Bioorganic & Medicinal Chemistry Letters 18 (2008) 4708–4712

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Allosteric FBPase inhibitors gain 10^5 times in potency when simultaneously binding two neighboring AMP sites

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ARTICLE INFO

Article history:
Received 29 May 2008
Revised 27 June 2008
Accepted 30 June 2008
Available online 5 July 2008

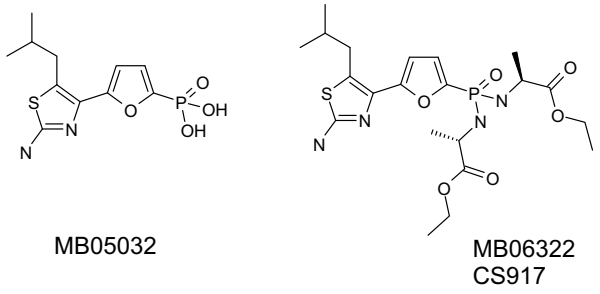
Keywords:
Fructose-1,6-bisphosphatase
Allosteric regulation
Cooperativity
Phosphate mimetic
Sulfonylureas

ABSTRACT

Human fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) is a key gluconeogenic enzyme, responsible for the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate, and thus presents an opportunity for the development of novel therapeutics focused on lowering the hepatic glucose production in type 2 diabetics. In its active form FBPase exists as a homotetramer and is allosterically regulated by AMP. In an HTS campaign aromatic sulfonylureas have been identified as FBPase inhibitors mimicking AMP. By bridging two adjacent allosteric binding sites using two aromatic sulfonylureas as anchor units and covalently linking them, it was possible to obtain dual binding AMP site inhibitors that exhibit a strong inhibitory effect.

The hydrophilic nature of AMP binding sites and their widespread use for allosteric regulation of enzymes in metabolic pathways has historically made the drug development of AMP mimetics difficult. By using a structure-based drug design strategy Metabasis discovered a series of furylphosphonates that mimic AMP (Scheme 1).[1,2] The lead compound, MB05032, exhibited high potency and specificity for human FBPase. Oral delivery of MB05032 was achieved by using the bisamidate prodrug MB06322 (CS-917), masking the strongly acidic phosphonate motif which hampers membrane permeation.[3]

As part of a program aimed at the discovery of FBPase inhibitors requiring no prodrug strategy, we performed a high-throughput screening campaign focusing on the allosteric AMP binding site. Inhibition of recombinant human liver FBPase activity was assayed using the Malachite Green procedure[4] to determine the inorganic phosphate release that results from dephosphorylation of fructose-1,6-bisphosphate.[5] By measuring FBPase inhibition with and without the catalytic site inhibitor fructose-2,6-bisphosphate (F-2,6-P) as well as by surface plasmon resonance competition experiments[6] with AMP,[7] we could identify a hit cluster of aromatic sulfonylureas that specifically bind to this site. The sulfide **1a** (Scheme 2) is a representative of this class and inhibits human liver FBPase with an IC_{50} value of 1.6 μM, which is further lowered by a factor 2.5 in the presence of F-2,6-P, characteristic of the cooperative binding of F-2,6-P and allosteric inhibitors.[8]



Scheme 1. Structure of the AMP mimicking FBPase inhibitor MB05032 and its prodrug CS917.

The high ligand binding efficiency of compound **1a** (0.47 kcal/mol/atom) in FBPase prompted us to further investigate this complex by X-ray crystallography. We were able to co-crystallize this compound with human liver FBPase and solve the complex structure to a resolution of 2.2 Å.[9] Figure 1 confirms that the sulfonylureas occupy all four allosteric AMP sites of the FBPase tetramer, each of which is ~30 Å away from the catalytic site of the respective monomer.

Interestingly, the structure reveals clear electron density between the thiol groups of two molecules **1a** that occupy the allosteric AMP sites of adjacent FBPase monomers. The two neighboring sulfur atoms are in covalent bond distance ($d = 2.02$ Å) suggesting a disulfide **1b** rather than two monosulfides **1a** occupy-

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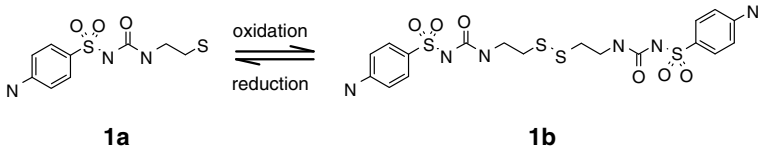
doi:10.1016/j.bmcl.2008.06.103



Scheme 2. Dimerization of sulfide **1a.**

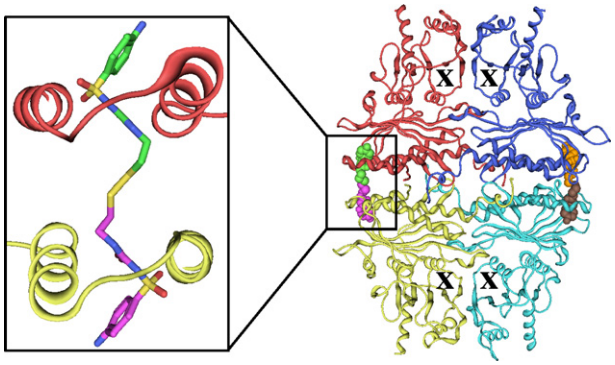
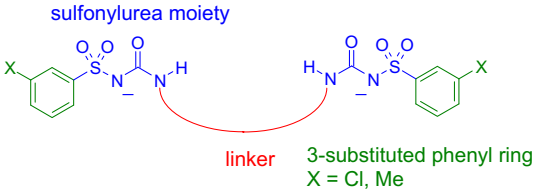


Figure 1. Tube diagram of the X-ray structure of human liver FBPase tetramer with bound **1b** (space filling representation) in the allosteric AMP sites. The four monomeric subunits are colored differently. X indicates the four catalytic sites of FBPase. The inset, rotated by 90° around the *y*-axis, shows the covalent S–S linkage of **1b** crossing the interface between two adjacent monomers.

ing the two neighboring AMP sites. Upon analysis by LC/MS the solid sample of the screening hit **1a** was found to be contaminated with about 5% disulfide **1b**, and the amount of disulfide in a 1:1 water/acetonitrile solution grew substantially within a few days at room temperature. This was assumed to be due to air oxidation of the thiol (Scheme 2).

Further analysis of the complex structure reveals that the aniline ring of the ligand sits in the hydrophobic cleft of the AMP binding site that is occupied by the adenine base in AMP (Fig. 2). The back part of this pocket is not filled and the para-amino group not engaged in polar interactions, indicating that a small meta-substituent could be a superior aromatic substitution for improving FBPase affinity. The polar sulfonylurea linker is engaged in several hydrogen bonds, with residues Thr31, Gly26, and a strongly interacting water molecule, and has the correct geometry to protrude through a narrow channel into the subunit interface region. This channel is formed by a short protein stretch Ala24–Gly28 connecting two adjacent α-helices. The phosphate recognition pocket of the AMP binding site is not occupied by **1b**, but by a water molecule, which interacts through four hydrogen bonds with one of the S=O groups of the ligand as well as Leu30, Lys112, and Tyr113. This is an elegant indirect way to fulfill the binding requirements of the very polar phosphate recognition pocket without the need for strongly acidic ligand functionalities, such as phosphates or phosphonates. A similar binding mode in which the ligand occupies both adenine binding site and interface region has been described recently for bisarylsulfonamides.[10,11] However, in this case the interaction between two ligand molecules of neighboring FBPase monomers is through non-covalent aromatic π–π interaction while our inhibitors span the interface by a covalent linkage.

Our experimental observation that two allosteric AMP binding sites can be occupied by a single molecule led us to explore a new inhibitor design concept for FBPase (Scheme 3). The concept relies on fairly optimized protein–ligand interactions in the classical AMP binding site with the search for an optimal covalent linker connecting two adjacent allosteric sites. It was hoped that the lower entropic cost of assembling only two instead of four ligands per FBPase tetramer could result in improved affinity and selectivity.[12] The concept of utilizing two symmetry-related identical binding sites in multimeric enzymes has been successfully employed in the design of ligands for, for example, 20S proteasome,[13] tryptase,[14] glutathione S-transferase,[15] AMPA receptor,[16], or glycogen phosphorylase (GP).[17] In the last example, two chloroindole amides in adjacent allosteric binding sites of the dimeric GP were covalently linked by an 8-atom 1,2-diethoxyethane chain, resulting in a 2000-fold potency improvement.

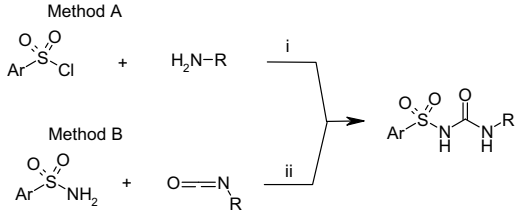


Scheme 3. General inhibitor design.

The synthesis of the test compounds was accomplished by known methods[18] (Scheme 4) starting from corresponding amines or diamines and reacting them with the complex derived from an aromatic sulfonylchloride obtained by reaction with sodium cyanate in the presence of pyridine (method A) or by the reaction of corresponding aliphatic isocyanates with sulfonamides in the presence of sodium hydride (method B).[19]

Physico-chemical properties of a symmetric, dianionic bissulfonylurea core structure as depicted in Scheme 3 are in a range (**6a**: pK_a: 5.35, log*D* (pH 7.4): −0.93, polar surface area: 128 Å^2; **7a**: pK_a: 5.12, log*D* (pH 7.4): −0.43, polar surface area: 128 Å^2) that might be expected to impair the pharmacokinetic performance of such compounds, especially with respect to oral bioavailability. Gratifyingly, oral bioavailability of $(CH_2)_6$ and $(CH_2)_7$ linked bissulfonylureas **6a** and **7b** was found to be almost quantitative in mice when applied as disodium salts.[20]

From the structural analysis of Figure 2 and work on other aryl sulfonylureas that bind to only one allosteric site, we identified 3-Cl and 3-Me substituted phenyls connected to sulfonylureas as good motifs to interact with the adenine binding site. As schematically drawn in Scheme 3, we investigated different linkers connecting these fragments with a focus on the influence of linker size, polarity, and rigidity. To quantify the change in inhibitory activity by covalent connection of two allosteric binding sites



Scheme 4. Preparation of mono and bissulfonylureas.

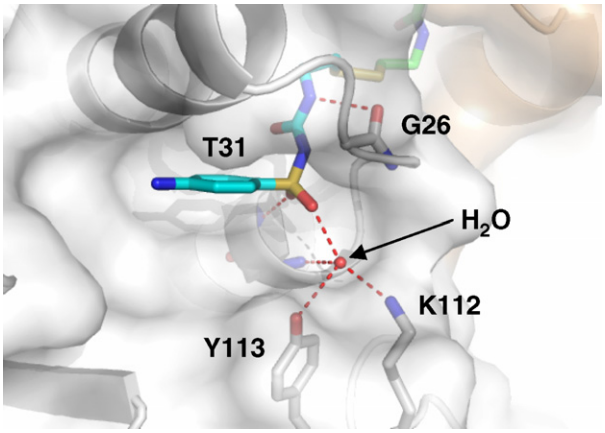


Figure 2. X-ray structure of the AMP allosteric site with bound inhibitor **1b**. Hydrogen bonds are displayed in dashed red lines.

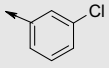
(termed 'dual-site binders'), control compounds that can only bind to one allosteric site (termed 'mono-site binders') but are otherwise similar to the dual-site binders were also synthesized.

Bissulfonylureas with alkyl linkers of varying chain length (C_2 to C_8) were first prepared, and show a consistent trend in FBPase inhibition (Table 1) for both the 3-chloro and 3-methylbenzene moiety. A U-shaped curve with an optimal linker length of 7 followed by 6 carbon atoms can be identified for both series (Fig. 3). This corresponds to the compounds **6a**,**b** and **7a**,**b**, which are among the most potent inhibitors of human liver FBPase to date with IC_{50} values in the low nanomolar range (9–24 nM). X-ray structures in the 3-chloro phenyl series could be solved for linker lengths C_5–C_8, and reveal a correlation of the linker length with the distances between the centers of the phenyl rings in the neighboring adenine binding sites. For C_5, the distance is only 17.1 Å, which is 0.7 Å below the average distance of the phenyl ring centers of mono-site sulfonylureas for which X-ray structures could be solved (data not shown). Obviously, a C_5 linker is too short to allow for relaxed, optimal interactions in the adenine binding site leading to an increase in IC_{50} (entry **5a**,**b** Table 1) compared to C_6 and C_7 linkers. On the other hand, the center distance seems too long (18.5 Å) for C_8 linkers.

Exemplary for the carbon-linked dual-site binders, Figure 4 shows the X-ray structure of bound **7a**.[9] As for the disulfide-bridged compound **1b**, the covalent linker spans the interface region and connects two adenine binding sites of adjacent FBPase monomers. As expected, the 3-chlorophenyl substitution points to the back of the adenine pocket to increase the number of protein–ligand contacts. The electron density in the linker region is less well defined than for the sulfonylurea anchors, indicating residual ligand flexibility in this region. This flexibility was incorporated into the coordinates using two low-energy conformations that were identified by a constrained conformational search of the linker.[22] The combined population of the two alternative linker conformations explains the observed electron density.

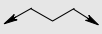
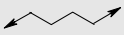
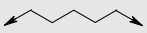
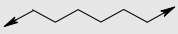
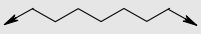
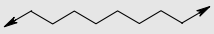
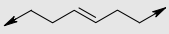
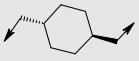
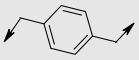
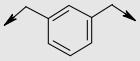
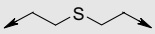
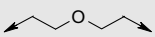
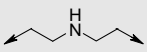
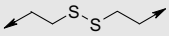
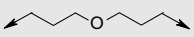
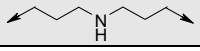
As demonstrated by our X-ray structures, the stoichiometry of binding for the bissulfonylureas is two ligands per FBPase tetramer

Table 1
FBPase inhibitory activity[21] of dual-site binding bissulfonylureas



x = a

x = b

Entry	Linker	HL Ic50 (μM)	HL Ic50 +F2,6P2 (μM)	HL Ic50 (μM)	HL Ic50 +F2,6P2 (μM)
2x		nd	nd	>100	>100
3x		nd	nd	>100	38.53
4x		2.755	0.264	3.396	0.542
5x		0.05	0.019	0.072	0.022
6x		0.017	0.008	0.024	0.012
7x		0.009	0.004	0.012	0.005
8x		0.045	0.017	0.035	0.008
9x		0.04	0.014	0.068	0.028
10x		nd	nd	0.095	0.012
11x		0.028	0.016	nd	nd
12x		0.006	0.004	0.003	0.002
13x		nd	nd	0.012	0.006
14x		nd	nd	0.07	0.018
15x		nd	nd	0.827	0.151
16x		nd	nd	>100	>100
17x		0.055	0.01	0.029	0.012
18x		0.125	0.057	nd	nd
19x		43.809	13.727	nd	nd

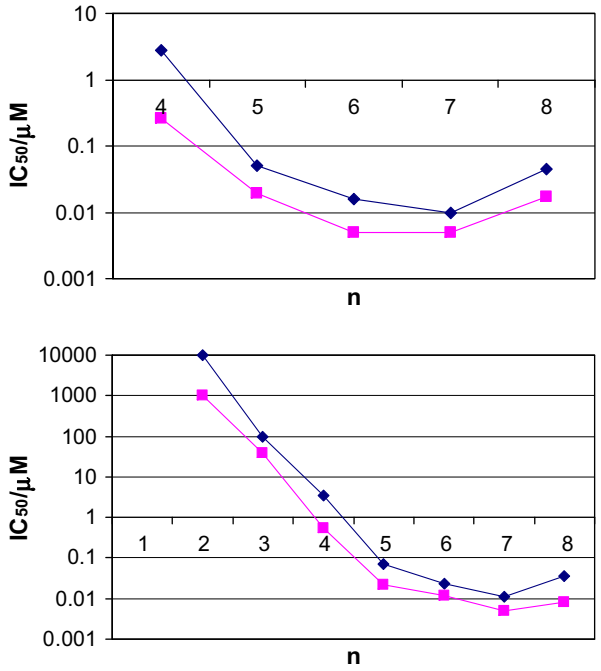


Figure 3. FBPase inhibitory activity (µM) vs. linker chain length (*n*) of dual-site binding bissulfonylureas with (square, magenta) and without (rhombus, blue) the catalytic site inhibitor fructose-2,6-bisphosphate (F-2,6-P). Both *m*-Cl phenyl (top) and *m*-CH3 phenyl (bottom) show a U-shaped curve. The concentration scale (*y*-axis) is logarithmic.

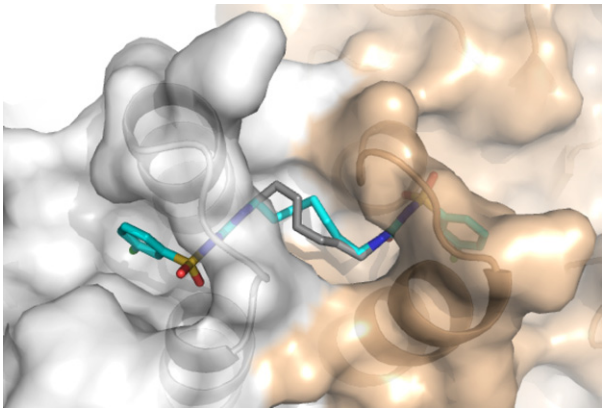


Figure 4. X-ray structure of the AMP allosteric site with bound inhibitor **7a**.

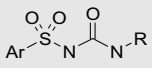
instead of four as seen so far for allosteric AMP site binders. The difference of two versus four binding events for dual-site and mono-site binders, respectively, is likely responsible for the qualitatively different cooperativity behavior that was observed in FBPase inhibition assays. The two types of inhibitors produced sigmoidal dose–response curves with a slope for dual-site binders that was consistently half of that for mono-site binders.[23]

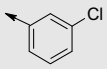
Substituting one or two carbon atoms of the linker by heteroatoms can considerably affect IC_{50} values (Table 1). The disulfide**s** **17a**,**b** are potent inhibitors, but are less active than their straight carbon analogs **6a**,**b**, suggesting that the disulfide moiety does not play a significant role in the binding apart from connecting the two sulfonylurea subunits. More polar replacements such as the ether oxygen in **15b** and **18a**, and in particular the positively charged amine nitrogen in **16b** and **19a**, lead to a significant decrease, or almost complete loss in potency. Inspection of Figure 4 shows that the binding site in the linker region is narrow enough that at least a part of the solvation shell of the linker heteroatom is stripped away without compensation by polar protein–ligand interactions. We hypothesize that the strong dependence on the polarity of the linker is caused by poorly compensated desolvation penalties for ether and amine linkers.

Preorganizing the predominantly bound ligand conformation through structure rigidification can be a successful strategy to improve binding affinities.[24] Keeping the chain length close to the optimum, we have explored double (entries **9a**,**b**) and triple bond (entry **10b**) insertions in the linker to investigate the transferability of this concept to our series. As can be seen from Table 1, all these conformationally restricted analogs reduce FBPase activity relative to the more flexible **6a**,**b**. Ring insertion to rigidify the linker was also assessed (entries **11**–**13**), and it either reduced (–CH_2-cyclohexyl-CH_2–) or only slightly improved (–CH_2-phenyl-CH_2–) the activity. In the comparison of **6a**,**b** with **11**–**13** it should be kept in mind that the latter compounds contain two additional heavy atoms that can profit from additional hydrophobic interactions. Overall, these results suggest that any possible preorganization is outweighed by the hindrance of an optimal geometrical orientation of the two anchor units. Moreover, our finding that several linker conformations rather than a predominant one should be present in the bound complex reduces the potential affinity gain through conformational restriction.

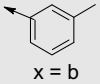
The comparison of the IC_{50} values for the dual-site binders with their respective control compounds (Table 2), which occupy only one allosteric binding site, reveals a dramatic difference. For example, the highly potent C_6 linked bissulfonylurea **6a** (17 nM) has a more than four orders of magnitude (4.1×10^4) greater inhibitory potency relative to the corresponding monomer (**20a**; 690 µM), which is virtually inactive. Surface plasmon resonance measurements of the dissociation constants of **6a** and **20a** to human liver

Table 2
FBPase inhibitory activity[21] of mono-site binders

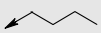
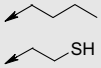
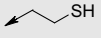




x = a



x = b

Entry	R	HL Ic50 (µM)	HL Ic50+F2,6P2 (µM)	HL Ic50 (µM)	HL Ic50 +F2,6P2 (µM)
20x		690.276	234.231	>1000	320.18
21x		196.207	134.18	177.856	86.763
22x		nd	nd	20.481	10.589
23x		514.756	277.143	nd	nd

FBPase confirm this drastic effect of covalent linkage, however, to a smaller extent (1.7×10^3). The observed differences in binding free energy of 6.3 and 4.4 kcal/mol, respectively, cannot be accounted for by significantly different intermolecular interactions as the atom count for the two monomers and the dimer are the same and the protein–ligand contacts in the linker region are relatively sparse (Fig. 4).

To better understand this finding, we performed MM-PBSA free energy calculations which can give an estimate of the different contributions to the free energy of binding.[25] The calculated difference in total binding free energy (6.2 kcal/mol)[26] for the human liver FBpase complex with four mono-site ligands **20a** and two dual-site ligands **6a**, respectively, is in good agreement with the experimental results. The individual free energy contributions suggest a more favorable entropy balance as the dominant contribution for the lower inhibition constants of the dual-site binders. This is due to the large translational and rotational entropy penalties for assembling four mono-site instead of two dual-site binders with one FBPase tetramer that are only partly compensated by vibrational entropy gains.

In summary, we discovered that the micromolar affinity of the screening hit **1a** to human FBPase was due to a contamination with the oxidative disulfide dimer **1b.** As shown by X-ray crystallography, the active compound **1b** spans two adjacent AMP binding sites in the FBPase tetramer. This finding led to a new inhibitor design concept covalently connecting two sulfonylurea moieties with linkers of appropriate lengths. Straight chain hexa- and hepta-methylene linkers proved optimal leading to nanomolar inhibitors of FBPase.

Acknowledgments

The authors gratefully acknowledge Urs Weiss for chemical syntheses, Tim Tetaz and Bruno Fol for enzyme preparation, as well as Annie Sellam and Tamara Codilupi for in vitro testing. Crystallographic data were collected on beam line X10SA at the Swiss Light Source.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.06.103.

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