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3-(2-Carboxy-ethyl)-4,6-dichloro-1*H*-indole-2-carboxylic Acid: An Allosteric Inhibitor of Fructose-1,6-bisphosphatase at the AMP Site

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Abstract—3-(2-Carboxyethyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid (MDL-29951), an antagonist of the glycine site of the NMDA receptor, has been found to be an allosteric inhibitor of the enzyme fructose 1,6-bisphosphatase. The compound binds at the AMP regulatory site by X-ray crystallography. This represents a new approach to inhibition of fructose 1,6-bisphosphatase and serves as a lead for further drug design.

Fructose-1,6-bisphosphatase (F16BPase) is an enzyme expressed predominantly in the liver and kidney, and is one of the rate limiting enzymes of hepatic gluconeogenesis. Liver F16BPase activity is elevated in insulin-deficient and insulin-resistant animal models of diabetes, highlighting the importance of this enzyme in the control of blood glucose.[1] The physiologically relevant form of F16BPase is a homotetramer,[2] which exists in distict conformational states (T and R), depending on the relative concentrations of active site and allosteric site ligands. It is subject to competitive substrate inhibition by fructose-2,6-bisphosphate[3] and to allosteric inhibition by adenosine-5-monophosphate (AMP), which afford the inactive T-state conformation.[4] The kinetically active conformer (R state) is stabilized by fructose 1,6-bisphosphate. The regulation and molecular basis of F16BPase enzyme activity has been deduced from crystallographic studies utilizing the recombinant human fructose-1,6-bisphosphatase protein.[5] A F16BPase inhibitor should reduce hepatic glucose output and lower blood glucose by inhibiting the elevated rate of gluconeogenesis present in diabetic patients, and would thus represent a useful therapy for the treatment of Type 2 diabetes. To this end, the naturally occurring AMP analogue ZMP (AICA-Riboside monophosphate)[6] as well as a number of synthetic purine and other heterocyclic phosphonic acids have been described as potential antidiabetic agents,[7] as have a series of piperazinediones.[8]

We sought an allosteric, low molecular weight (<500) inhibitor of F16BPase that was not a phosphonic acid or phosphate ester. Recently, we reported[9] that a series of anilinoquinazolines, currently of interest as inhibitors of tyrosine kinases, were identified as allosteric inhibitors of F16Bpase by screening a library of compounds known to be AMP and/or ATP competitive enzyme inhibitors against purified recombinant human F16BPase.[10] As a result of subsequent file screening, we identified 3-(2-carboxyethyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid (MDL-29951, **1**), an antagonist of the glycine site of the NMDA receptor,[11] as an inhibitor of F16Bpase (Fig. 1).

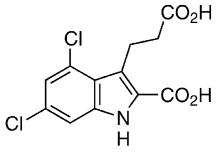

Figure 1. Compound **1**.

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F16BPase activity was assayed by measuring the inorganic phosphate hydrolyzed from fructose-1,6-bisphosphate by the enzyme. The phosphate released was quantified spectrophotometrically as a complex with ammonium molybdate and malachite green.[12] The assay was run under saturating concentrations of substrate (500 μM) due to the low K_m of F16BPase for its substrate and the sensitivity of the phosphate detection method. Under these conditions, the assay was linear with time and enzyme concentration, and it was able to detect inhibition of F16BPase by AMP ($IC_{50} = 0.8$ μM). MDL-29951 (**1**) was found to inhibit the human F16Bpase under these conditions ($IC_{50} = 2.5$ μM).

It was considered unlikely that **1** binds at the F16BPase active site in as much as enzyme inhibition is observed at substrate concentrations well above saturation. In order to better understand the binding mode of our compounds, we co-crystallized recombinant porcine kidney F16BPase with **1** in the presence of manganous chloride, and fructose-6-phosphate.[13] The space group of these crystals at 2.15 angstron resolution was found to be C2 wth unit cell dimensions $a = 118.87$, $b = 73.45$, $c = 78.02$ Å, $\beta = 106.2°$ with two monomers of FBPase in the crystallographic asymmetric unit. The structure was solved by molecular replacement using the program AmoRe,[14] as implemented in the CCP4 suite of software.[15] The starting model for molecular replacement was the structure of T-state F16BP complexed with AMP and F6P (pdb reference 1FBP).[16] Examination of difference Fourier maps, after rigid body and individual atom positional refinement of the protein atoms showed clear density for inhibitor and F6P at the allosteric and substrate binding sites respectively. Two Mn^{2+} ions could also be placed in the electron density. The structure was refined using REFMAC[17] to a final *R*-factor of 0.208 and free *R*-factor of 0.283.

Our structure reveals that **1** binds at the same site as the allosteric inhibitor AMP (Fig. 2), and presumably achieves inhibition by the same mechanism as AMP. The indole ring of the inhibitor occupies the same shallow hydrophobic pocket as the purine ring of AMP, and the indole nitrogen N1 makes the same hydrogen bond with Thr31 as N1 of AMP. The pocket occupied by the inhibitor is deeper than that occupied by AMP. This appears to occur because Met177 adopts a rotamer different from that found in the AMP bound structure, which allows the 6-chloro substituent on the indole to be completely buried.

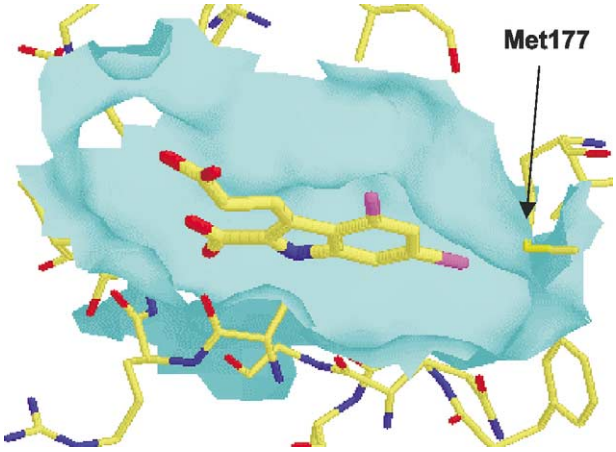


Figure 2. X-Ray crystal structure of **1** in porcine kidney F16BPase. **1** is colored by atom type.

The indole 2-position carboxyl group makes a hydrogen bond with a backbone amide nitrogen of Gly28, and also makes a hydrogen bond to a water molecule disposed between the carboxyl group and the side chain of Tyr113. The indole 2-position carboxylic acid extends in the direction of, but does not occupy, the AMP phosphate binding region (Fig. 3). The carboxylic acid residue extending from the indole 3-position is in an extended conformation, pointing out to the solvent channel normally occupied by the ribose ring of AMP in the T-state enzyme, and making a hydrogen bond to the phenolic hydroxyl group of Tyr113.

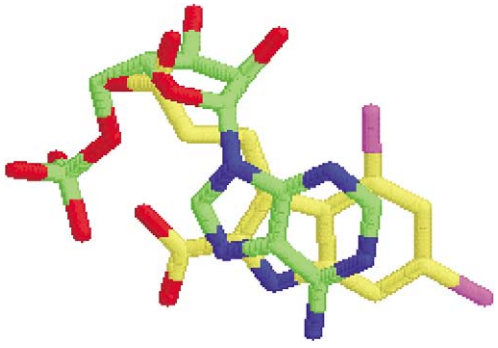
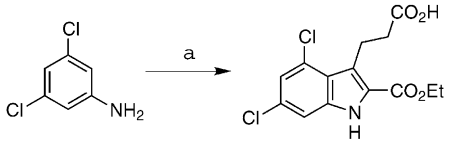


Figure 3.

Some derivatives were prepared to examine the effects of modification at the two carboxylic acid containing residues. MDL-29951 (**1**) and analogues were prepared from 3,5-dichloroaniline by Japp–Klingmann indole synthesis and subsequent standard functional group manipulation of the differentially protected intermediate **10** as reported in the literature (Scheme 1).[18] All products were characterized by ^1H NMR and APCI MS.

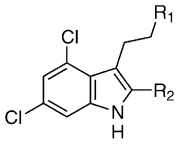


10

Scheme 1. (a) (i) $NaNO_2$, HCl, H_2O, 0 °C; (ii) NaOAc, ethyl 2-oxocyclopentanecarboxylate, 15 °C; (iii) Et_3N, H_2O, reflux; (iv) p-TsOH, toluene, reflux.

The carboxypropyl side chain at the indole 3-position was found to be relatively tolerant of functional groups that retained a polar hydrogen bonding array at this position (Table 1, entries 1–6). For example, the amides **2** and **3**, the hydroxamic acid **4**, and the alcohol **6** all were similar in potency to **1**. In contrast, the carboxyl group at the indole 2-position was found to be very restrictive (Table 1, entries 7–11). Only the carboxylic acid group was tolerated at this position.

Table 1. Effect of carboxyl modification on F16BPase inhibition



Compd	R_1	R_2	F16BPase IC_{50}, μM[a]
1	CO_2H	CO_2H	2.5
2	$CONH_2$	CO_2H	2.2
3	$CONMe_2$	CO_2H	1.7
4	CONHOH	CO_2H	1.5
5	CO_2Me	CO_2H	7.0
6	CH_2OH	CO_2H	2.7
7	CO_2H	$CONH_2$	≫10
8	CO_2H	$CONMe_2$	≫10
9	CO_2H	CONHOH	≫10
10	CO_2H	CO_2Et	≫10
11	CO_2H	CH_2OH	≫10

[a]Values are means of three experiments. An IC_{50} of ≫10 indicates that no curve was noted in the dose response up to 10 μM.

Methylation of the indole nitrogen to furnish **12** also eliminated F16BPase inhibitory activity (IC_{50}≫10 μM) (Fig. 4).

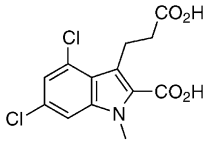


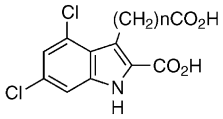
Figure 4. Compound **12**.

The length of the alkyl chain extending from the indole 3-position was modified to afford both chain-shortened and chain-elongated analogues of **1** (Table 2). One methylene unit (**13**) was not sufficient to retain activity, whereas longer chains (**14** and **15**) had inhibitory properties similar to those of **1**.

In general, the sequences of F16BPase are conserved between mammalian species (e.g., identity of human and rabbit = 91%). A comparison of the enzyme inhibitory potency of **1** against some F16BPase isozymes revealed that **1** inhibited the human liver (IC_{50} = 2.5 μM), porcine kidney[19] (IC_{50} = 1.0 μM), and rabbit liver[20] (IC_{50} = 0.21 μM) isoforms of the enzyme, but was significantly less potent against the rat liver[21] isoform (IC_{50} = 11 μM).

3-(2-Carboxyethyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid (**1**) represents the first low molecular weight (< 500) AMP site allosteric inhibitor of fructose 1,6-bisphosphatase that is not a phosphorus-based AMP mimic. Given that **1** is a small molecule with known drug-like properties, it represents a new approach to inhibition of F16BPase. Enzyme inhibition is achieved by binding the AMP binding site, with a similar hydrogen bond network but deeper hydrophobic pocket than found in the AMP bound structure. These compounds represent potential leads for improving potency and selectivity for fructose 1,6-bisphosphatase inhibition.

Table 2. Effect of linker length modification on F16BPase inhibition



Compd	*n*	F16BPase IC_{50}, μM[a]
13	1	≫10
1	2	2.5
14	3	1.0
15	4	1.2

[a]Values are means of three experiments. An IC_{50} of ≫10 indicates that no curve was noted in the dose response up to 10 μM.

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