the lack of selectivity toward different families and isoforms.

Experimental

Herein, we describe the identification of a chemically new class of CAIs characterized by the presence of new Zinc Chelating Group (ZCG), which preferentially interact with microbial CA active sites over the human ones. The hit compound identified was used as starting point for the hit expansion. The second series of synthesized compounds were tested against a large panel of different classes of CAs, allowing us to identify a small sub-set of compounds that inhibit microbial CAs with a selectivity fold greater than 60.

Results and discussion

Our results highlight for the first time that is possible to design and synthetized compounds that selectively target different classes of CAs. Indeed, the newly reported inhibitors preferentially interact with the β- and η-CA family over the α one. The most promising compounds, in terms of affinity and selectivity, were then tested against an important human pathogen revealing that our compounds are also able to inhibit microbial CA in cells and the mechanism of action of our CAIs was also confirmed.

Conclusions

Finally, our compounds represent a new class of CAIs able to selectively inhibit in cells microbial CAs, furnishing for the first time a robust proof of concept that microbial CAs can be suitable drug targets in antimicrobial therapies.

Carbonic anhydrase at the host-interactive surface of the human blood fluke *Schistosoma mansoni*

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Schistosomes are parasitic flatworms (commonly called blood flukes) that cause a chronic, debilitating disease afflicting over 200 million people in over 70 countries. There is no vaccine to prevent infection and a single drug (praziquantel) that has been in use for over 30 years is the mainstay of control. New drugs are needed.

The research emphasis of our laboratory is the biochemistry of the schistosome surface (the tegument). We have identified a novel tegumental carbonic anhydrase (CA) enzyme at the surface of intravascular *Schistosoma mansoni* (which we designate SmCA). Immuno-staining and enzyme assays confirm that living worms express SmCA at the surface. This activity is significantly diminished following specific SmCA gene suppression using RNA interference. Importantly, few juvenile parasites whose SmCA gene has been suppressed survive following infection of experimental animals. This shows that SmCA performs an essential function for the mammalian-stage parasites *in vivo*. We hypothesize that targeting SmCA with an inhibitory compound will diminish parasite enzyme function (mimicking the RNAi effect) and debilitate the worms.

We have generated a codon-optimized version of SmCA (containing a poly-his domain) and produced high levels of functionally active enzyme in the mammalian CHO-S cell expression system. Recombinant SmCA has been purified using conventional immobilized metal affinity chromatography (IMAC). A collection of known CA inhibitors (and chemical variants) have been tested for their ability to inhibit SmCA activity using a stopped-flow, CO_2 hydrase assay. Several potent SmCA inhibitors have been identified and these are currently being tested for their ability to block the native enzyme on the surface of live parasites as well as for their ability to cure experimental animals infected with schistosomes.

The Role of β-carbonic anhydrases in Calcium Deposition by *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic human pathogen causing life-threatening infections in patients with cystic fibrosis and infective endocarditis, which can be associated with calcification at later stages. The mechanisms of calcification are not well defined and may involve contributions of bacterial pathogens. We have shown that *P. aeruginosa* produces extracellular deposits of calcium (Ca^{2+}) when grown at elevated Ca^{2+} levels. We hypothesized that carbonic anhydrases are responsible for the deposition by providing carbonate, the product of CO_2 hydration. Earlier we have identified and characterized three functional β-carbonic anhydrases (CAs): psCA1, psCA2, and psCA3, produced by *P. aeruginosa*. Here we show that the transcription of *psCA1* is increased in the presence of elevated Ca^{2+}. To study the role of these genes in calcification, we generated single, double, and triple deletion mutants. We also obtained and verified transposon mutants with individually disrupted *psCA* genes. While the deletion of *psCA1* or both *psCA1* and *psCA2* did not affect the growth, deletion of all three *psCA* genes delayed *P. aeruginosa* growth at ambient air by 4 hours. No impact on growth of the mutants at 5% CO_2 was registered. Measuring both deposited and free Ca^{2+} in liquid medium for the wildtype showed that growth at no shaking with 5% CO_2 favored Ca^{2+} deposition more than that at shaking and ambient air. Deletion of *psCA1* alone caused ~2 fold decrease in Ca^{2+} deposition and almost abolished free Ca^{2+} removal from the medium at both 5 and 10 mM Ca^{2+}. The results show that psCA2 also contributes to Ca^{2+} deposition at 10 mM Ca^{2+}, but psCA3 plays no role in this process. Earlier we have identified several compounds that inhibit the activity of two of the three heterologously expressed carbonic anhydrases, psCA1 and psCA3 at nanomolar level. Since psCA1 appears to play a major role in Ca^{2+} deposition, we tested the effect of 3-aminobenzenesulfonamide inhibiting the enzyme activity with K_I of 19.2 nM. So far, the results showed 58% inhibition of the deposition. Finally, we hypothesized that the formation of Ca^{2+} extracellular deposits may enhance biofilm formation of the pathogen by providing support and strengthening the extracellular matrix. Quantification of biofilm growth by the wildtype and *psCA* mutants showed decreased biofilm formation in the mutant lacking *psCA1* as well as *psCA1-psCA2* double and *psCA1-psCA2-psCA3* triple mutants during growth at 5 and 10 mM Ca^{2+}. Currently, we are testing whether inhibiting psCAs activity would have an impact on biofilm growth and whether psCA-dependent calcium deposition plays role in the virulence of *P. aeruginosa.*

An overview of the bacterial carbonic anhydrases

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The bacterial carbonic anhydrases (CAs, EC 4.2.1.1) represents a valuable member of new macromolecules affecting the growth of microorganisms or making them vulnerable to the host defense mechanisms. By equilibrating CO_2 and bicarbonate, these metalloenzymes interfere with pH regulation and other crucial physiological processes of these microorganisms [1]. In the last years, bacterial CAs from pathogenic and non-pathogenic bacteria has been subject to extensive studies either from pharmacological or environmental viewpoints [2].

The following is observed:

CA-classes: Bacteria encode for enzymes belonging to α-, β-, and γ-CA classes and show an intricate CA distribution pattern. In fact, some of them encode CAs belonging to only one family, whilst others encode those from two or even three different genetic families.

CA-binding site: The α- and β-CAs are metalloenzymes, which use the Zn(II) ion as a catalytic metal; γ-CAs are Fe(II) enzymes, but they are also active with bound Zn(II) or Co(II) ions. The metal ion from the CA active site is coordinated by three His residues in the α- and γ-classes, and by one His and two Cys residues in the β-class. The fourth ligand is a water molecule/hydroxide ion acting as a nucleophile in the catalytic cycle of the enzyme.

CA-structure: The three-dimensional structure of the bacterial α-CAs generally resembles those of human α-CAs. The crystallized α-CAs are active as monomers and dimers, show a more compact structure and have an active site larger with respect to the mammalian counterpart. The bacterial β-CAs crystallized so far are active as dimers or tetramers, with two or four active sites. Bacterial γ-class crystallizes as a trimer with three zinc-containing active sites, each located at the interface between monomers. The γ- CA is only active as a trimer.

Inhibition studies: Several classes of CA inhibitors (CAIs) are known to date, among which sulfonamide, sulfamide and sulfamate are the most investigated ones. Certain pharmacological CAIs were able to highly inhibit most of the bacterial CAs. The inhibition profile with simple and complex anions, as well as small molecules inhibiting other CAs, showed that the most efficient inhibitors detected so far are sulfamide, sulfamate, phenylboronic acid, and phenylarsonic acid. Moreover, it has been demonstrated in vivo that the inhibition of bacterial CAs influences the pathogenicity and/or the growth of the microorganism. These promising data on live bacteria allow us to propose bacterial CAs inhibition as an approach for obtaining antiinfective agents with a new mechanism of action compared to classical antibiotics.

Activation studies: An interesting feature of the CA superfamily is that they can bind within the middle-exit part of the active site molecules known as “activators” (CAA). In contrast to the CAIs, the CA activators (CAAs) were much less investigated. Most of the activators belong to the amino and/or amino acid chemotypes and determine efficient proton shuttling processes between the active site and the environment. Some activators are able to enhance the k_{cat} of the bacterial CAs up to one order of magnitude compared to the enzyme without activators.

Phylogenetic studies: Phylogenetic analysis of carbonic anhydrases identified Gram-positive and negative bacteria showed that the ancestral CA is represented by the γ-class. In fact, the γ-CA is the only CA class, which has been identified in Archaea.

Localization and Physiological Role: The α-CA, characterized by a signal peptide, are able to convert the CO_2 to bicarbonate diffused in the periplasmic space of the Gram-

negative bacteria. On the contrary, β- or γ-classes have a cytoplasmic localization and are responsible for CO_2 supply for carboxylase enzymes, pH homeostasis, and other intracellular functions.

Biotechnological studies: Since CAs are very effective catalysts for the conversion of CO_2 to bicarbonate, they might be involved in the capture/sequestration of CO_2 from combustion gases. To accomplish this, the *Escherichia coli* cells have been engineered to overexpress and anchor a bacterial thermostable CA on their bacterial surface.

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Are carbonic anhydrases involved in the protection from oxidative damage?

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Among the cytosolic isoforms of the alfa-carbonic anhydrase (CA) family, CA III and VII are two of the less investigated. Despite the high sequence identity and similarity of three dimensional structure, they have very different catalytic efficiency when comparing their specific activity for CO_2 hydration-dehydration reaction, with CA VII being much more active than CA III. Recently, CA III and CA VII have been proposed to play a role as scavengers in cells where oxidative damage occurs.

Here, we will examine the functional and structural features of these two isoforms highlighting the factors affecting similarities and differences among the two enzymes.

Carbonic Anhydrase Activators: mechanism of action, drug design and pharmacologic applications

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Introduction

Mammalian carbonic anhydrases of which 16 isoforms are known, are involved in important physiological functions. Their inhibition is exploited pharmacologically for the treatment of many diseases (glaucoma, edema, epilepsy, obesity, hypoxic tumors, neuropathic pain, etc.) but the activators were less investigated [1]. In recent years, attention and interest have been captured by the identification, structural characterization and understanding of the physiological and pharmacologic roles that CA activators may play [2,3].

Experimental

*CA activation mechanism***:** After a rather long period of controversy dated till the early 1990s, when work with highly purified enzymes and very precise techniques, such as the stopped-flow assay, undoubtedly demonstrated that the CAAs exist and that they take part to the catalytic cycle [1,4,5]. In 1990, a general mechanism of action for the CAAs has been proposed based on Equation 1.

$$EZn^{2+}-OH_2+A \Leftrightarrow [EZn^{2+}-OH_2\text{ - }A] \Leftrightarrow [EZn^{2+}-HO^-\text{ - }AH^+] \Leftrightarrow EZn^{2+}-HO^-+AH^+$$
enzyme - activator complexes

CAAs drug design studies: Interesting drug design studies of CAAs were reported in the last 20 years, which led to the discovery of many low nanomolar activators for most isoforms. Many of these derivatives were obtained using histamine or histidine as leads. Other amino acid/oligopeptide activators have also been prepared and showed highly interesting activity against the physiologically dominant CA isoforms, such as CA I, II, IV, VA, VII, IX, etc. Some of the very recent studies allowed for the identification of highly effective, isoform-selective activators based on histamine Schiff base derivatives [1,6].

Pharmacologic applications of CAAs: Although there are CA-deficiency syndromes for many isoforms, among which CA I, II, IV, VA, XII and XIV, the activators were not yet employed for the treatment of these conditions. A rather interesting field is constituted, by the use of CAAs for memory therapy and there may be a future of the CAAs also for the tissue engineering field, with the possibility to obtain artificial bone fragments, as it has been recently demonstrated that the presence of CAAs in the medium enhances the formation of the inorganic salts (involved in the biomineralization process [1,6].

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Unravelling the role of carbonic anhydrases on fear memory extinction

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Introduction

Fear extinction is an active form of learning defined by the attenuation of a learned response following non-reinforced exposure to a previously fearful stimulus. This phenomena seems to be mediated by similar neural circuits in rodents and humans. For that reason, the interest in fear extinction as model for translating basic research into clinical leads is growing. Recent evidences provided by us [1] and others [2,3] indicates a role for central carbonic anhydrases (CAs) in fear memory aquisition and consolidation, but nothing is know about its role on extinction. Thus, the aim of this study was to investigate the impact of CAs activation on fear memory extinction.

Experimental

The effects of CAs inhibitors acetazolamide and compound 18 or the CA activator D-phenylalanine were evaluated using the extinction of contextual fear conditioning paradigm in male rats. The protocol consists of 3 sessions: acquisition, training and test performed with 24h inter-trial intervals. In the first session, the animals were placed in the conditioning chamber and 3 foot shocks (0.5 mA, 2 s) were delivered at 30 s interval. During extinction, animals were placed again in the same apparatus for a extinction training (lasting 15 or 30 min depending on the experiment), in the absence of punishments. In the final session, the animals were placed again in the same conditioning chamber for a 3 min retention session. Compounds were administered immediately after the acquisition session and the time the animals spent freezing (defined as the complete absence of somatic motility, with the exception of respiratory movement) was manually recorded by a trained researcher.

Results and discussion

Systemic administration of acetazolamide (30 mg/Kg, i.p.) impaired, while injection D-phenylalanine (300 mg/kg, i.p.) facilitated extinction memory consolidation. Co-treatment with acetazolamide prevented D-phenylalanine-induced effect. No behavioural alterations were observed after systemic treatment with Compound 18 (30 mg/kg, i.p.), a CA inhibitor that is unable to cross the blood brain barrier. Extinction deficits were also observed following acetazolamide (10 nmols/side) infusion into the CA1 region of the hippocampus, the basolateral amygdala or the ventromedial prefrontal cortex. On the contrary, acetazolamide was ineffective when infused into the substantia nigra.

Conclusions

Here we provided the first demonstration of the involvement of central CAs in fear memory extinction. Therefore, CAs can be considered an innovative target for the developement of new compounds for the treatment of disorders characterized by maladaptative fear responses.

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Carbonic Anhydrase Inhibitors as strategy for chronic pain treatment: effects of new synthetic inhibitors

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Persistent pain is an unpleasant sensory and affective experience that is the complex sum of injuries and sensory stimuli mediated by individual emotions and expectations. Current pharmacological approaches recommend to start with the conventional oral analgesics during the first treatments, as Nonsteroidal-Anti-Inflammatory-Drugs (NSAIDs), up to the used of weak and strong opioids as codeine, dihydrocodeine and morphine when no other drug is satisfactory. However the management of persistent pain remains inadequately treated claiming for new therapeutic strategies.

Several studies demonstrated important role of human Carbonic anhydrase (hCAs; EC 4.2.1.1) in a variety of physiological and pathological process. Consequently, the 12 catalytically active hCA isoforms have become an interest target for the design of inhibitors with biomedical application and in recent years much efforts were dedicated to the development of new inhibitors that, although presenting lower affinity for the CA active site, would be able to be more selective toward the different isoforms.

Aim of the study was to individuate and test new hCAs molecules active against persistent pain induced by different origins. In these experiments were used Complete Freund's Adjuvant (CFA) and oxaliplatin to reproduced rheumatoid arthritis and neuropathic pain in rats and mice, respectively. In rats with articular pain, a series of dual inhibitors incorporating both a CA-binding moiety of the sulfonamide type and a cyclooxygenase inhibitor of the NSAID type (such as naproxen, ketoprofen, sulindac and diclofenac) were tested. On day 14, after CFA injection, the molecules (namely 2A, 3A, 6A and 8A) were per os administered (0.1 – 10 mg kg^{-1}) for evaluating their acute pain relieving effect. All compounds were significantly active taking effect 15 min after treatment; in particular 6A and 8A fully reverted articular pain. On these bases, 6A and 8A were deeply analyzed and after a repeated daily treatment prevented the development of rheumatoid arthritis-related pain. 6A and 8A (1 mg ml^{-1}) were also directly intra-articularly administered (50 µL) 7 days after CFA injection and were able to counteract mechanical hypersensitivity and improved motor coordination.

In mice with oxaliplatin-induced neuropathy, we tested a series of novel selenides bearing benzenesulfonamide moieties (AA20-13; AA20-22; AA20-24) showing a potent inhibitory action against hCA VA, VII and IX in the low nanomolar range and compounds possessing sulfamate group as zinc binding group which are isosteres of sulfonamides (MB8-468A; MB8-459A; MB9-497A; MB9-498A and MB9-503A). Compounds were more potent with respect to acetazolamide, active in a range doses of 10 – 30 mg kg^{-1} in reducing allodynia measured by a thermal non noxious stimulus using the Cold plate test.

In conclusion, these new hCAs molecules are a promising tools for the treatment of persistent pain originating from inflammatory states as rheumatoid arthritis or neuropathies induced by chemotherapeutic agents like oxaliplatin.

Carbonic Anhydrase Activity, Inhibition and Activation: How do measurements in vitro and in vivo inform our understanding?

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Many of the isozymes of carbonic anhydrase have activities that are among the highest known in the animal kingdom when measured in vitro; thus inhibition in many physiological processes often requires > 99% reduction in activity to alter physiological function. Yet, in some situations of metabolic and physiological stress or disease states concentrations of CA isozymes may be increased or decreased by 2-3 fold. This raises the seeming paradox of why if it takes > 99% inhibition to alter a CA-dependent function (an almost hundred-fold reduction in enzyme activity) would increasing an already high activity be useful or a fall in 50% be critical. Most activators of CA only increase activity by about 2-fold, but are being considered useful in some disease states. The answer to these questions are related to fundamental uncertainties about the actual state and microenvironments in which CA operates in vivo, actual in vivo activity as opposed to activities measured in very simplified in vitro conditions, and access of inhibitors and activators to the enzymes in membranes and in the very complicated and restricted environments of the cell cytoplasm and organelles, in which they must function, sometimes in intimate association with other proteins involved in the same process- so called metabolons. Little also is known as to the extent like many other enzymes, they undergo phosphorylation, nitroso-thiolation and other secondary and tertiary structural changes that may alter their activity and sensitivity to inhibition in vivo. Although in vitro measurements of CA activity, inhibition, and activation provide initial important information, better methods of making these determinations in more realistic recreations of in vivo conditions and ideally in vivo are clearly needed.

Nanoscale ion emitters in native mass spectrometry: Rapid and accurate determination of ligand-protein binding affinities

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Electrospray ionization (ESI) mass spectrometry (MS) has emerged as an important technique in the analysis of intact proteins and macromolecular complexes in their native structures. However, salt and non-volatile and semi-volatile molecules can readily adduct to proteins, which broadens peaks, lowers signal-to-noise values, and can make it difficult to impossible to assign mass spectral peaks in native MS [1]. Moreover, many druggable protein targets are only stable at high salt concentrations that mimic the intra- and extra-cellular environments. Here, we use ESI emitters with exceedingly narrow tip diameters (250 nm) compared to conventional native MS tips (2000 nm) to significantly reduce the extent of adduction of salt and non-volatile molecules to protein complexes. The formation of smaller ESI generated droplets using nanoscale ESI emitters results in significantly less enrichment of salts and non-volatile molecules during droplet desolvation and ion formation than by use of larger tips. The effects of ion emitter size on the native mass spectra of ligand-protein complexes of the druggable protein target, human carbonic anhydrase, and six small molecule inhibitors that have known binding constants of between 1 and 38500 nM. By use of the smallest emitter size and a novel analytically-derived equation, Kd values can be accurately obtained for all six ligands directly from the abundances of the unbound and bound ligand-protein complexes from a single mass spectrum, which is not possible using conventional native MS emitters. Moreover, ligand-protein binding constants can be directly, and accurately measured with high accuracy using buffered solutions at ionic strengths that mimic the extracellular environment by native MS for the first time. Thus, the accurate and high-throughput screening of small molecule libraries against many different proteins in solutions that maintain their native-like structures with ultrahigh sensitivity using ESI MS is now feasible.

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Membrane permeant but not membrane impermeant carbonic anhydrase inhibitors dilate pre-contracted porcine retinal arteries

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Introduction

Carbonic anhydrase inhibitors (CAIs) used for the treatment of glaucoma, such as acetazolamide and dorzolamide have also been found to cause vasodilation of retinal arteries [1], and to elevate retinal and optic nerve PO_2 [2]. However, the mechanism for this effect is not fully understood. The purpose of this study is to identify where the carbonic anhydrase isoforms that produce vasodilation in retinal arteries are located (i.e. cytosolic or on the surface of cell membranes). We have examined the effects of a series of membrane permeable and impermeable CAIs on the vascular tone in pre-contracted porcine retinal arteries.

Experimental

Dissected segments of porcine retinal arteries were mounted in a DMT 630MA wire myograph to measure contractile activity, and pre-contracted with 10^{-6} M U-46619, a prostaglandin analog, added to the organ bath. When the vascular tone stabilized the CAI tested was applied to the bath, and its effects on vascular tone recorded. Results are presented as mean ± SEM percentage of the maximum vasodilation, compared to the vasoconstriction induced by 10^{-6} M U-46619. Two-tailed Student's t-test was used for statistical analysis of the results.

Results and discussion

A series of five relatively membrane impermeable CAIs were tested on isolated arteries pre-contracted with 10^{-6} M U-46619. The pyridinium and membrane impermeable derivative FC5-207A (10^{-3} M) had no significant effects on the vascular tone of retinal arteries pre-contracted by U-45519. Three other membrane impermeable CAIs, the sulfonamides MB9-527R2A, MB9-523R9A and MB9-512B were applied to pre-contracted retinal arteries at 10^{-3}M. The mean relaxation induced by MB9-527R2A was 9.7±2.4%, which was not significant (p=0.8), by MB9-523R9A 5.6±3.4% (p=0.9), and MB9-512B 3.3±1.5% (p=0.7). Benzolamide, shown to enter red blood cell membranes [3], induced a significant mean relaxation of 85 ± 8% ($P< 0.01$) at 10^{-3}M after vasoconstriction. The effect of benzolamide was dose-dependent. Dorzolamide induced 76 ± 8% relaxation ($P< 0.02$). A series of three other lipophilic CAIs were tested similarly on retinal arteries, and sulfonamides EB3-217B, EB3-177A and AA-20-22 all induced relaxation (85.5%, 96% and 83%, respectively).

Conclusions

All the known membrane permeant carbonic anhydrase inhibitors tested induce vasodilation in pre-contracted porcine retinal arteries, while membrane impermeable

inhibitors do not have a significant effect on vascular tone. This suggests that cytosolic isoenzymes of carbonic anhydrase are involved in mediating the vasodilation induced. The results further suggest that benzolamide is likely a membrane permeable CAI.

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Development of a fingerprint scoring function for the prediction of the binding mode of Carbonic Anhydrase inhibitors

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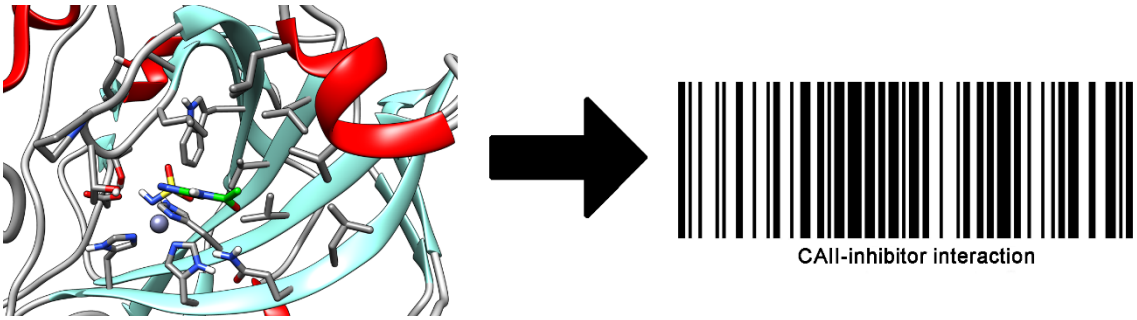
Introduction

The application of docking strategies has become a widespread method for drug lead discovery and optimization. There is a large number of different docking software but they are all characterized by the same strategy: an algorithm searching for possible ligand binding poses and a scoring function predicting which should be the most energetically favored binding pose.

A wide variety of different approaches employed for the development of scoring functions have been reported in literature; however, although the large amount of data about the Carbonic Anhydrase (CA) inhibition, no specific scoring functions for the analysis of CA inhibitors have been developed so far.

Results and discussion

Through the analysis of the ligand interactions detected in all the crystallographic structures of the CAII-ligand complexes, we have developed a fingerprint scoring function specific for the CAII ligands binding pose prediction. Interestingly, the application of this scoring function to the AutoDock4 docking results has led to an improvement in the accuracy of binding pose prediction of about 35%. Furthermore, the application of this scoring function to ligand-CAII complexes can also be used for clustering ligands on the basis of their interactions with the protein, thus providing an alternative approach to the ligand chemical similarity analysis.



Young researchers

Investigation of new analogues of SLC-0111

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Carbonic anhydrases (CAs, EC 4.2.1.1) are a metalloenzyme superfamily presented in all living organisms. CAs catalyze the reversible conversion of carbon dioxide to bicarbonate and protons. Through this simple reaction CAs are involved in physiological and pathological processes such as pH homeostasis, bone resorption and biosynthetic reactions (gluconeogenesis, ureagenesis, lipogenesis, etc.), calcification, edema, glaucoma, obesity, tumorgenicity, arthritis, neuropathic pain and cerebral ischemia [1-2].

CA Inhibitors (CAIs) are clinically used for decades as diuretics, anti-obesity agents, antiglaucoma agents and for the treatment of altitude sickness [1]. An ureido-sulfonamide CAI (i.e. **SLC-0111**) is a selective inhibitor of the tumor associated isoforms CA IX and XII and very recently successfully completed phase I clinical trials to treat solid hypoxic tumors [3]. Our group investigated the potencies of thioureido and selenoureido derivatives of **SLC-0111** [4-5]. As follow up, here we report the novel sulfonamide derivatives possessing aliphatic/ aromatic ureido tails as selective inhibitors of tumor associated isoforms CA IX and XII as promising drug candidates.

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Zebrafish: an animal model for testing of novel carbonic anhydrase inhibitors against multidrug resistant tuberculosis

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Introduction

Zebrafish is considered a suitable model for disease and phenotypic based drug discovery and has been used for studying developmental toxicity and modeling various aspects of human tuberculosis (TB) [1, 2]. We studied the toxicity and *in vivo* inhibition properties of several carbonic anhydrase inhibitors (CAIs) using zebrafish larval model.

Experimental

In the present study, we screened 25 carbamates, 10 coumarins and 10 sulfonamides and their derivatives using 1-5 day post fertilized (dpf) zebrafish. We determined minimal inhibitory concentrations (MICs) of several CAIs using *M. marinum.* Similarly, we determined *in vivo* effects of CAIs for inhibition of *M. marinum* infection in zebrafish larvae.

Table 1. Carbonic anhydrase inhibitor screening in zebrafish.

CA inhibitors	Total[a]	MIC[a]	*In vivo*[a]	Safe[a]	Toxic	LC^{50}
Carbamates	25	8	8	22	3	300-500µM
Coumarins	10	2	-	8	2	125-250µM
Sulfonamides	10	-	2	10	-	15µM-2mM

[a]Indicates number of CAIs tested.

Results and discussion

The toxicity screening showed that 88% of the carbamates, 80% of the coumarins and all the sulfonamides showed no apparent toxicity below the LC_{50} as shown in the table 1. Using F14-584b we successfully inhibited the growth of *M. marinum* in 1-5dpf zebrafish larvae. These results suggest that zebrafish represents an excellent preclinical model for testing both efficiency and toxicity of novel drug candidates.

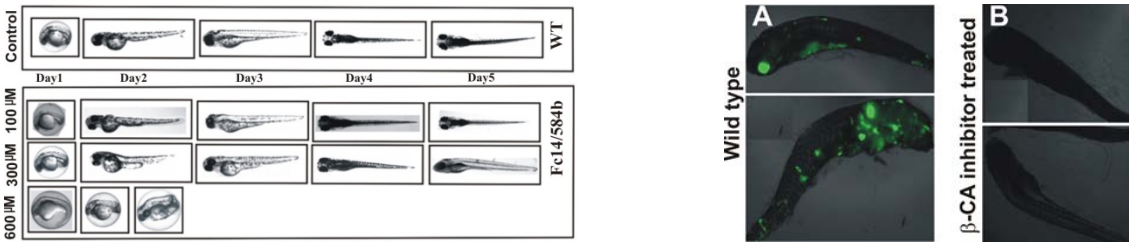

Fig. 1. Example of toxicity and *in vivo* inhibition studies using dithiocarbamate F14-584b.

Conclusions

The toxicity and *in vivo* inhibition studies carried out in our laboratory showed that the zebrafish is a suitable model for developing CAIs against multidrug resistant-TB.

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First activation studies with amines and amino acids of β-, γ-, δ-, ζ- and η-carbonic anhydrases

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Carbonic anhydrases (CAs, EC 4.2.1.1) are an ubiquitous family of enzymes that catalyse the rapid interconversion between CO_2 and water to bicarbonate and protons[1]. These enzymes are grouped in seven genetically distinct families, named α-, β-, γ-,δ-, ζ-, η- and θ-CAs, and although they share a low sequence similarity and protein three dimensional structure, all of them possess a high efficiency as catalysts for the transformation of the metabolically crucial gas CO_2 into soluble products, HCO_3^- and H^+ ions[1]. The metal ion from the CA active site is crucial for catalysis, and the rate determining step in the CA catalytic cycle is the formation of the metal hydroxide species of the enzyme from the acidic one in which a water molecule is coordinated as the fourth ligand to the metal centre. This process is usually assisted by amino acid residues placed in the middle or at the rim of the active site, which can shuttle protons between the metal centre and the reaction medium by means of moieties possessing a pKa in the region of 6–8 pH units[1]. Thus, compounds able to intervene in such proton transfer processes are known as CA activators (CAAs) and they were rather well investigated for mammalian α-CAs[1], but much less for the other families of CA. The main interest of this study is to understand the role activation of β-, γ-, and η-carbonic anhydrases from different phatogenic bacteria[2-4] for a better understanding factors connected to the invasion and colonisation of the host during infection. Moreover, the activation of δ-, ζ-CA from diatoms[5] may lead to a more complete understanding the role of nature amines and amino acids in the modulation of CO_2 fixation in phytoplankton.

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Computational analysis of transcription factor binding sites in cancer related carbonic anhydrases

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Motivation

Transcription factors (TFs) are the primary actors of gene expression and regulation, and a genome-wide understanding of their targets would have significant benefits in many biological studies. However, due to the time and monetary cost of direct experimentation we possess little knowledge as to which TFs initiate the expression of most genes. Computational prediction of these sites bypasses these significant hurdles at the cost of accuracy. One reason for this inaccuracy is that due to the large size of our genome, and relatively small binding site size for most TF proteins, there are many sequence locations that satisfy TF binding requirements. Conservation of nucleotides, or binding likelihood in this case, across orthologous sequences of related species represents an expenditure of energy which implies some level of functionality. α-carbonic anhydrases are a protein family which are present in humans and fulfill a variety of roles dependent on their catalysis of the reversible reaction of hydration of carbon dioxide to bicarbonate and H^+. Importantly, several members of this family are directly involved in, or markers of, a variety of human cancers.

Results

We have analyzed the promoters of all 76,165 Ensembl database annotated human protein-coding transcripts, from 18,317 genes, for which there is sequence data for multiple mammal species. The conserved TFBSs results are presented graphically and in tables in our database TFBSs.org. We present a brief analysis of the promoters of the cancer-associated carbonic anhydrase proteins.

Availability

Freely available on the web at www.TFBSs.org. The website is implemented in MySQL, PHP, and WordPress, with all major browsers supported. The custom tools used to perform all analyses are available at https://github.com/thirtysix/TFBS_footprinting.

Deciphering the mechanism of human carbonic anhydrases inhibition with sulfocoumarins: synthesis, inhibition and computational studies of novel 6- and 7-substituted benzo[e][1,2]oxathiine 2,2-dioxide

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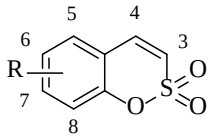

The discovery of the coumarin bioisosters 1,2-benzoxathiine-2,2-dioxides, also named sulfocoumarins, as novel and selective inhibitors of the zinc metalloenzymes α-carbonic anhydrases (CAs, EC 4.2.1.1) is the result of intense efforts towards the development of innovative compounds as potential tools for the treatment of various diseases.[1,2] In analogy to the coumarins, the sulfocoumarins are not *per se* able to inhibit the CA activity. Instead they interfere with the enzyme by acting as efficient substrates for the esterase activity, generating the actual inhibitor *in situ*.[2]

Herein the reaction mechanism of the carbonic anhydrase-mediated hydrolysis of sulfocoumarins to sulfonic acids has been investigated on an enzyme cluster model using the B3LYP hybrid density functional theory (DFT) and the QST procedure for the Transition State (TS) search.[3] A multistep process was highlighted, with the rate determining step identified in the initial dual nucleophilic/acidic attack of the zinc bound-hydroxide ion to the sulfocoumarin sulfur atom and to the C3-C4 double bond. The reported multi-step process, combined to SAR analysis on a new set of derivatives, highlighted unprecedentedly considered mechanistic aspects of the CA-mediated prodrug activation, which in turn do possess relevant consequences to the isoforms-selective inhibition profiles reported by such a class of compounds.

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Synthesis and Evaluation of Carbonic Anhydrase Inhibitors with Carbon Monoxide Releasing Properties

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Carbon monoxide (CO) is a gas endogenously produced through heme catabolism and is classified as a vital cell signalling mediator and regulator [1]. CO has been reported to induce vasorelaxation and exhibits anti-inflammatory as well as cytoprotective effects [1]. Gaseous CO is difficult in handle, and *di per se* doesn't show selectivity against any tissue or biochemical target. In this context the controlled and site-specific delivery of CO has reached enormous interest among the scientific community. In particular transition metal carbonyls (i.e. Co) have emerged as the preferred candidates also in consideration of the straightforward preparation procedures and identification [2]. Human Carbonic Anhydrase (hCA) is an ubiquitous enzyme present in fifteen active isoforms in human body [3]. Some of them are constitutively expressed, whereas others are inducible: for example, CA IX is overexpressed in various pathological conditions connected with ischemia, such as tumors, fibrosis, vascular remodeling and inflammation [4]. All these conditions are also sensitive to the action of CO. Here, we report for the first time a series of CORMs consisting of a Carbonic Anhydrases inhibitor (CAI) entity and a transition metal carbonyl centre based on cobalt. All the compounds have been characterized and tested for their *in vitro* inhibition activity against hCA I, II, IV, IX, XII. Some of these derivatives showed a good selectivity profile against the isoforms of interest (IV, IX and XII) and so their CO releasing profile have been investigated by means of the standard myoglobin assay [5].

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2-Benzylpiperazine: a new scaffold for potent human Carbonic Anhydrase inhibitors. Synthesis, enzyme inhibition, enantioselectivity, computational and crystallographic studies and in vivo activity for a new class of intraocular pressure lowering agents

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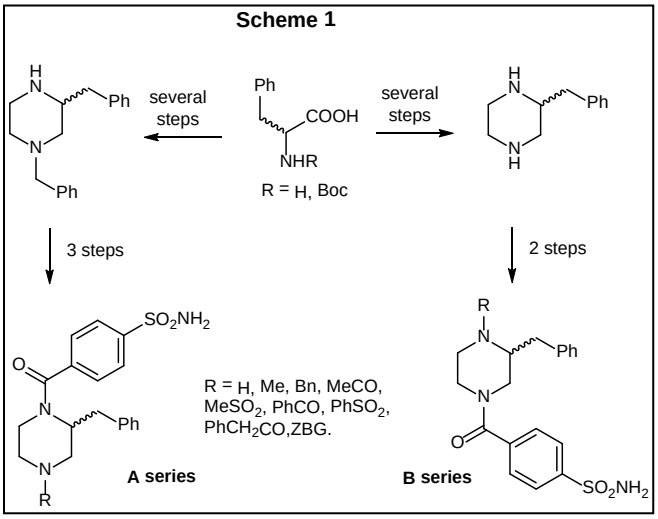
The hydration of CO_2 into bicarbonate and protons and the optimal equilibrium between these chemical species is essential for the vitality of organisms in all life kingdoms [1]. This reaction is catalyzed by the metalloenzyme Carbonic Anhydrase (CA), one of the most efficient enzyme known in nature, evolved in seven genetically different families (α-θ). A large number of isoforms are described among the different organisms, their presence being crucial for pH regulation, secretion of electrolytes and for other essential physiological or pathological processes [2]. For these reasons, CAs are important targets for drugs that can be used for different pathologies, providing that it could be possible to exploit the existent differences between families or isoforms to achieve a selective activity. This may not be an easy task, since in human the coding genes are well conserved between the sixteen existing α isoforms (I-XVI), creating a very similar catalytic site.

Aiming to find new potent and selective CA inhibitors, two series of 2-benzylpiperazines (**A** and **B, scheme 1**) have been prepared and tested for the inhibition of four physiologically relevant isoforms of human-CA I, II, IV and IX. Thes new compounds are decorated on one N atom a sulfamoylbenzoate group as Zinc-chelating moiety, and on the other different alkyl/acyl/sulfonyl groups. The synthesis started from from D and L-Phenylalanine, through the intermediate compounds 2-benzyl- and 1,3-dibenzyl-piperazine [3, 4]. The majority of these compounds showed Ki values in the low-medium nanomolar range against hCA I, II and IV, but not IX. The binding mode has been studied by means of X-ray crystallography and molecular modelling. Two compounds have been evaluated in rabbit models of transient and stable glaucoma, showing a significant reduction in intraocular pressure.

Scheme 1



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The role of non-covalent interactions in design of potent and selective inhibitors of carbonic anhydrases

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Introduction

The objective of this study is to decipher the causes of CA IX/II selectivity of some of its inhibitors from an *in silico*, structure-based drug design perspective, since hCA IX was known to correlate with oncogenesis in humans, while CA II inhibition is undesirable. One new chemotype, sulphocoumarin, has become a promising selective CA inhibitor class. Numerous studies have attempted to optimize this lead using various "tails" *via* click chemistry. [1–7] However, until now there was no systematic target-oriented study to explain the CA IX/II selectivity of this chemotype, as well as its inhibition mechanism. This work aims to address this using *in silico* methods: molecular docking and molecular dynamics.

Experimental

Grid-based induced-fit and rigid molecular docking analyses were performed on a series of ligands from coumarins and sulphocoumarins chemotypes to determine the best docked conformation of the ligands in CA II (PDB ID: 3KS3) and CA IX (PDB ID: 5DVX) receptors. The select resulting best poses were then analysed *via* 20 ns molecular dynamics simulation to check for the non-covalent interactions indicated from the docking results.

Results and Discussion

Molecular docking study performed in this study revealed that the formation of hydrogen bonds with **(i) hydroxide anion bound to the zinc prosthetic group, and (ii) His200, which was responsible for the proton-transfer shuttle process, as well as (iii) π-π stacking with His226**, were important in terms of potency against hCA IX. **These interactions were often absent in hCA II protein-ligand complexes, thus leading to the hCA IX/II selectivity.** The results also revealed that several protein-ligand complexes formed novel interaction of halogen bonding with residues of hCA IX. Furthermore, trajectory analyses from MD simulations confirmed the stability of the protein-ligand complexes and confirmed the hypotheses from molecular docking results.

Conclusions

In conclusion, this study provided structural insights to guide further lead optimization process for drug development targeting hCA IX. For better description of halogen bonding, the extra-point charge model could be implemented, but it is out of the scope of this paper and a part of future work.

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Targeting Carbonic Anhydrase-IX as a novel approach to overcome hypoxia-mediated drug resistance in FLT3/ITD mutated AML

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Introduction

FLT3/ITD$^+$ AML is a highly aggressive form of leukemia that carries a dismal prognosis [1]. Evidence suggests that this poor outcome is due to the survival of a subset of leukemic cells (LCs) that elude therapy and survive in hypoxic niches of the bone marrow (BM) [2,3]. Effective targeting of LCs under hypoxic conditions is thus critical to cure AML. Our study examines the anti-leukemic potential of targeting Carbonic anhydrase-IX (CA9), a transmembrane protein which functions to maintain a neutral intracellular pH (pH_i) under hypoxic stress conditions [4,5], against AML cells. Targeting CA9 is of particular interest as its expression is confined to only a few normal tissues [6] and may therefore show relatively few side effects compared to standard therapies.

Experimental

Molm14 (M14) and primary cells (PC) from FLT3/ITD$^+$ AML patients were incubated under normoxic (21% O_2) and hypoxic (1% O_2) conditions in the presence or absence of Quizartinib (Q), Cytarabine (Cy) or the CA9 inhibitor FC531 (FC). After 48h proliferation and apoptosis were determined per MTT assays, annexin V/PI staining and FACS analysis. Cells were assessed for CA9 mRNA expression as well as pH_i levels via RT-PCR and fluorescent imaging, respectively. FLT3/ITD$^+$ AML xenografts were generated by injecting M14 cells in the tail vein of NSG mice. Tissue samples were fixed in 10% NBF, embedded in paraffin and stained with H&E and CA-IX for pathologic evaluation.

Results and discussion

Cy and Q were significantly less effective against M14 cells under 1% compared to 21% O_2. CA9 mRNA was upregulated in M14 cells (4.5±0.9-fold; n=4, $p<.01$) and in FLT3/ITD$^+$ AML PCs from newly diagnosed (n=5) and relapsed/ refractory (n=3) patients (up to 529- and 73-fold, respectively) under 1% compared to 21% O_2. Immuno-histochemical staining of the BM and spleen obtained from AML xenografts showed multifocal CA-IX staining of LCs that was localized on the cell membrane. Under hypoxic conditions, FC but not Cy or Q, significantly induced apoptosis and showed dose-dependent growth inhibitory effects against M14 cells (n=3). In addition, treatment of M14 cells with FC under 1% O_2 resulted in strong pH_i acidification ($\Delta pH=-2.7\pm0.6$, $p<.05$, n=3). Moreover, FC combined with Cy synergistically inhibited M14 cell growth *in vitro* and effectively eliminated LCs from the BM and spleen compared to Cy only treated mice. Accordingly, spleen size and weight were significantly reduced in mice treated with FC and Cy compared to single agent Cy (n=3).

Conclusions

1. Hypoxia blunts the effects Cy and Q in FLT3/ITD$^+$ AML. 2. CA9 is induced in FLT3/ITD$^+$ AML cells under hypoxic conditions. 3. The CA9 inhibitor FC confers anti-leukemic activity against FLT3/ITD$^+$ AML cells under hypoxic conditions via pH_i

acidification and acts synergistically with Cy. 4. CA9 inhibition has the potential to target LCs residing in hypoxic niches and may be of value as an adjunct to chemotherapy for FLT3/ITD^{+}AML therapy.

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Poster communications

New Perspectives in Rheumatoid Arthritis Treatment

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On the basis of the use of small molecule hybrids composed of Nonsteroidal-Anti-Inflammatory-Drugs and Carbonic-Anhydrase-Inhibitors of the coumarin type (NSAIDs−CAIs) as *proof-of-concept* for the management of ache symptoms associated to inflammatory diseases such as rheumatoid arthritis (RA), [1,2] herein the new perspectives in this field will be discussed. In particular this contribution will consider state-of-the-art paharmacological approaches based on the use of small molecules as well as the latest technological applications.

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Targeted Drug Delivery of Carbonic Anhydrase IX liposomes Mediated by a Novel Peptide for Pancreatic Cancer Therapy

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Carbonic anhydrase isoform IX (CA IX) is highly overexpressed in many types of cancer where its expression is induced by hypoxia-inducible factor 1 (HIF1). CA IX plays a crucial role for tumour growth and metastasis, including pH regulation, survival and adhesion/migration. The pivotal functions of CA IX in tumours under hypoxia together with its tissue-specific expression have evoked interest in CA IX as a potential target for anticancer therapy. A broad range of small molecular inhibitors specifically inhibiting CA IX have been developed, including the sulfonamide inhibitors and its isoesters sulfamates. Ureido- substituted sulfamate (S4) that was synthesized with high selectivity for CA IX. S4 has shown significant anti-proliferative efficacy in vitro in a panel of breast cancer cell lines [1, 2]. The combination of current cancer therapeutics with S4 has improved the therapeutic outcome in small cell lung cancer [3]. However, S4 has poor water solubility and low bioavailability, which affects its potential therapeutic use. One approach to tackle this limitation is to encapsulate S4 into liposomes. liposomes are considered to be the most successful drug-carrier system due to their biocompatibility, biodegradability, low toxicity, and the ability to trap both hydrophilic and lipophilic drugs [4]. To further enhance targeting S4 loaded liposomes, a peptide ligand will be attached to the liposome surface. We have synthesized a pentaptide that bind to the neuropeptide receptor :Gastrin Releasing Peptide Receptor (GRPR), which is expressed in numerous cancers [5]. The ligand peptide with D Trp (N-butyl) modification displayed a significant enhancement in cytotoxicity against small cell lung cancer [6]. This dual targeting approach will enhance the solubility, stability and achieve better accumulation of S4 into CA IX and GRPR expressing pancreatic cancer cell lines. S4 was successfully loaded into liposomes and we are currently evaluating its effectiveness in a range of pancreatic cell lines.

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Synthesize of new Histamine Schiff base derivatives as an isoform-selective and potent carbonic anhydrase VII activators

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Introduction

Carbonic anhydrase has been a therapeutic target for many years and their inhibitors are clinically used/investigated as diuretics, anticonvulsant, antiobesity, antiglaucoma and more recently antitumor and anti-infective agents. However, the activators of CAs (CAAs), although investigated simultaneously with inhibitors, do not have pharmaceutical applications, yet. Indeed, it has been proposed that some CAAs might have applications in the neurodegenerative disorder of memory and cognitive function (Alzheimer disease) since it has been shown the level of brain CAs significantly diminished in the brain of Alzheimer disease and older rats as compared to normal and young brain of animals.

Experimental

In this study, a series of twenty new histamine Schiff base derivatives were synthesized by reaction of histamine (well known carbonic anhydrase activator pharmacophore) with substituted aldehydes. The histamine Schiff base derivatives **H(1-20)** were obtained by condensation of histamine with aromatic/heterocyclic aldehydes in EtOH and assessed as an activators of several selected CA isozymes which are hCA I, hCA II, hCA IV, hCA VII and hCA IX.

Results and discussion

The histamine Schiff base derivatives were obtained and the CA activation assay proved an excellent activation profile for the new compounds with activation constants K_A as long as 1.8 nM. The structure-activity relationship for activation of these isoforms with the new histamine Schiff base derivatives will be discussed in detail.

Conclusions

All the compounds showed a better potency than the histamine against isozymes CA I and CA VII with a distinct activation profile. Many of the compounds showed nanomolar potency against isozyme CA VII (K_As in the range of 6 nM to 24.6 µM) which is a key CA isoform involved in brain metabolism.

Acknowledgement

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Keywords

Alzheimer, Carbonic Anhydrase Activators, Isozymes, Histamine, Schiff bases

Increasing Lipophilicity of Inhibitors Induces Conformational Changes in Human Carbonic Anhydrase II and IX

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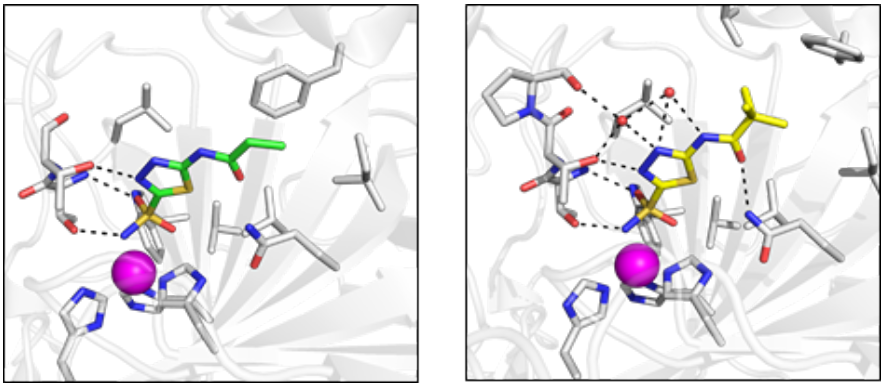
Introduction

Human carbonic anhydrase IX (CAIX) is a widely-studied cancer therapy target due to its elevated expression in cancer cells. Previous studies have shown that knockdown or inhibition of CAIX reduces cancer growth and invasion [1]. Specific inhibitors for human carbonic anhydrase IX are needed to obtain a therapeutic effect while not inhibiting or interfering with the other CA isoforms. The first step toward selective inhibitors is identifying unique residues between the isoforms and how they interact with drug moieties. This work examines the effects of a series of inhibitors with lengthening lipophilic tails to identify the effect of steric hindrance on the surrounding residues of the active site, specifically residues 91 and 131.

Experimental

CAIX mimic and CAII were co-crystallized with a series of 5 sulfonamide based inhibitors with a growing lipophilic tails named I1 through I5. Data was collected at -180ºC at Cornell High Energy Synchrotron Source (CHESS) and Stanford Synchrotron Radiation Lightsource (SSRL), using a Pilatus 6M detector.

Results and discussion



Here we describe the interactions of these inhibitors in the active sites of human CAII and CAIX. In general, the addition of lipophilic groups induces a conformational change in human CAII, shifting residue P13. Larger, 3D tails induce movement of this residue, while planar groups and rings retain the phenylalanine in its native state.

Conclusions

This work shows the lengthening tail results in additional interactions in both isoforms while moving bulky residues in the human CAII active site.

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Organochalcogenide as new modulator of Carbonic Anhydrase activity

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During the last decades, organochalcogenide compounds were the subject of intense interest, especially from the point of view of public health [1]. Oxidative stress is known to activate several transcription factors including hypoxia inducible factor HIF-1α, peroxisome proliferator-activated receptor (PPAR)-γ, both of which modulate the expression of numerous genes involved in immune and inflammatory responses, carcinogenesis, and metastasis. In this context, selenium has a long history of association with human health and it plays an important role in biological systems as part of the active site in many proteins [2]. The unique redox properties of selenium may also be considered a double-edged sword in terms of possessing antioxidant as well as pro-oxidant properties that may be beneficial or harmful depending upon the form and dose being used in a normal or cancer setting [3]. In the last years, organoselenium derivatives were studied as inhibitors of carbonic anhydrases (CA, EC 4.2.2.1) and they showed antioxidant, antitumor [4] and antiviral [5] action. On the other hand, Tellurium is a rare element and, unlike the other group VI members (e.g., O, S, and Se), which have many biological applications, this element has not any known such function [6]. At the same time, a range of organotellurium compounds were developed, being shown that they possess a range of unique properties such as interesting activity against pathogenic microorganisms, inhibition of cancer cells growth and antioxidant activity, which are often superior to those of the selenium analogues [7]. In this particular contest, different scaffolds contain chalcogen atoms were tested *in vitro* for their inhibition activity against the physiologically relevant hCAs and, some of these isoforms are involved in serious diseases, thus leading to possible applications as therapeutic agents for many chalcogen-containing derivatives [8,9].

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Is three better than two? Structural development of the tail approach by incorporation of three tails on benzenesulfonamide scaffolds.

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Being sulfonamides the best zinc-binding group to design inhibitors of the metallo-enzyme carbonic anhydrases (CAs, EC 4.2.1.1), the main drawback that prevents a wider use as therapeutic agents is the significant lack of selectivity against human isoforms of the enzyme.[1,2] To overcome this issue, the "tail" approach was proposed. It consists in appending proper moieties (tails) to the main inhibitory scaffold of the ligands in order to diversely interact with the middle and outer rim of the binding site pockets, which are the most variable among the 15 human isoforms identified to date.[1,3] An extension of this approach has been developed, with two tails of varied nature being concomitantly incorporated to aromatic sulfonamide scaffolds to enhance interactions with the two-halves divided hCAs binding site (hydrophobic and hydrophilic).[4] In the present study, we propose the incorporation of an additional tail designed *in silico* as tool to efficiently fill the complex and diverse architecture of the different hCAs active sites. Varied combinations of molecular fragments with variable hydrophilic and/or hydrophobic features were considered to enhance ligand/target complementarity, with *in vitro* inhibition studies performed against 5 hCAs, namely I, II, IV, IX and XII. Crystallographic studies further established the potential of the three tail-approach to develop isoform-selective inhibitors as candidates for the treatment of diseases, such as glaucoma, stroke, epilepsy, arthritis, and tumors.

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Design, synthesis and stability study in plasma of New Nonsteroidal Anti-Inflammatory Drugs and Carbonic Anhydrase Inhibitors Hybrids

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Rheumatoid arthritis (RA) is a chronic inflammatory disease caused by a faulty autoimmune response [1,2]. Recently, it was reported that some human carbonic anhydrases (CAs) isoforms are overexpressed in inflamed synovium of RA patients [3,4]. The new CA inhibitors (CAI) incorporating both the CA-binding moiety and the cyclooxygenase inhibitor tail (NSAID type) [1]. The NSAID−CAI hybrids were shown to be highly promising tools for RA treatment in an animal model of the disease [1]. The aim of this work is the evaluation of the chemical stability of NSAID−CAI hybrids towards spontaneous or enzymatic hydrolysis (phosphate buffer solution, human and rat plasma) by application of appropriate LC-MS/MS methods [5]. Since the panel of NSAID−CAI hybrids are positional isomers pairs of 6-hydroxycoumarin or 7-hydroxycoumarin, a mathematical tool (LEDA) to distinguish the isomers was developed. The LEDA algorithm was applied, also from unresolved chromatographic peaks, to assign the correct abundance of the isomer present in the sample at ng mL^{-1} level [5]. Its reliability was checked (>90%), being proved the effectiveness that allows the correct assignment of the isomer present in the sample and ensuring its proper monitoring. The proposed LC-MS/MS approach was suitable for the determination of studied compounds at ng mL^{-1} level in PBS or human or rat plasma samples, for describing their degradation profiles [5].

Since the NSAID−CAI hybrids linked by an amide linker resulted stable in all the tested matrices and therefore likely reach the target tissues unmodified, an additional set of hybrid compounds containing NSAIDs and Coumarin portion were designed and synthesized by bioisosteric substitution of the amide moiety with the ester one.

Promising data open new perspectives regarding a further development of these compounds as anti-inflammatory drugs.

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The crystallographic structure of human ca vii provides insights into the complicated puzzle of carbonic anhydrase catalytic mechanism

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Carbonic Anhydrases (CAs) are ubiquitous metallo-enzymes present in most living organisms [1]. In humans fifteen isoforms have been identified, which differ for tissue distribution, catalytic activity and cellular localization. These enzymes catalyze a simple but fundamental physiological reaction, the reversible hydration of CO_2 to HCO_3^- and H^+, following a two-step mechanism. The first step consists of the nucleophilic attack of the Zn^{2+}-bound hydroxide on CO_2 with consequent formation of HCO_3^-. The second step, which is the rate-limiting one, consists of the regeneration of the zinc-bound hydroxide, through a proton transfer reaction from the zinc-coordinated water molecule to the bulk solvent [2]. In the majority of the human (h) CA isoforms, a histidine residue placed in the middle of the active site cavity, namely His64, assists this step acting as a proton shuttle [3]. Among the different hCAs, we recently focused our attention on hCA VII, an enzyme mainly present in brain, where it functionally participates as a molecular switch for GABAergic excitation [4]. Catalytic assays demonstrated that this enzyme is able to catalyze the CO_2 hydration reaction, with slightly less efficiency compared to the most active isoform, i.e. hCA II [5]. Thus, with the aim to understand the structural factors responsible of the lower catalytic efficiency of hCA VII with respect to hCA II, we have undertaken a detailed structural study and theoretical pKa calculations on a hCA VII variant [6]. In particular, our studies showed that His64 in CA VII presents a higher pK_a with respect to that of the same residue in hCA II inducing a predominant *out* conformation of its side chain. Consequently the network of ordered water molecules, which connects the zinc bound solvent molecule to the proton shuttle His64, is altered, making less efficient the catalytic mechanism.

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Personalized therapies in cancer: Carbonic anhydrase peptide- based motifs as templates for biomarkers

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Carbonic anhydrase (CA) plays a major role in abnormal cellular proliferation and might serve as a novel biomarker for cancer [1]. Cellular level studies of several CA isoforms for CA inhibition potential had shown the role of CA in cancer pathways. Distinct tumor-associated CA isoforms identified in humans may be targets for potential approach in cancer therapeutics. The main objective of study is to develop carbonic anhydrase based biomarkers with use in detection and therapy of cancer. Novel biomarkers of CA isoforms based on gene data and experimental results retrieved form literature to model a 3D protein sequence were developed using computational strategies [2]. Descriptors used to describe each CA (I-XIV) were computed using Schrodinger, Mathematica software. In computing these descriptors, each CA isoform was converted into a molecular graph using HyperChem and TopoCluj software. A prediction model was developed using multiple linear regression. Specific activity of human CA (s^{-1}) was set as dependent variable; the activity for each CA isoform (I-IXIV) was retrieved from literature [3]. The proposed model showed reliable predictive ability. A scoring algorithm was designed to elect specific therapy for each case.

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New SLC-0111 enaminone analogs as selective subnanomolar inhibitors of the tumor-associated carbonic anhydrase isoform IX

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Herein we present the design, synthesis, and biological evaluation of novel series of sulfonamides (**5a-o**) incorporating substituted aryl moieties (as tails) linked to benzenesulfonamide (as zinc anchoring moieties) through enaminone linker. The synthesized sulfonamides were evaluated *in vitro* for their inhibitory activity against the following human (h) carbonic anhydrase (hCA, EC 4.2.1.1) isoforms, hCA I, II, IV and IX. All these isoforms were inhibited by the sulfonamides reported here in variable degrees. hCA I was inhibited with K_Is in the range of 9.3–>10000 nM, hCA II in the range of 0.48–472.8 nM; hCA IV in the range of 6.5–>10000 nM, whereas hCA IX in the range of 0.21–7.1 nM.

Heterocyclic analogues of sulfocoumarin as a potent inhibitors of carbonic anhydrases

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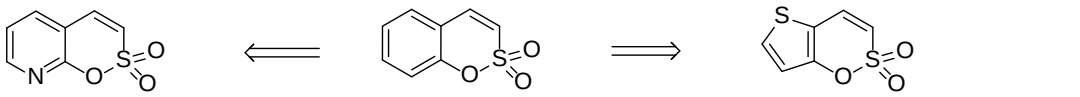
Carbonic anhydrases (CA) are ubiquitous zinc containing enzymes which catalyze reversible hydration of carbon dioxide and provide pH regulation and CO_2 transport in cells. Looking for selective inhibitors of tumor associated CA IX and CA XII, good inhibitory activities were demonstrated for sulfocoumarin derivatives [1, 2]. In a search for new CA IX and CA XII inhibitors we turned our attention to heterocyclic analogues of sulfocoumarin – thieno[2,3-*e*][1,2]oxathiine 2,2-dioxides and [1,2]oxathiino[6,5-*b*]pyridine 2,2-dioxides



[1,2]oxathiino[6,5-*b*]pyridine 2,2-dioxide Sulfocoumarin Thieno[2,3-e][1,2]oxathiine 2,2-dioxide

The synthesis of sulfocoumarin heterocyclic analogues - thieno[2,3-*e*][1,2]oxathiine 2,2-dioxides and [1,2]oxathiino[6,5-*b*]pyridine 2,2-dioxides will be discussed.

Acknowledgements
We would like to thank to ERDF project Nr.1.1.1.2/VIAA/1/16/235 for financial support.

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Specific carbonic anhydrase inhibitors effect on hypoxic cells – a quantitative assay

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Introduction

Dynamic cell-based biosensing platforms were proven [1] to complement cell-free and end-point analyses, and support the process of design and evaluation of proposed inhibitors of Carbonic anhydrases, important orchestrators of hypoxic tumour environment fostering selection of effective compounds towards valid therapeutic targets.

Experimental

We assess the effectiveness of recently emerged CA IX inhibitors (sulfonamides and sulfocoumarins) and their antitumour potential using a novel biosensing platform [1,2].

Results and discussion

According to Fig. 1 both the continuous cell dynamics and early time point (6 h) assessment were demonstrated to enable quantitative assessment of specific CAIs effect on live hypoxic cells. The analysis allows discriminating between the inhibitory capacities of the compounds and their inhibition mechanisms.

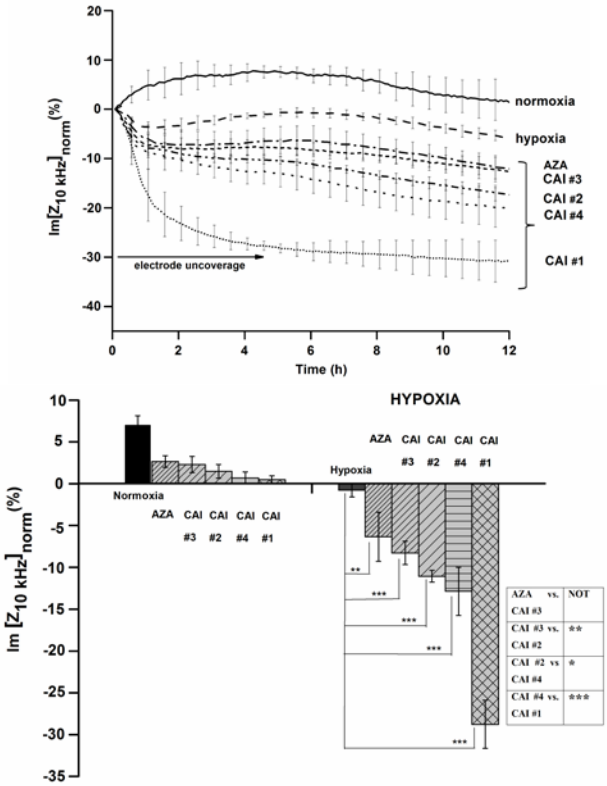

Fig. 1. (A) The dynamics of impedance data during hypoxia as function of CAIs exposure (100 μM). (B) Characteristic impedance changes after 6 h of treatment revealing CAIs potency under normoxia and hypoxia. mean ± SD for n=6. Inset statistical significance of impedance data for discrimination between CAI's potency *p<0.05, **p<0.01 and ***p<0.001 vs. specific inhibitors based on paired t-test.

Conclusions

This study bridges the gap between classic end-point analyses and dynamic cell assessment and highlights process parameters relevant for assessing CA inhibition effectiveness. It demonstrates exquisite sensitivity of electric cell substrate impedance for the evaluation of CAIs potency in preclinical testing of novel anticancer drugs targeting CA.

Acknowledgements

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A novel library of saccharin and acesulfame derivatives as potent and selective inhibitors of carbonic anhydrase IX and XII isoforms

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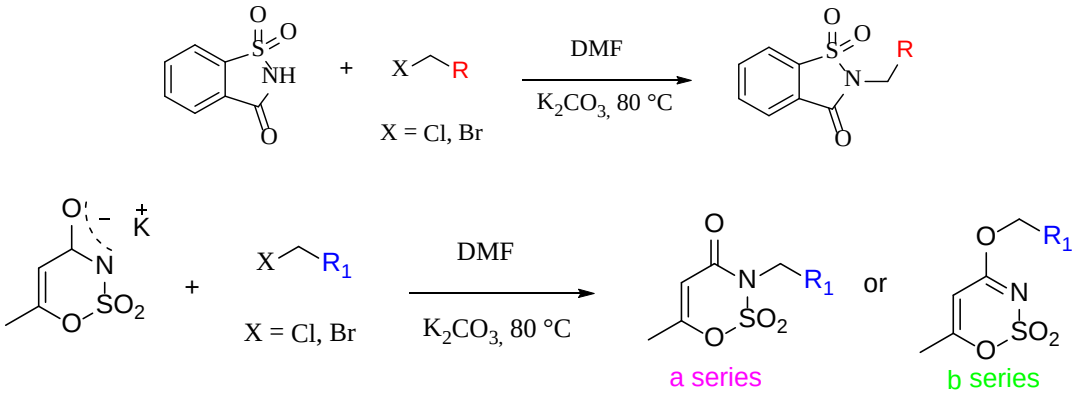
Nowadays, the involvement of human carbonic anhydrase isoforms IX and XII in tumorigenicity have been recognized, therefore, selective inhibition of these enzymes is emerging as a promising therapy tool for the treatment of solid tumors. Classical inhibitors of human carbonic anhydrases (hCAs) possess primary or secondary sulfonamide moiety as zinc binding group that gives potent inhibitory activity but, on the other hand, frequently reduces isoform selectivity. With this in mind, we developed saccharin and acesulfame derivatives (respectively tertiary sulfonamide and tertiary sulfamate), that showed inhibitory activity mainly on isoforms IX and XII of hCA [1,2].

With the aim to increase our knowledge about these molecules, we synthesized (**Scheme 1**) and tested a new library of *N*-substituted saccharin and *N*-/*O*-substituted acesulfame derivatives as atypical and selective inhibitors of four different isoforms of human carbonic anhydrase (hCA I, II, IX and XII).



Scheme 1. Synthesis of novel saccharin and acesulfame derivatives.

All these compounds inhibited hCA XII in the low nanomolar range (3.9 nM ≤K_{Is}≤ 340 nM) and hCA IX between 19 and 2482 nM, whereas they were poorly active against hCA II (K_Is >10 µM) and hCA I (K_Is ranging between 318 nM and 50 µM). In the light of the above, these scaffolds confirm the activity and selectivity towards the two tumor-related isoforms because most of the compounds were not active towards hCA I and II, while they were very effective against hCA IX and XII. A molecular modelling approach was also applied to corroborate these data.

These derivatives could represent an important starting point for the development of new antitumor agents based on the atypical inhibition of the cancer-related isoforms of human carbonic anhydrase overexpressed in hypoxic tumors.

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Salvianolic Acids From the Roots of *Salvia miltiorrhizza* as Effective Carbonic Anhydrase Inhibitors

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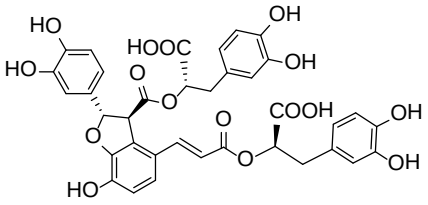
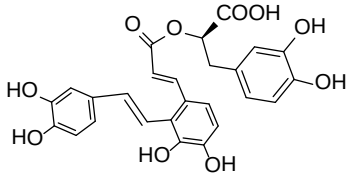
Salvia miltiorrhiza Bunge (Lamiaceae) is a very important herbal drug of traditional Chinese medicine [1]. Among the active principles are the hydrophilic depsides which constitute up to 4–5% of the dried herbal drug. They are derivatives obtained by condensation of caffeic acid units or by combination of caffeic acid plus 3,4-dihydroxyphenyl lactic acid in the form of dimers, trimmers, and tetramers. Main representatives are Salvianolic B and its salts with magnesium and ammonium potassium, Salvianolic acid A, Lithospermic and Rosmarinic acids. In the framework of our research for natural products with carbonic anhydrase inhibitory activity, salvianolic acids were selected based on the observation that simple caffeic acid is an effective CA inhibitor [2].

A fast isolation protocol combining adsorption resins and size exclusion chromatography on Sephadex LH 20 was developed. Targeted isolations were achieved by means of HPLC-DAD-MS and compound identification was confirmed by 1 and 2D NMR. The compounds were tested for their *in vitro* inhibition of human (h) CA isoforms hCAs I, II, IV, VII and XII, as well as β-carbonic anhydrases from *Malassezia globosa*. A stopped flow carbon dioxide hydration assay was performed.

Salvianolic acids A and B were active against the hCAIV with K_I values 66.6 and 65.5 nM, respectively, comparable to the standard AAZ (acetazolamide) with K_I of 74.0 nM. The tumor related HCA XII was efficiently inhibited by Lithospermic acid with K_Is of 4.8 nM (vs AAZ 5.7nM). Most importantly, all compounds were efficient inhibitors of the fungal beta MgCA with K_I at the range of 0.9-3.3 μM 20-80 fold higher than AAZ (74 μM). Further investigations are needed in order to elucidate the binding modes of the selected compounds on the appropriate hCA isoforms.

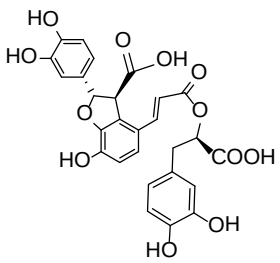


Salvianolic acid B



Salvianolic acid A

Lithospermic acid



Acknowledgements
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Novel benzenesulfonamides as selective carbonic anhydrase IX inhibitors exhibit functional effects to reduce hypoxia-induced acidification and clonogenicity in cancer cell lines

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Introduction

Human carbonic anhydrase (CA) IX has emerged as a promising anticancer drug target and an attractive diagnostic biomarker for a broad range of hypoxic tumors.

Experimental

Affinities of novel benzenesulfonamides VR16-09, VD11-4-2, and VD12-09 toward 12 human CA isoforms were determined and their crystallographic structures in complex with CA IX were solved. CA IX and CA XII expression levels were assessed in HeLa, H460, MDA-MB-231, and A549 cells using Western blotting. The impact on hypoxia-induced extracellular acidosis was evaluated by measuring the pH of culture medium and by mass spectrometric gas-analysis. EC_{50} viability ratios were determined by Alamar Blue assay. Non-hypoxic and hypoxic H460 3D spheroids were exposed to VR16-09 for 24 h and plated for clonogenic survival.

Results and discussion

Compounds exhibited K_d values toward CA IX of 160 pM, 50 pM, and 1.1 nM for VR16-09, VD11-4-2[1], and VD12-09[1], respectively. VR16-09 was the most selective for CA IX. Crystal structures of CA IX with VD11-4-2 and VD12-09 showed a conventional coordination bond between the sulfonamide amino group and Zn(II) in the active center of the enzyme. Western blot analysis demonstrated increased CA IX expression levels in response to hypoxia in all investigated cell lines. Studies of mass spectrometric gas-analysis revealed nanomolar IC_{50} values for all 3 tested inhibitors to inhibit extracellular CA activity in cell suspensions. Compounds significantly reduced hypoxia-induced extracellular acidification in a dose-dependent manner ($P<0.05$), whereas the effect on extracellular pH under normoxic conditions was negligible. This functional effect was the most pronounced for VR16-09 at 50 µM dose. Cell viability EC_{50} values were lower in normoxia than hypoxia in 2D cells. These results correlate with previously published cytotoxicity profiles of benzenesulfonamides, including SLC-0111, which showed the more effective cell kill in normoxia than hypoxia[2]. In contrast, hypoxia-dependent effects on clonogenic survival of the lead compound VR16-09 were observed in H460 3D spheroids.

Conclusions

Novel benzenesulfonamides exhibited high affinity and strong selectivity toward recombinant human CA IX and reached nanomolar functional effect in hypoxic cancer cells.

Interestingly, hypoxia-dependent reduction of clonogenicity was observed only in spheroids, highlighting the importance of testing CA IX-targeting compounds in 3D cell models resembling naturally occurring hypoxic microenvironment with clonogenic survival as endpoint. Overall, our newly designed compounds appear to be promising agents for CA IX-specific therapy.

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Sweetener-Based Inhibition of Carbonic Anhydrase

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Introduction

Carbonic Anhydrase IX (CA IX) overexpression aids in the regulation of pH in hypoxic tumors and is therefore recognized as a therapeutic target for the treatment of various cancers. Building upon recent studies that demonstrate the selectivity of sweeteners for CA IX, the X-ray crystal structure of CA IX-mimic in complex with sucralose is presented. Furthermore, this structure is compared to sucrose, saccharin, and acesulfame potassium binding in order to determine active site properties of CA IX that promote selectivity.

Results and discussion

CA IX-mimic crystals in complex with sucralose diffracted to 1.5Å resolution. Sucralose was determined to bind at the entrance of the active site, stabilized through hydrogen bonds with hydrophilic active site residues (Fig. 1A). Inhibition is hypothesized to result from obstruction of substrate entry on the hydrophobic side of the active site (Fig. 1B).

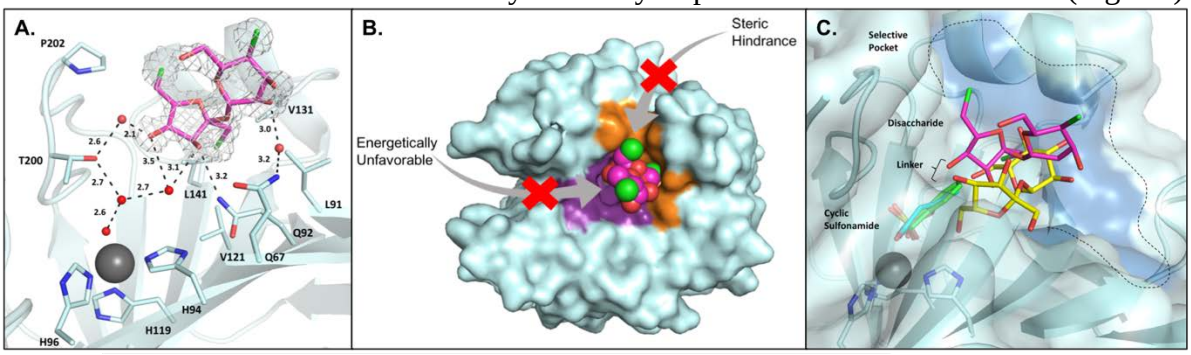

Fig. 1. A. Sucralose binding in CA IX-mimic (2Fo-Fc electron density map is contoured to σ=0.8). B. Overlay of sweeteners in complex with CA IX-mimic. Sucralose (pink), sucrose (yellow), saccharin (green), and ace K (blue).

Conclusions

The insights gained from the structural comparisons of saccharin, ace K, sucrose and sucralose binding can be utilized to rationalize structure-guided drug design of CA IX specific inhibitors. As fragmentation studies show that the most potent inhibitors result from the combination of small molecules, the combination of a cyclic sulfonamide (such as saccharin or ace K) linked to a disaccharide (such as sucralose or sucrose) has the potential to produce efficacious CA IX inhibitors. The chemical linker would provide the spacial requirements to optimize interactions with residues in the selective pocket of the active site, improving selectivity for CA IX and preventing off target inhibition of CA II. Such compounds would simultaneously inhibit activity through two mechanisms- displacing the ZBS essential for catalysis and occluding the active site for substrate binding (Fig. 1C).

Carbonic anhydrase inhibition and anti-inflammatory effects of a new class of NSAIDs in the airways remodeling of a murine model of bleomycin-induced lung fibrosis

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Introduction

Fibrosis of lung tissue is characterized by chronic inflammation that determines a pathological remodelling of the parenchyma. The animal model obtained by intra-tracheal administration of bleomycin in C57BL/6 mice is one of the most validated murine model, which is characterized by inflammation of the airways and subsequently lung fibrosis [1].
The carbonic anhydrase (CA) family includes 16 catalytically active zinc metallo-enzymes that catalyze the reversible interconversion of carbon dioxide and water to bicarbonate and protons. CA inhibitors, besides antitumour activity [2], exhibit anti-inflammatory effects in rats with permanent middle cerebral artery occlusion [3].

This study investigated the effects of a new class of drugs endowed with CAIX and COX-2 inhibitory activity in the modulation of inflammation and the remodeling of lung parenchyma.

Experimental

C57BL/6 mice were treated with bleomycin (0.05 IU) or saline, intratracheally, to induce lung fibrosis. Immediately after, mice were treated with vehicle, ibuprofen (0.5 mg/kg b.wt.), acetazolamide (0.5 mg/kg b.wt.) or compound 1e (1 mg/kg b.wt.), a molecule endowed with a NSAID portion and a sulfonamide group, at equimolar doses, released by micro-osmotic pumps for 21 days.

Histochemical and biochemical parameters to evaluate TGF-β/SMAD signalling pathway with alpha-smooth muscle actin (αSMA) deposition and the levels of a number of inflammatory markers (TNF-α, IL-1β, IL-10) were performed. Moreover, DHE staining and 8-OH*d*G analysis were done to evaluate reactive oxygen species (ROS) production in lung tissue and caspase-3 expression to evaluate apoptosis in lung tissue homogenates.

Results and discussion

Bleomycin administration increased lung stiffness, TGF-b levels, pSMAD3 expression, αSMA deposition, and content of inflammatory markers. Compound 1e attenuated all these physiological, biochemical and histopathological parameters. Our findings support the proposal that these compounds, endowed with a dual COX-2 and CAIX inhibition, have a therapeutic potential in reducing the progression of signs and symptoms of the disease by decreasing TGF-β expression and the TGF-β/SMAD transduction pathway.

Conclusions

The results here reported demonstrate that these new class of compounds could be a novel therapeutic strategy for lung inflammatory and fibrotic diseases.

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Acesulfame K Displays Alternative Binding Modes Dependent on Carbonic Anhydrase Isoform

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Introduction

Human carbonic anhydrase IX (CA IX) is upregulated in neoplastic tissues and has been studied as a drug target for anticancer chemotherapy. Inhibition of CA IX has been shown to be therapeutically favorable in terms of reducing tumor growth. Recently, it been observed that sweetener bind and selectively inhibit CA IX over other CA isoforms.

Experimental

Using the NANUQ^{TM} automated freezing and hand freezing techniques along with X-ray crystallography, we expanded on these findings and performed a structural characterization on acesulfame potassium (Ace K), a commonly used artificial sweetener, and Ace K derivatives that displayed at least 60-fold selectivity for the CA IX over ubiquitous forms [1].

Results and discussion

It was found that Ace K bound directly to the catalytic zinc in CA IX (mimic) and through a bridging water in CA II, which accounts for the 10-fold selectivity of Ace K for CA IX (mimic) when compared to CA II (Fig. 1) [2]. Ace K derivatives (N-alkylated and O-alkylated) displayed another binding mode with N-alkylated derivatives binding to the catalytic zinc through the aromatic oxygen instead of the aromatic nitrogen previously seen.

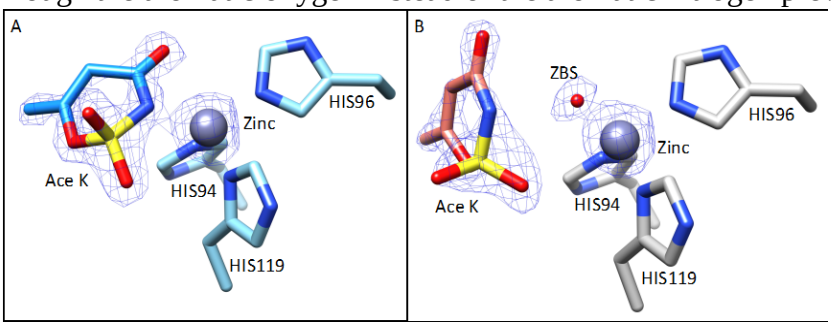

Fig. 1. Ace K in complex with A) CA IX (mimic, PDBID 5WGP) and B) CA II (PDBID 5WG7). The $2|F_o| - |F_c|$ electron density map (blue) contoured at 1.0σ.

Conclusions

In this study, we present the X-ray crystal structures of Ace K and Ace K derivatives in complex with cancer-related and off-target CA isoforms displaying alternative binding modes not seen with classical CA inhibitors which could lend itself to increased selectivity to CA IX (mimic).

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Carbonic anhydrase IX: a promising therapeutic target for cancer treatment

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Introduction

Many solid tumors are characterized by hypoxia, and it is a prognostic indicator of a poor clinical outcome for patients [1]. Many hypoxia-targeted cancer therapies are based on carbonic anhydrase IX (CA IX), a membrane protein which is highly overexpressed in numerous cancers, but is largely absent in normal tissues. Several inhibitors selective to CA IX have been synthesized at Vilnius University Prof. D. Matulis group. Sulphonamidic compounds VD11-4-2 and VD12-09 possessed a high affinity and excellent selectivity towards CA IX [2]. We tested both CA IX inhibitors in cancer cell migration in invasion assays *in vitro*, also evaluated antimetastatic effect in triple-negative breast cancer model in mice.

Experimental

Anticancer effect *in vitro* has been evaluated by testing compound effect on tumor spheroid growth. Tumor spheroids were formed from different breast cancer cell lines using 3D Bioprinting method. Effect on cancer cell migration and invasion has been tested by 3D inverse invasion and 3D invadopodia formation assays. Antimetastatic effect has been evaluated in highly metastatic triple-negative breast cancer model in mice by analysing metastases in lungs.

Results and discussion

Compounds reduced the size of spheroids made from human breast cancer HCC38, BT474 and MCF-7 cells, also highly aggressive and metastatic mouse breast cancer cell line 4T1 and human cell line MDA-MB231 T1AS. VD12-09 was the most active compound in all experiments, and VD11-4-2 showed lower activity, while the growth of spheroids in both control group and the group treated with acetazolamide, was almost the same.
Also, compound VD11-4-2 inhibited the migration of human triple-negative breast cancer cells MDA MB231 T1AS in hypoxia conditions. Both CAIX inhibitors inhibited the formation of 3D spheroid invadopodia and reduced the migration of cells through them.
Compounds did not show significant effect on tumor growth in mice. However, both of them significantly reduced metastasis formation in mouse lungs.

Conclusions

CA IX inhibitors are active in different tumor 3D models and those compounds could be developed as antimetastatic agents.

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Benzolamide is protective in cardiac ischemia: Role of endothelial nitric oxide (NO) synthase-mediated NO production

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Background

Recent studies from our laboratory have demonstrated the cardioprotective action of benzolamide (BZ, a carbonic anhydrase inhibitor) against ischemia-reperfusion (IR) injury. However, the mechanisms involved have not been fully elucidated.

Objective

To examine the role of the endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) pathway in the effects of BZ in a model of regional myocardial ischemia.

Methods

Isolated rat hearts perfused by the Langendorff technique were subjected to 40 min of coronary artery occlusion followed by 60 min of reperfusion. Other hearts received BZ 5 Mm during the first 10 min of reperfusion in absence or presence of LG-nitro-L-arginine methyl ester (L-NAME, an eNOS inhibitor). The infarct size and the post-ischemic recovery of myocardial function were measured. Oxidative damage was assessed by reduced glutathione (GSH) content and thiobarbituric acid reactive substances (TBARS) concentration. Nitrosative damage was assessed by increases in 3-nitrotyrosine. The expression of the phosphorylated form of Akt and eNOS, (PeNOS and P-Akt) and the concentration of inducible nitric oxide synthase (iNOS) were also determined.

Results

BZ significantly decreased total infarct size (6.0 ± 0.5% vs. 34 ± 4%), improved post-ischemic contractility, preserved GSH levels and diminished TBARS and 3-nitrotyrosine content. The expression of PeNOS and P-Akt decreased and iNOS increased in control hearts. After BZ addition, the levels of PeNOS and P-Akt increased and iNOS decreased. Except for the changes in iNOS expression, all observed changes were abolished by L-NAME.

Conclusions

Our data demonstrate that the treatment with BZ at the onset of reperfusion after 40 minutes of ischemia reduces cell death by 80%, prevents contractile dysfunction and oxidative/nitrosative damage produced by coronary artery occlusion. These beneficial actions achieved by BZ appear in part to be mediated by eNOS/NO-dependent pathways.

Novel Atg4B-inhibitors and dual [Atg4B-Carbonic Anhydrase] inhibitors for interfering with cytoprotective mechanisms of cancer cells in the acidic tumor micro-environment

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Inadequate perfusion, oxygen limitation and cell metabolic changes, are key factors contributing to the formation of an acidic microenvironment in tumors [1]. Two pivotal adaptations of tumor cells, related to maintaining intracellular pH and homeostasis in an acidic environment, have recently received significant attention: (1) the presence of chronic autophagy and (2) the overexpression of tumor-associated carbonic anhydrases (CAs), CA IX and CA XII [1].

Autophagy becomes abundant during exposure to several types of cellular stress. In acidic culture, inhibition of autophagy has been shown to promote tumor cell death and to increase sensitivity to cytostatics [2]. All known autophagy inhibitors however have low intrinsic potency and act in a non-specific manner, mostly interfering upstream of autophagosome formation and affecting a variety of processes. Several of these autophagy inhibitors are nonetheless being clinically evaluated in oncology. In our own approach autophagy is targeted in a specific manner, via the cysteine protease Atg4B. As demonstrated *in vitro* and *in vivo* (HT-29 cells and xenografts) we have reported benzotropolones as a novel class of Atg4B inhibitors [3]. However this group of compounds still needs to be optimized further to increase potency and improve biopharmaceutical parameters. CAs on the other hand are zinc metalloenzymes commonly present in eukaryotic and prokaryotic organisms. They are reversibly catalyse the hydration of carbon dioxide to bicarbonate and a proton. In vivo studies have shown that silencing of both tumor-associated CA isoforms resulted in 85% reduction of tumor growth [4]. A CA IX/XII inhibitor (SLC-0111) is currently investigated clinically.

We will report on new bezotropolone-derived autophagy inhibitors. Also, compounds with potentially increased antitumoral potency will be discussed that combine the benzotropolone pharmacophore with a CA IX/XII targeting pharmacophore. Such compounds could be particularly useful to target autophagy in the acidic tumor micro-environment

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The production and characterization of novel beta-carbonic anhydrase of *Trichomonas vaginalis*

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The objective was to produce and characterize first of the two β-CAs of *Trichomonas vaginalis*, a protozoan parasite responsible of one of the world's most common sexually transmitted infections, trichomoniasis[1-2]. The β-CA of *T. vaginalis*, described in the current manuscript as TvaCA1 (Uniprot Entry id. A2ENQ8[3]), was expressed recombinantly in *Escherichia coli* using a multipromoter plasmid[4] as the cloning vector. The protein was purified with immobilized metal affinity chromatography (Ni^{2+}-NTA), and confirmed with SDS-PAGE (Figure 1), Western blotting and sequencing (Seq; in Fig. 1). Protein structure was determined by X-ray crystallography. Kinetics and inhibition profile was investigated using a wide range of sulfonamides and anions. X-ray crystallography revealed the protein structure of TvaCA1 as a homodimer (Fig. 2).

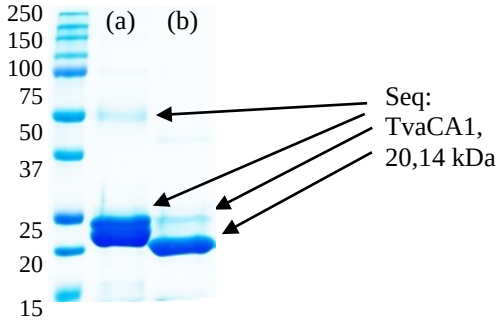

Figure 1. SDS-PAGE image of TvaCA1, (a) with (6,4 mg/ml), and (b) without His-tag (5,2 mg/ml).

Figure 2. Crystallographic structure of TvaCA1; monomers visualized in green and magenta.

The kinetic studies showed that TvaCA1 had significant catalytic properties for the hydration of CO_2 to bicarbonate and protons, with the following kinetic parameters: k_{cat} of 4,9 x 10^5 s^{-1} and a k_{cat}/K_M of 8.0 x 10^7 M^{-1} s^{-1}. The enzyme activity was inhibited in the nanomolar range by sulfonamides and some other small molecules such as boronic and phosphonic acids. Further work to detect more potent TvaCA1 inhibitors is warranted.

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Alfabetical Index of Authors

A

Abdel-Aziz, H.A. 64
Abdul Malik, A.-T. 16, 55
Afanasjevas, D. 78
Ai, L. 25
Akdemir, A. 68
Akgul, A. 16
Akocak, S. 21, 57
Alexander Donald, W. 38
Alghamdi, A. 56
Álvarez, B. 79
Ames, S. 24
Andring, J.T. 7, 58
Angeli, A. 16, 27, 36, 39, 46, 49, 59, 81
Annunziato, G. 27
Aojula, H. 56
Aspatwar, A. 44
Augustyns, K. 80

B

Baranauskienė, L. 72
Barboiu, M. 11
Barker, H.R. 44, 47, 81
Bartolucci, G. 16, 49, 50, 61
Bazzicalupi, C. 48
Becker, H.M. 24, 72
Berrino, E. 39, 49
Bilia, A.-R. 70
Blandina, P. 34
Bonardi, A. 39, 50, 60
Bozdag, M. 39, 43, 49
Bruno, A. 27
Bua, S. 50, 60, 61
Buonanno, M. 62

C

Capasso, C. 30
Carardori, S. 15, 68
Carta, F. 16, 25, 34, 36, 39, 43, 44, 48, 49, 55, 75
Cavalli, A. 9
Chen, F. 52
Chen, Z. 25
Chiaramonte, N. 50
Ciocci Pardo, A. 79
Costa, A. 34
Costantino, G. 27

D

D'Ambrosio, K. 62
Da'dara, A.A. 28
David, J. 51
David, S. 66
de Carvalho Myskiw, J. 34
De Meyer, G.R.Y. 80
De Simone, G. 8, 62, 81
Dedhar, S. 22
Dei, S. 50
Deitmer, J.W. 24
DI Cesare Manneli, L. 16, 36
Di Fiore, A. 62, 81
Diudea, M.V. 63
Dubois, L.J. 72
Dudutienė, V. 72
Durante, M. 50, 75
Durgun, M. 57

E

Eldehna, W.M. 64
Elisée, E. 19
Eysteinsson, T. 39

F

Fantinelli J., 79
Ferraroni, M. 9, 43, 50
FIseher Z 7
Frost, S.C. 25

G

Gallaway, E. 29
Gao, Y.-G. 10
Gaspari, R. 9
Geniston, L.A. 63
Ghelardini, C. 16, 36
Gheorghiu, E. 66
Gheorghiu, M. 66
Gieling, R. 56
Giovati, L. 27
González Arbeláez, L. 79
Grandane, A. 17, 65
Gratteri, P. 48, 50, 60
Gudmundsdottir, H. 39
Guglielmi, P. 68
Guzel, O. 36

H

Hammaren, M. 44
Hardarson, A.Ö. 39
Heldermon, C. 25
Hjelmeland, A.B. 23
Hytönen, V.P. 81

I

Ilies, M.A. 13, 58
Iorga, B.I. 19
Ivan, M. 52
Ivanova, J. 17
Izquierdo, I. 34

J

Jamali, S. 24

K

Karioti, A. 70
Kayastha, B.B. 29
Kazokaitė, J. 72
Kim, C.U. 7
Konig, H. 52
Koskinen, S. 44
Krasavin, M. 12
Kuuslahti, M. 81

L

Lambin, P. 72
Langella, E. 62
Langer, S. 24
Lanzi, C. 75
Leitāns, J. 72
Lolak, N. 57
Lomelino C.L. 7, 60, 74, 77
Lotlikar, S. 29
Lucarini, L. 50, 75
Lungu, C.N. 63
Luukinen, B. 44

M

Magliani, V. 27
Mahon B.P. 7
Manetti, D. 50
Martinet, W. 80
Masini, E. 50, 75
Matulienė, J. 72
Matulis, D. 72, 78
Mboge, M.Y. 25
McKenna, R. 7, 25, 58, 60, 74, 77
Menicatti, M. 49, 61
Micheli, L. 36
Monti, S.M. 32, 62, 81
Mosca, S. 79
Murat, B. 36
Murray, A.B. 74, 77

N

Nguyen, G.T.H. 38
Niemans, R. 72
Nocentini, A. 48, 49, 50, 60, 61, 64
Noor, S.I. 24

P

Parikka, M. 44
Parkkila, S. 44, 47, 81
Passani, M.B. 34
Patrauchan, M.A. 29
Pavone, M.L. 27
Petrikaitė, V. 78
Pieroni, M. 27
Poli, G. 41
Polonschii, C. 66
Prekeris, R. 78
Provensi, G. 34
Pustenko, A. 17

R

Rani, B. 34
Rogozea, A.L 52
Romanelli, M.N. 50

S

Sandusky, G. 52
Schmidt, S.D. 34
Scozzafava, A. 9, 16, 55
Secci, D. 68
Selleri, S. 48
Sgambellone, S. 75
Skelly, P. J. 28
Stanica, L. 66
Stroobants, S.G. 80
Supuran, C. T. 6, 9, 16, 25, 27, 28, 29, 33, 34, 36, 38, 39, 43, 44, 46, 48, 49, 50, 51, 55, 56, 57, 58, 59, 60, 61, 62, 64, 65, 66, 68, 70, 74, 75, 77, 80, 81
Swenson, E.R. 37, 79

T

Takhsha, F.S. 80
Tanc, M. 48, 80
Tārs, K. 72
Teodori, E. 50
Thibaudeau, S. 18
Tolvanen, M.E.E. 47
Tu, C. 25
Tuccinardi, T. 41

U

Urbański, L. 81

V

Van Der Veken, P. 80
Vangestel, C. 80
Vozny, I. 17
Vullo, D. 16, 28, 33, 36, 49, 57

W

Winum, J.-Y. 14
Wong, M.W. 51
Yaromina, A. 72

Z

Zalubovskis, R. 17, 65
Zhang, Z. 25
Zubrienė, A. 72
Žuvela, P. 51