CHEMISTRY
A European Journal

A Journal of
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Accepted Article

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To be cited as: *Chem. Eur. J.* 10.1002/chem.201805039

Link to VoR: http://dx.doi.org/10.1002/chem.201805039

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10.1002/chem.201805039

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Phosphorus vs sulphur: discovery of benzenephosphonamidates as new versatile sulfonamide-mimic chemotypes acting as carbonic anhydrase inhibitors

Alessio Nocentini,[a,b] Paola Gratteri,*[a] and Claudiu T. Supuran *[b]

Abstract: The first zinc binding group (ZBG) to have been identified as inhibitor of the metallo-enzymes carbonic anhydrases (CA, EC 4.2.1.1) was the sulfonamide. From then on several classes of zinc-binders have been described. Herein we propose the benzenephosponamidates as a new chiral aromatic sulfonamide-mimic ZBG able to meet the requirements for effectively binding the enzyme active site. Several low micromolar CA I, II, VII, IX inhibitors were thus detected. Kinetic studies, QM-polarized ligand docking and MM-GBSA *in silico* methods were used to characterize this newly identified CA inhibitor chemotype.

Searching for new chemotypes able to yield enzymatic inhibition is a main feature of the ongoing research regarding the metallo-enzymes carbonic anhydrases (CA, EC 4.2.1.1).[1-4] Their role in cells as well as in complex organisms, is primarily related to the ability to reversibly catalyse the carbon dioxide hydration reaction, affording bicarbonate and a proton.[5] Such an equilibrium is crucial for a vast array of physio/pathological processes, with ligands targeting both human (h) and pathogens isozymes possessing pharmacologic applications in the management of human diseases such as glaucoma, edema, obesity, epilepsy, hypoxic tumors, neuropathic pain, or arthritis, and for the development of anti-infectives with a new mechanism of action.[2,3,6] Up to now, seven distinct genetic families have been identified, namely the α-, β-, γ-, δ-, ζ-, η- and θ-CAs.[2,7,8] All CAs known so far are metal ion-dependent enzymes, with a metal-hydroxide species within the active site cavity acting as a nucleophile in the catalytic cycle. The metal ions identified in different CAs gather Zn(II), Cd(II), Co(II) or Fe(II), with the first ion being the cofactor present in α- and β-isozymes.[2,7,8] In humans, 15 α-CA isoforms are known, CA I−CA VA, CA VB, CA VI−CA XIV.[2]

A crucial event mainly occurring in the enzyme inhibition process is the inhibitor binding to the metal ion. Most CA inhibitors owe their activity to zinc binding groups (ZBGs) of the sulfonamide type, which displaces the zinc-bound nucleophile (water molecule or hydroxide ion).[9] The majority of the clinically used CAIs are sulfonamides. Structural resolutions of ligand-CA adducts highlighted that the sulfonamide group is an ideal ligand for the CA active site owing to the combination of the coordination bond between the negatively charged nitrogen with the positively charged zinc(II) ion, and of the presence of one proton on the coordinated nitrogen atom satisfying the hydrogen bond acceptor character of Thr199 side chain (Figure 1).[3] A main drawback related to sulfonamides is a low membrane-permeability and likely allergic reactions aroused in patients,[2,3] as well as their non-selective inhibition of many CA isoforms.

As result of a straightforward ligand-based drug design process, sulfamides and sulfamates arose as sulfonamide most related isosters.[9] An additional heteroatom confers to the zinc-binding group a more extended H-bond network forming ability. In the last period, some of us ventured out from the sulfonamides shell presenting additional CA-ZBG, among which carboxylates, hydroxamates, phosphonates and lately crucial advances have been made in this field, with mono- and dithiocarbammates, xanthates, thioxanthates and boroles identified as novel zinc-binders.[10-16]

Most of these inhibitory chemotypes bind in deprotonated form, as anions, straightly coordinating the Zn(II) ion from the enzyme active site.[17] There is overwhelming X-ray crystallographic evidence showing this type of binding for sulfonamides, sulfamates, sulfamides, dithiocarbamates, and their derivatives, hydroxamates, some carboxylates, one phosphonate, and some boroles.[3,10-16]

A recent comparison between carbon- and sulphur-based ZBGs provided for mechanistic and structural insights over a pattern of chemotypes featuring rather diverse hybridizations and electronic properties of the central element.[17] Of note, phosphorus compounds did not enjoy an analogue attention and investigation as that dedicated to carbon and mainly sulphur compounds, thus being only scarcely investigated as CAIs. However, among pseudopeptides containing a phosphorus atom, phosphonamidates of the $RNHPO_2^-$ types are considered the closest analogues of the high energy tetrahedral transition state of the amide bond hydrolysis and such compounds were mostly investigated as protease inhibitors.[18,19] Phosphonamidates and phosphinamidates have gained increasing interest not only for the unique structures but also the distinguished properties in pharmaceutical applications and material science.[20,21] In the context of CAs, it should be stressed that only a few phosphonates have been investigated as inhibitors, and just one compound, foscarnet was crystallized bound to hCA I, witnessing the zinc-binder character of such an acidic function.[16]

The electronic properties of phosphorus, that belongs to group V, do elicit interesting different structural features for a phosphonamidate compared to the lead sulfonamide, though likewise in this latter, a tetrahedral geometry is maintained for the central phosphorus atom. Conversely, carbon-based ZBGs exhibit a trigonal planar geometry due to the sp^2 hybridization (Figure 1). Comparing phosphonamidates and sulfonamides, one of the S=O

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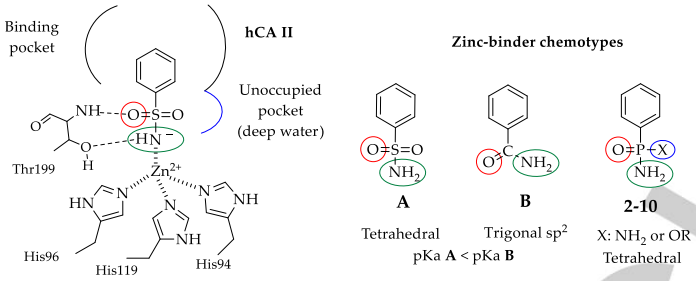
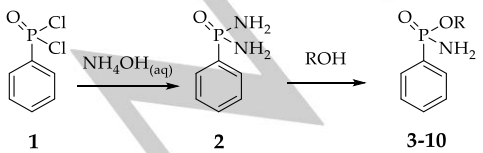


Figure 1. Schematic representation of the binding mode of carbon, sulphur and phosphorus zinc-binders chemotypes.

double bonds of the sulfonamide is substituted by a single P-X bond which can be exploited to incorporate an additional amino function or alternative substitutions, such as alkyl phosphoesters. This implies that an additional functionalization can be appended to the ZBG maintaining pivotal elements present in the lead, such as the NH_2 and one P=O (S=O in the lead), thus likely extending the interaction network with the enzymatic counterpart (Figure 1). Indeed, it should be noted that the so-called "deep water" area [3] in the hydrophobic half of the hCAs active site, is not reached by the sulfonamide moiety, thus leaving an empty targetable-pocket that is defined by residues Trp9, Val121, Val143 and Leu198 (taking CA II as representative isoenzyme).[3] Moreover, as a result of the electronic properties of the central atom and of the geometrical features, the acid-base properties of benzenephosphonamidates **2-10** considerably differ from those of the sulfonamide lead. A remarkable difference indeed exists between benzenesulfonic and phenylphosphonic acid (pKa of -2.8 and 1.85, respectively), that is even more evident when compared to benzoic acid pKa (4.2)[17] Likewise, benzenesulfonamide (pKa of 10.2) acts as stronger acid than benzamide (pKa of 14.5). In this context, it has been long debated how the pKa of the ZBG influences ligands CAI properties, with the conclusion being that it is not the most influential factor, although it is a relevant one.[17] Indeed, the sulfonic acid is a weaker CAI than the corresponding sulfonamide.[17] It is well established that a zinc-binder must be deprotonated to efficiently bind the zinc ion, but a medium-range dissociation is preferable over too strong or too weak ones.[17] In this context, a brand-new crystallographic study outlined the binding mode of weak acid to CA II, namely benzylcarbamate.[22] Mimicking bicarbonate binding, the carbamate moiety directly coordinates the catalytic zinc ion. Although the difference between the pKa of carbonic acid and the carbamate is huge, the deprotonation of the -NH_2 group is promoted into the CA active site.[22]



Scheme 1. Synthesis of benzenephosphonamidate derivatives.

Considering what mentioned above and our interest in the development of novel classes of CAIs, alternative to the sulfonamides, we have explored the capability of phenylphosphonic diamide **2** and alkyl phosphonamidates **3-10** to inhibit a pattern of hCAs (i.e. hCA I, II, IV, VII, and IX) and studied *in silico* their binding mode within the enzyme active site.

A straightforward strategy was used to synthesize the compounds starting from phenylphosphonic dichloride **1**. Phenylphosphonic diamide **2** was obtained by reacting **1** with an aqueous solution of 35% ammonia. Derivative **2** was the common intermediate to easily obtain alkyl phosphonamidates **3-10** by reaction with the proper alcohol, as described in the literature.[23] Unlike the diamide derivative **2**, all alkyl phosphonamidates **3-10** were obtained as racemic mixtures of both R and S optical isomers. All the obtained derivatives were properly characterized by means of ^{1}H-NMR, ^{13}C-NMR, ^{31}P-NMR as well as MS (ESI).

Table 1: Inhibition data of human CA isoforms I, II, IV, VII and IX with phenylphosphonic diamide **2** and alkyl phosphonamidates **3-10** and the standard sulfonamide inhibitor acetazolamide (AAZ) by a stopped flow CO_2 hydrase assay.[26]

		K_I (μM)[a]				
Cmp	**R**	**CAI**	**CA II**	**CA IV**	**CA VII**	**CA IX**
2	-	77.8±4.2	32.8±2.6	346.9±22	39.1±3.0	7.6±0.6
3	$-CH_3$	145.6±8.3	39.8±3.2	610.2±34	51.9±3.6	16.9±0.9
4	$-CH_2CH_3$	338.6±22	160.6±9.8	>1000	98.0±5.3	45.3±2.8
5	$-(CH_2)_2CH_3$	589.9±31	459.4±28	>1000	247.6±18	116.2±8.5
6	$-CH(CH_3)_2$	730.0±45	348.8±20	>1000	406.2±23	212.0±13
7	$-(CH_2)_3CH_3$	876.3±51	750.0±48	>1000	516.1±28	123.5±7.2
8	$-(CH_2)_2OCH_3$	961.2±54	520.1±28	>1000	727.3±41	250.9±15
9	$-(CH_2)_2Cl$	322.2±23	95.4±7.2	916.6±48	160.5±9.1	146.3±8.9
10	$-CH_2CCH$	575.8±36	465.3±30	>1000	602.8±35	323.3±19
AAZ	-	0.25	0.01	0.07	0.01	0.03

a. Inhibition data are expressed as means ± SEM of 3 different assays.

Among the twelve catalytically active hCAs, the isoforms chosen to work out our proof-of-concept were the cytosolic hCA I and II (widespread house-keeping enzymes involved in a host of physiologic processes), the membrane-bound hCA IV (involved in glaucoma, retinitis pigmentosa, stroke and rheumatoid arthritis), hCA VII (drug-target for epilepsy), and the tumor-associated hCA IX (abundant in hypoxic tumors and recently validated as antitumor target).[2,24,25] Phosphonamidates have been shown to be fully stable up to pH 7 with significant hydrolysis evidenced at pH below 6.20. A longer CA/inhibitor preincubation (1h) than that

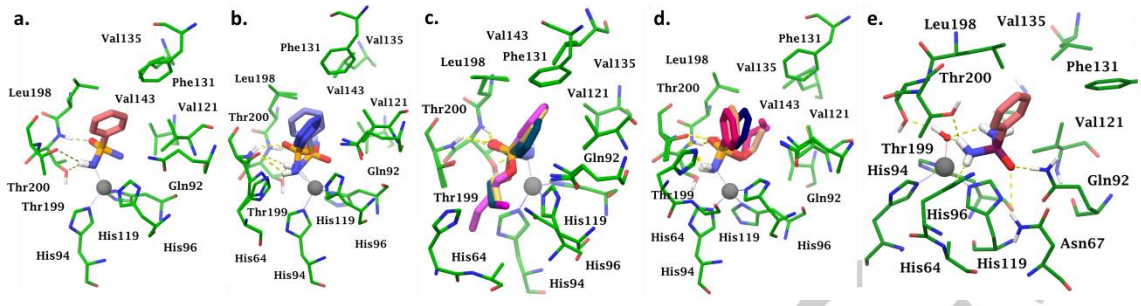


Figure 2. QPLD/MM-GBSA predicted poses of the studied compounds with hCA II: a) **2** (pink), b) **3**-(R) (plum) and **3**-(S) (violet), c) **4**-(R) (yellow), **7**-(R) (magenta), **9**-(R) (dark green) and d) **4**-(S) (pink), **7**-(S) (purple), **9**-(S) (blue). e) docking of not dissociated **2** within hCA II active form (Zn-bound hydroxide ion).

usually used for sulfonamide derivatives (15 min) was necessary to observe the CAs inhibitory efficacy of phosphonamidates derivatives **2-10**. The data of Table 1 show that most screened derivatives inhibit hCAs in the micromolar range depending on the substitution pattern on the phosphorus atom. The simplest and less sterically hindered compounds **2** and **3** arose as the best inhibitors against all the evaluated isoforms. These compounds showed target-promiscuity, comparably inhibiting hCA II, VII and IX, which were the most affected isoforms, with inhibition constants (K_Is) in the range of 32.8-47.1 µM, 39.1-51.9 µM and 7.6-20.7 µM, respectively. Phenylphosphonic diamide **2** exhibited a low micromolar efficacy against hCA IX with a K_I of 7.6 µM and a greater than four-fold selective action for the tumor-associated isozymes over the remaining considered ones. However, the introduction of bulkier substituents of the phosphoester type on the phosphorus atom leads to a noticeable change in the CA inhibitory properties, with the further elongation of the aliphatic chain being deleterious for the inhibition almost unambiguously against all isozymes. Indeed, derivatives **4-10** showed a feeble worsening of efficacy against isozyme IX, with K_Is settling in the range 45.3-323.3 µM. The roomier active site of hCA IX likely better accommodates the ligands rearrangement due to the presence of increasingly bulkier motifs. Elongation of the R group elicits a wider binding worsening to other isoforms, with the case of hCA IV being noteworthy since **4-10** do not inhibit the isozyme up to 10 mM. A diverse trend was observed for the chloroethyl derivative **9**, being the inhibition data reported in Table 1 not consistent with what mentioned above (K_Is of 322.2, 95.4, 916.6, 160.5, 146.3, respectively). Although **9** incorporates one of the unwieldy substituents on the central ZBG atom, only a slight activity lowering is observed in comparison to equally long motifs-bearing congeners, such as **6-8**. Plausible halogen-bond interactions could take place within the binding site cavities and justify the exhibited trend. A covalent bond involving the chlorine atom could also occur, although it should be verified that this hypothesis may take place.

The phosphonamidate was designed as ZBG to likely increase the binding within CAs active site by additional functionalization of the ZBG. The micromolar inhibitory profiles of derivatives prove the capability of the newly designed molecules to act as CAIs. To clarify the binding mode and the unexpected inhibitory trend of Table 1, computational investigations were undertaken. Quantum-polarized ligand dockings (QPLD) were performed on hCA II preparing both enantiomers of phosphonamidates **3-10** in the negatively-charged form and the selected poses were submitted to MM-GBSA calculations (ΔG bind data, Table 2). The combination of QPLD and GBSA methods has been recently shown to provide reliable docking/scoring results with low deviations from ligand/target crystallographic geometries.[27,28]

It is worth noting that phenylphosphonic diamide **2** assumed a sulfonamide-like binding mode with the negatively charged nitrogen coordinating the Zn ion and the NH donating a H-bond to Thr199 side chain hydroxyl group. Moreover, The P=O moiety accepted a H-bond from the same residue backbone NH. The other NH_2 of **3** slightly protrudes into the internal pocket lined by Val143, Trp9, Val121, Leu198. The aromatic ring is π-alkyl stacked by Val121 and Leu198 side chains (Figure 2A).

Not surprisingly, two alternative and clearly distinct binding modes were found for **3-10** according to the R/S configuration of the ZBG central atom. With the exception of the less sterically hindered methyl P-phenylphosphonamidate **3** (Figure 2B), the alkyl phosphoester groups accommodate within two distinct pockets of hCA II active site depending on the considered configuration. The stabilization of a unique binding mode for enantiomer was driven by the common necessity to bind the Zn through the NH moiety and to establish a proper network of H-bonds with the residues nearby. As a result, the (R) enantiomers assumed an orientation whereby the coordinating NH protrudes towards the target pocket and the P=O is in H-bond distance with Thr199, thus exposing the alkyl groups to the pocket defined by Ala65, Asn244, His64 (Figure 2C). In a completely opposite manner, the (S) enantiomers maintain mostly the same set of interactions, except for the alkyl moieties that occupy the target “deep water” area lined by the hydrophobic Val121, Trp9, Leu198 and Val143 side chains (Figure 2D). According to literature reports, the aromatic ring positioning is deeply influenced by the ZBG nature, namely by the metal coordination pattern and by the network of H-bonds formed, with a 90° rotation occurring between the (R) and (S) subsets. The small methyl group of derivative **3** allowed intermediate binding modes for both enantiomers than those above

depicted (Figure 2B), since the methyl groups do not point to any precise pocket.

Table 2: MM-GBSA ΔG bind data of derivatives **2-11** with hCA II

Cmpd	MM-GBSA ΔG bind (kcal/mol)	
2	-36.732	
	(R)	**(S)**
3	-49.611	-41.460
4	-41.379	-46.549
5	-47.993	-54.298
6	-33.819	-52.174
7	-46.702	-54.590
8	-50.216	-48.379
9	-49.410	-55.858
10	-46.762	-47.840

The binding free energy data (Table 2) clearly ascribed to (S)-enantiomers the role of eutomers within the racemic mixtures. It should be considered that, unlike the "deep water" cleft, the pocket that fits the alkyl moieties of (R) enantiomers could be occupied by water molecules that are actually present in almost all X-ray solved hCAs/ligand structures. The displacement of such water molecules could be associated with unfavourable entropic contribution to the binding free energy. The poses depicted in Figure 2 and the data in Table 2 confirm that the stereochemistry undeniably possesses a significant role in the ligand/target recognition, but does not hinder the ZBG character of the studied chemotype to the CAs.

Owing to the poor correlation between the data in Table 1 and the binding free energy estimations in Table 2, further aspects need to be considered to properly typify such a new binding chemotype.

In fact, both the enantiomer R and S of compounds **3-10** possess better free energy values than compound **2**, thus contending their worse *in vitro* inhibitory efficacy. Moreover, *in vitro* inhibition values of phosphonamidates are several orders of magnitude lower than sulfonamides despite an almost common binding mode which is additionally strengthened by interaction with the "deep water" pocket.

To shed light on these issues, pKas of derivatives **2-10** were computed by QM techniques. To validate the found values, the experimentally known pKas for benzenesulfonamide **A** and benzamide **B** were compared to those obtained applying the same QM procedure. The comparison showed the almost 100-fold less acidic character of **2-10** (pKa 12.5±0.5) than lead benzenesulfonamide **A** (pKa 10.2) thus suggesting the poor deprotonation degree associated with these compounds and, as a consequence, a not sufficiently strong Zn-binder character. This is what the difference in inhibition efficacy of sulfonamides vs phosphonamidates is probably due. Indeed, it has been shown the importance of the ZBG anionic form to effectively displace the Zn-bound nucleophile. In other words, it is likely that phosphonamidates predominantly exist in the not-dissociated form within the site, exhibiting a diminished zinc-binding efficacy (as in the case of benzylcarbammate) [22] or alternatively an anchoring binding mode as depicted in Figure 2e rather than coordinating the Zn ion the anionic form. In this latter case, the efficacy of binding might be considerably reduced and should be re-considered because of the lacking coordination to the metal ion.

In conclusion, altogether the data here reported provide evidence that phosponamidates can be efficiently used as a new class of CAIs, presenting interesting inhibition properties and a new, chiral binding mode to the CAs active site. It might be worth to evaluate such new chemotypes for inhibition of different metallo-enzymes, since phosphonates more effectively act against other enzymes, such as peptidases, compared to sulfonamides.[18,19] Insights on the experimental pKa and kinetic studies on single enantiomers will be helpful to precisely work out the reported SAR as well as crystallography studies to validate the proposed binding mode.

Acknowledgements

Ente Cassa di Risparmio di Firenze, Italy, is gratefully acknowledged for a grant to A.N (ECR 2016.0774).

Conflict of interest

The authors declare no conflict of interest.

Keywords: metallo-enzymes, carbonic anhydrase, zinc-binder, phosphonamidate, inhibition, QPLD, MM-GBSA.

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10.1002/chem.201805039

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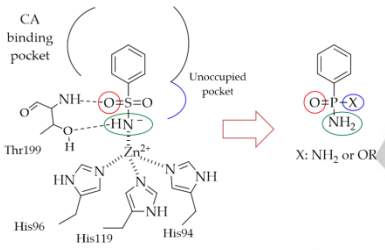
COMMUNICATION

There is a lack of studies that investigate phosphorus derivatives as Carbonic Anhydrase inhibitors (CAIs). In the present study, we started to fill this gap demonstrating that phosphonamidates can effectively inhibit human CAs as sulfonamide-mimic chemotypes by using kinetic studies and QPLD/MM-GBSA *in silico* methods.



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