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

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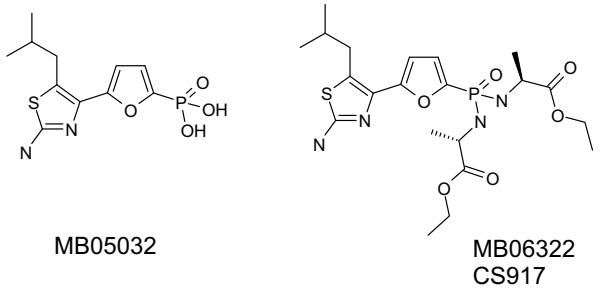
ABSTRACT

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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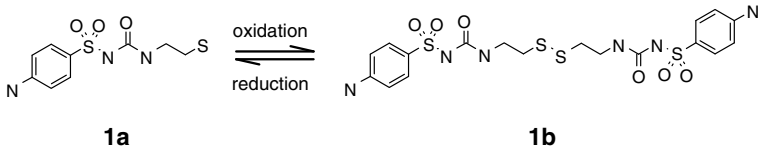


Scheme 2. Dimerization of sulfide **1a.**

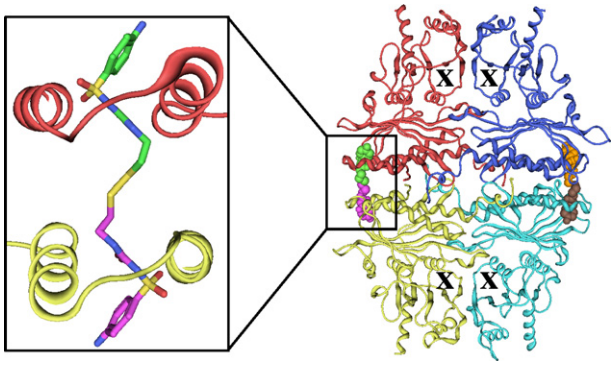
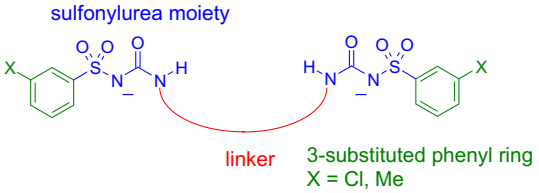


Figure 1. Tube diagram of the X-ray structure of human liver FBPase tetramer with bound **1b** (space filling representation) in the allosteric AMP sites. The four monomeric subunits are colored differently. X indicates the four catalytic sites of FBPase. The inset, rotated by 90° around the *y*-axis,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

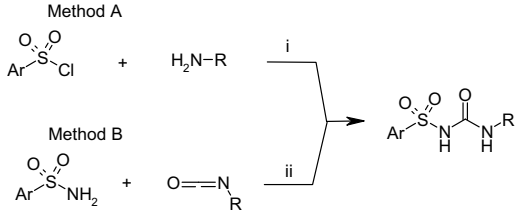


Scheme 3. General inhibitor design.

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.