Phosphonamidates are the first phosphorus-based zinc binding motif to show inhibition of β-class carbonic anhydrases from bacteria, fungi, and protozoa

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ABSTRACT
A primary strategy to combat antimicrobial resistance is the identification of novel therapeutic targets and anti-infectives with alternative mechanisms of action. The inhibition of the metalloenzymes carbonic anhydrases (CAs, EC 4.2.1.1) from pathogens (bacteria, fungi, and protozoa) was shown to produce an impairment of the microorganism growth and virulence. As phosphonamidates have been recently validated as human α-CA inhibitors (CAIs) and no phosphorus-based zinc-binding group have been assessed to date against β-class CAs, herein we report an inhibition study with this class of compounds against β-CAs from pathogenic bacteria, fungi, and protozoa. Our data suggest that phosphonamidates are among the CAIs with the best selectivity for β-class over human isozymes, making them interesting leads for the development of new anti-infectives.

1. Introduction
Virulence is labelled as the ability of a microorganism to infect the host and cause a disease. Virulence factors are molecules that assist the pathogen to colonise the host at the cellular level. These factors can be either secretory, membrane associated or cytotoxic and facilitate the microorganism metabolic, physiological, and morphological adaption in the host.

Virulence factors are deeply implicated in the onset of pharmacological resistance to anti-infectives. Preventing the implementation of these processes is a strategy deemed successful to date to overcome drug-resistance. Targeting virulence factors might indeed reduce, eliminate, and/or reverse the evolutionary selective pressures that induce the pathogen to develop resistance, which represents a main threat to human health nowadays. The metalloenzymes carbonic anhydrases (CAs, EC 4.2.1.1) have been shown to play critical roles in the virulence of many pathogens among which bacteria, fungi, and protozoa.

CAs are a superfAMILY of phylogenetically ubiquitous metalloenzymes, present in Prokaryotes and Eukaryotes, classified in seven genetically unrelated families, α, β, γ, δ, ε, η, and δεη. By catalysing the reversible hydration of CO2 to HCO3- and H+, these enzymes are implicated in many physiological processes which are basic for life. In microorganisms, CAs are involved in photosynthesis (cyanobacteria), biosynthesis of DNA, amino acids and fatty acids, and proliferation, survival and differentiation of pathogens both in the hosts and in the environmental niches. Compelling data exist in the literature, which strongly indicate that interference with CA activity in various parasites leads to an impairment of their growth, which in turn leads to a significant anti-infective effect.

Unlike mammals and human whose genome uniquely encode for α-class CAs, the genome of many pathogenic bacteria (e.g. Vibrio cholerae, Francisella tularensis, Burkholderia pseudomallei, the Gram-negative bacteria provoking cholera, tularemia, and melioidosis), fungi (e.g. Candida glabrata, Cryptococcus neoformans, or Malassezia globosa, respectively responsible of candidiasis, cryptococcosis, and dandruflf, and protozoa (e.g. Leishmania donovani chagasi which provokes leishmaniasis) encode for β-class enzymes. Actually, the seven different CA classes do not show significant structural homology with each other. Furthermore, CAs of the same class but belonging to genetically distant species, show differences at the level of their catalytic site. Therefore, a probable and realistic possibility to develop selective CA inhibitors exists. β-CAs exist as oligomers formed by two or more identical subunits such as dimers, tetramers, and octamers.

Sulphonamides and bioisosteres are the most potent class of CA inhibitors (CAIs) and have been, probably exhaustively, exploited to date in the design of inhibitors against almost every known CA. Nonetheless, these chemotypes commonly show a weak isomorph-selectivity both within the subset of human CAs and between isoforms from distinct classes, such as α- and β-CAs. As a result, many other chemotypes (phenols, mono- and dithiocarbamates, boroles, carboxylates, N-nitrosourea) have been investigated as inhibitors of β-CAs to identify selective modulators for targeting the pathogens encoding for enzymes of
this class. Among these, N-nitrosulfonamides, phenols, and natural polyphenols stood out as selective inhibitors for $\beta$-class CAs from pathogens over human ubiquitous CAs$^{20,21,27}$.

A recent paper by us showed for the first time that phosphonamidates are able to inhibit human CAs in the micromolar range acting a sulphonamide biomimetics additionally possessing a chiral binding mode (Figure 1)$^{28}$. Up to now, no one scheduled testing phosphorus based binding motifs against $\beta$-class CAs. Considering the interesting results reported earlier against these enzymes, here we report an inhibition study with benzenephosphonamidates against $\beta$-CAs from pathogenic bacteria, fungi, and protozoa working out their structure–activity relationship (SAR) in comparison to hCA I and II previously reported inhibition.

2. Methods

2.1. Chemistry

The synthesis of phenylphosphonic diamide 2 and alkyl phosphonamidates 3–10 was reported earlier by our group$^{28}$.

2.2. Carbonic anhydrase inhibition

An Sx.18Mv-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic activity of various CA isozymes for CO$_2$ hydration reaction$^{29}$. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5, for $\alpha$-CAs) or TRIS (pH 8.3, for $\beta$-CAs) as buffers, and 20 mM Na$_2$SO$_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalysed CO$_2$ hydration reaction for a period of 10–100 s. The CO$_2$ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled–deionised water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 1 h at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3 and the Cheng–Prusoff equation, as reported earlier, and represent the mean from at least three different determinations$^{30,31}$. All CA isoforms were recombinant ones obtained in-house as reported earlier$^{32,33}$.

3. Results and discussion

3.1. Chemistry

A straightforward strategy was used to synthesise the compounds starting from phenylphosphonic dichloride 1$^{28}$ (Scheme 1). Phenylphosphonic diamide 2 was obtained by reacting 1 with an aqueous solution of 35% ammonia. Derivative 2 was converted to alkyl phosphonamidates 3–10 by reaction with the proper alcohol, as described in the literature$^{34}$ (Scheme 1). Unlike the diamide derivative 2, all alkyl phosphonamidates 3–10 were obtained as racemic mixtures of R and S optical isomers.

3.2. $\beta$-Class carbonic anhydrase inhibition

Phenylphosphonic diamide 2 and alkyl phosphonamidates 3–10 were assayed as inhibitors of a panel of $\beta$-class CAs from pathogenic bacteria, fungi, and protozoa: VCh$\beta$ from V. cholerae, Ftu$\beta$CA from F. tularensis, Bps$\beta$CA from B. pseudomallei, Can$\beta$ from C. neoformans, CgNce from C. glabrata, MgCA from M. globosa, and LdcCA from L. donovani chagasi. A stopped flow CO$_2$ hydrase assay was used including acetazolamide (AAZ) as standard
inhibitor. A longer preincubation between compounds 2–10 and the enzymes (1 h) was necessary to observe their maximum inhibitory activity in comparison to the 15 min incubation which is commonly used for sulphonamides inhibitors. It was previously shown that phosphonamidates possess a significantly lower acidity (more than 2 orders of magnitude) than the bioisosteres sulphonamides which presumably induce an extended time needed to form the E–I complex.

The inhibition profiles against the human ubiquitous CAs I and II are displayed for comparison. The following structure–activity relationships (SAR) can be assembled from the inhibition data reported in Table 1.

What immediately stands out from the data in Table 1 is that benzenephosphonamidates 2–10 act as significantly stronger inhibitors against the tested β-class CAs than CA I and CA II. In fact, low to submicromolar inhibition constants (Ks) were measured against VChβ (Ks = 0.5–64.9 μM), Ftuβ/CA (Ks = 6.9–55.3 μM), BpsβCAβ (Ks = 2.1–32.7 μM), Can2 (Ks = 0.02–10.4 μM), CgNce (Ks = 0.05–20.2 μM), and LdcβCA (Ks = 0.8–36.5 μM), whereas solely MgCA was inhibited in a medium micromolar range (Ks = 26.1–265.7 μM). In contrast, CAs I and II had been shown to be target of compounds 2–10 in a medium to high micromolar scale spanning from 77.8 to 961.2 μM and 32.8 to 520.1 μM, respectively.

Using in silico techniques, phosphonamidates have been reported to act as zinc-binding group in the interaction with the active site of hCA II (Figure 1) and thus it is not unexpected that the inhibition activity in comparison to the 15 min incubation which is commonly used for sulphonamides inhibitors. It was previously shown that phosphonamidates possess a significantly lower acidity (more than 2 orders of magnitude) than the bioisosteres sulphonamides which presumably induce an extended time needed to form the E–I complex.

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Using in silico techniques, phosphonamidates have been reported to act as zinc-binding group in the interaction with the active site of hCA II (Figure 1) and thus it is not unexpected that the incorporation of gradually bulkier substituents on the phosphorus atom might decrease the inhibitory effectiveness of this class of compounds because of steric hindrance induced nearby the zinc ion. Nonetheless, the reduction of inhibition potency against β-class CAs upon increasing the length of the alkyl chain was not as significant as that reported against hCAs, and thus other binding factors appear to take place within the binding site of β-CAs. In detail, phenylphosphonamide 2 and methyl phosphonamide 3 indistinctly showed the best inhibitory potency against VChβ (Ks of 0.9 and 0.5 μM), BpsβCAβ (Ks of 2.5 and 2.1 μM), Can2 (Ks of 0.03 and 0.02 μM), CgNce (Ks of 0.05 and 0.2 μM), MgCA (Ks of 28.4 and 26.1 μM), and LdcβCA (Ks of 21.1 and 0.8 μM) among the reported compounds. As a unique exception, Ftuβ/CA was found to be most potently inhibited by the propargyl phosphonamide 10 which shows a Ks of 6.9 μM. In the cases of BpsβCAβ and Can2, the ethyl derivative 4 showed a comparable inhibition to compounds 2 and 3, with Ks of 4.6 and 0.05 μM, and further showed an interesting submicromolar Ks of 0.8 μM against CgNce. Branching of the alkyl chain from propyl (5) to isopropyl (6) always produced a positive effect on the inhibition potency, which was two-fold against VChβ, Ftuβ/CA, BpsβCAβ, and CgNce and nine-fold against Can2 (Ks from 0.9 to 0.1 μM). Switching the propyl (6) to a propargyl (10) produced instead a positive effect uniquely in the case of Ftuβ/CA (Ks from 35.6 to 69.1 μM). Enhancement of inhibitory potency against Ftuβ/CA (Ks from 35.6 to 15.1 μM) and Can2 (Ks from 0.9 to 0.6 μM) is also provoked by swapping the terminal CH3 of the propyl of 5 with a chlorine atom as in 9, whereas the two compounds inhibit CgNce almost equally (Ks of 1.3 and 1.8 μM).

The standard AAZ is a more effective inhibitor of all tested β-CAs than phosphonamidates 2–10 here investigated (Ks in the range 0.01–0.77 μM), with the exception of MgCA, against which it showed a Ks of 40 μM, whereas the most potent compounds 2 and 3 act with Ks of 28.4 and 26.1 μM. However, AAZ also inhibits CA I and II in a medium to low nanomolar range, while Ks of compounds 2–10 do not go below 30 μM against these human isozymes. In Table 2, the selectivity index (SI) for the target β-CAs over hCA II is reported. With the exception of MgCA, all tested phosphonamidates show a remarkably selective inhibitory action against all β-CAs over the most relevant human isozyme. While the SI for VChβ, Ftuβ/CA, LdcβCA and BpsβCAβ (over hCA II) span between 2.7 and 79.6, it is noteworthy stressing the up to four-digits SI calculated for a subset of compounds against Can2 (1093.3–3488.0) and the three-digits SI that compounds 2–7 showed against CgNce (141.5–656.0).

AAZ weakly inhibits MgCA from M. globosa whereas phosphonamidates 2–10 inhibit this enzyme less effectively than other screened β-CAs and almost comparably with hCA II, leading to SI ranging between 0.8 and 7.3. Selectivity indexes for the target β-CAs over hCA I are obviously even higher than those depicted in Table 2 because hCA I is less inhibited than hCA II by derivatives 2–10.

Significantly higher SI have been calculated with most phosphonamidates for β-class CAs over human isoform II in comparison to sulphonamide derivatives, which are here represented by AAZ (SI in the range <0.01–1.0, Table 2). These data include this new class of compounds among the CAs that are most selective for...
β-class over human isozymes known to date. One could speculate that the narrower active site pocket of β-CAs better accommodates benzenephosphonamidates than the roomier binding cavity of human CAs in terms of binding interactions. Indeed, a recent paper by us showed that the binding mode of benzoazaboroles within the active site of β-CAs Can2 and MgCA is strongly driven by π–π interactions involving aromatic residues solely present in β-class enzymes. Analogue binding interactions might exist with benzenephosphonamidates, which increase their inhibition effectiveness against β-CAs. Novel insights are emerging by novel crystallographic studies currently ongoing with phosphonamidates and hCAs, which will definitely clarify the binding mode of such a chemotype with these metalloenzymes. Therefore, this knowledge will be extended to visualise the reasons for benzenephosphonamidates selective action against β- over α-class enzymes.

4. Conclusions

The robust spread of antimicrobial resistance represents a main threat to human health. A primary strategy to combat it consists in the identification of novel therapeutic targets and anti-infectives with alternative mechanisms of action. The inhibition of the metalloenzymes carbonic anhydrases (CAs, EC 4.2.1.1) from pathogens among bacteria, fungi, and protozoa was shown to produce an impairment of the microorganism growth and virulence, whose targeting is deemed to prevent the evolutionary selective pressures that induce the pathogen to develop resistance. Unlike mammals and thus human, the genome of many pathogenic bacteria, fungi, and protozoa encode for β-class enzymes, which show significant structural diversities compared to α-class isozymes. As phosphonamidates have been recently validated as human α-CA inhibitors (CAIs) and no phosphorus-based zinc-binding group have been assessed to date against β-class CAs, herein we report an inhibition study with this class of compounds against β-CAs from pathogenic bacteria, fungi, and protozoa. Compounds 2–10 showed low to submicromolar Ks against most tested β-CAs, namely VChβ (Ks = 0.5–64.9 μM), FtuβCA (Ks = 6.9–55.3 μM), BpsCAβ (Ks = 2.1–32.7 μM), Can2 (Ks = 0.02–10.4 μM), CgNce (Ks = 0.05–20.2 μM), and LdcCA (Ks = 0.8–36.5 μM). As human isoforms CAs I and II are instead solely inhibited in a high micromolar range (32.8–961.2 μM), these data include phosphonamidates among the CAs most selective for β-class over human isozymes known to date, making them interesting leads for the development of new anti-infective agents. Crystallographic studies currently ongoing with phosphonamidates 2–10 and hCAs will definitely clarify the mechanism of action of this class of compounds probably shedding light on their selective efficacy against β- over α-class enzymes.

Disclosure statement

No potential conflict of interest was reported by the authors.

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