Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 17–21

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Allosteric Inhibition of Fructose-1,6-bisphosphatase by Anilinoquinazolines

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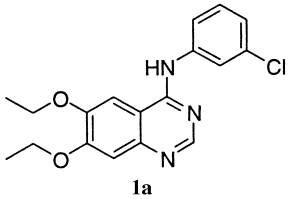
Received 8 August 2000; accepted 6 October 2000

Abstract—Anilinoquinazolines currently of interest as inhibitors of tyrosine kinases have been found to be allosteric inhibitors of the enzyme fructose 1,6-bisphosphatase. These represent a new approach to inhibition of F16BPase and serve as leads for further drug design. Enzyme inhibition is achieved by binding at an unidentified allosteric site.

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 is subject to competitive substrate inhibition by fructose-2,6-bisphosphate[3] and to allosteric inhibition by AMP.[4] 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 has 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. We screened a library of compounds known to be AMP and/or ATP competitive enzyme inhibitors against purified recombinant human F16BPase.[9] 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.[10] 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 ($IC_{50} = 0.8\ \mu M$). As a result of this effort we identified the anilinoquinazoline **1a** as an inhibitor of F16BPase ($IC_{50} = 1.6\ \mu M$). Compound **1a** and numerous similar anilinoquinazolines have already been reported to be extremely potent, nanomolar inhibitors of the epidermal growth factor receptor (EGFR) family of tyrosine kinases (Fig. 1).[11]



1a

Figure 1.

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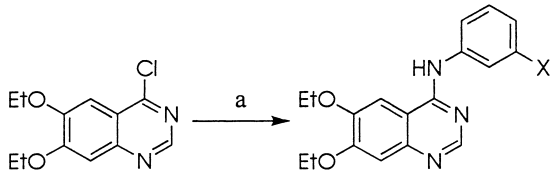
0960-894X/01/$ - see front matter
PII: S0960-894X(00)00586-2

Chemistry

Analogues of **1a** were prepared using parallel synthesis in the conventional manner from the appropriate chloroquinazolines **2** and anilines **3** in ethanol at reflux (Scheme 1).[12] The products of these reactions precipitated from the reaction mixture in analytically pure form in almost all cases and were isolated by filtration and washing with additional ethanol. All products were characterized by ^{1}H NMR and APCI MS.



Scheme 1. (a) $H_2NC_6H_4X$ (**3**), EtOH, reflux.

Biological Results

We prepared analogues to examine the effects of substituents at various parts of the molecule. Our initial efforts focused upon the SAR of the aniline ring. This position of the molecule was found to be intolerant of any substituents other than chlorine (**1a**), fluorine (**1b**), bromine (**1c**), iodine (**1d**), and alkyne (**1e**) (Table 1). Surprisingly, even a nitrile could not be substituted for halogen or alkyne at this position. Entry **1g** demonstrates that the halogen atom is required for activity. Likewise, analogues of **1a** were prepared which incorporated one or more substituents in addition to the *meta* chlorine substituent. Of these, only one active compound was identified (**1h**), with a fluorine substituent adjacent to the *meta* chlorine substituent. This compound was somewhat more potent than the parent **1a**. Other groups, such as methyl or chloro, at the *para* position were not tolerated, either singly or in combination with a halogen atom at the *meta* position (for example, **1i** and **1j**, Fig. 2, Table 1). No groups were tolerated at other ring positions. These results, remarkably similar to those previously observed for tyrosine kinase inhibition by these compounds, suggest that this ring may fit into a small hydrophobic pocket at the binding site.

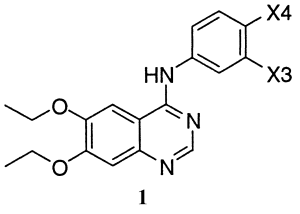


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Figure 2.

Table 1. Effect of aniline ring substituents on F16BPase inhibition

Compound	X3	X4	F16BPase IC_{50}, μM[a]
1a	Cl	H	1.6
1b	F	H	9.2
1c	Br	H	1.7
1d	I	H	2.1
1e	CCH	H	2.4
1f	CN	H	>10
1g	H	H	>10
1h	Cl	F	0.53
1i	Cl	Me	>10
1j	Cl	Cl	>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.

A set of 45 analogues in which the *N*-phenyl bond was interrupted by one or more sp^3 carbon atoms was prepared using a variety of substituted benzylamines, phenethylamines, and phenylpropylamines. All of these analogues were inactive. In contrast to results observed with tyrosine kinase inhibitors, the NH residue was found to be critical for F16BPase inhibition. Replacement of the NH group, either by alkylation as an *N*-methyl group or by incorporation into an indoline ring, resulted in the loss of activity.

The quinazoline 6- and 7- positions were more flexible in their SAR requirements (Fig. 3, Table 2). A variety of alkoxy substituents were found to be tolerated in various combinations, although the SAR at this position was more restrictive than that required for potent tyrosine kinase inhibition. Potency was greatest with a 6,7-dimethoxy pattern. Other substituents besides alkoxy groups were not well tolerated. Substituents at the quinazoline 5- and 8- positions were not tolerated singly or in combination with substituents at the other quinazoline ring positions.

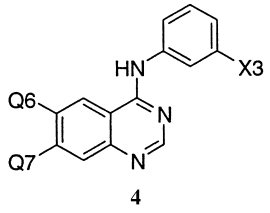

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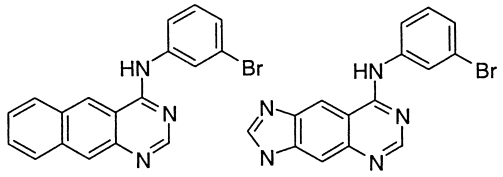
Figure 3.

Table 2. Effect of quinazoline ring substituents on F16BPase inhibition

Compound	X3	Q6	Q7	F16BPase IC_{50}, μM[a]
1a	Cl	EtO	EtO	1.6
1c	Br	EtO	EtO	1.7
4a	Cl	MeO	MeO	1.3
4b	Br	MeO	MeO	1.0
4c	Br	2-PrO	H	>10
4d	Br	*n*-BuO	H	>10
4e	Br	MEE[b]	H	2.9
4f	Br	MEE	MeO	5.5
4g	Br	MEE	MEE	>10
4h	CCH	MeO	MeO	0.90
4i	Br	H_2N	Cl	11.9
4j	Br	H	CO_2H	>10
4k	Br	NO_2	Cl	>10
4l	Br	NO_2	EtO	3.6
4m	Br	NO_2	MeO	>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.
[b]MEE = 2-Methoxyethoxy, $CH_3OCH_2CH_2O$.

The fusion of an additional ring onto the quinazoline ring gave diminished potency as compared to 6,7-dialkoxy substitution. For example, the fusion of an additional benzene ring (**4n**, $IC_{50} = 7.5\,\mu M$) or an imidazole ring (**4o**, $IC_{50} = 4.0\,\mu M$) gave compounds with reduced potency compared to the diethoxy (**1c**) and dimethoxy (**4b**) analogues (Fig. 4).



4n **4o**

Figure 4.

A relatively restrictive SAR was observed at the quinazoline 2-position. The addition of a methyl group at this position (**5a**) diminished activity, while substituents other than H or methyl at the quinazoline 2-position resulted in the loss of F16BPase inhibitory activity (Fig. 5, Table 3). Again, this closely parallels the tyrosine kinase inhibitor SAR, where any substitution at the quinazoline 2-position eliminates activity.

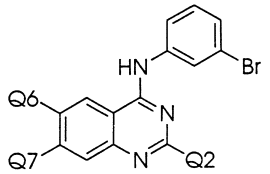


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Figure 5.

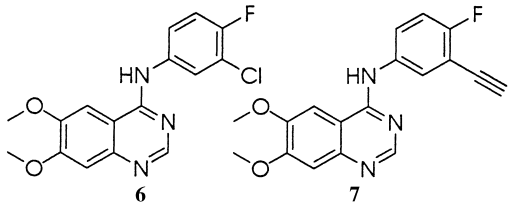
Table 3. Effect of quinazoline ring 2-substituent on F16BPase inhibition

Compound	Q2	Q6	Q7	F16BPase IC_{50}, μM[a]
5a	Me	MeO	MeO	4.5
5b	NEt_2	MeO	MeO	>10
5c	Bn	EtO	EtO	>10
5d	Ph	EtO	EtO	>10
5e	*i*-Bu	EtO	EtO	>10
5f	CH_2Cl	EtO	EtO	>10
5g	CH_2SO_2Me	EtO	EtO	>10
5h	CH_2NEt_2	EtO	EtO	>10
5i	CH_2morpholine	EtO	EtO	>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.

Optimum potency was achieved by the addition of a fluorine substituent to the dimethoxy quinazolines **4a** and **4h**, to afford **6** ($IC_{50} = 0.29\,\mu M$) and **7** ($IC_{50} = 0.25\,\mu M$), respectively (Fig. 6).

It is unlikely that the anilinoquinazolines bind at the F16BPase active site in as much as enzyme inhibition is observed at substrate concentrations well above saturation. These compounds have been shown to inhibit the



6 **7**

Figure 6.

EGFR tyrosine kinase through competition at the ATP binding site.[13] Binding of **4b** to the ATP site is accomplished by the formation of two hydrogen bonds to the kinase and the placement of the bromoanilino substituent into a deep hydrophobic pocket adjacent to the ATP binding site which is not used by ATP. Similar binding to the ATP binding sites of kinases by inhibitors which bind primarily by the occupancy of unused hydrophobic space have been reported for p38 mitogen-activated protein kinase and for the VEGF tyrosine kinase.[14] It therefore seemed reasonable to consider the possibility that these compounds were binding to the AMP allosteric site in F16BPase, by analogy to their binding at the ATP site in the tyrosine kinases. Unlike the tyrosine kinases, however, competitive inhibition studies with AMP cannot be carried out with F16BPase, as the enzyme is negatively regulated by AMP. X-ray crystallography was unsuccessful due to the low solubility of these compounds. NMR experiments designed to demonstrate that anilinoquinazolines such as **1a** bind to the AMP allosteric site in human F16BPase were performed. AMP binding to the enzyme was observed, and furthermore, ZMP was shown to compete with AMP for allosteric site binding, in agreement with published observations.[15] Compounds such as **1a** appeared to compete with AMP at low concentrations (0.1 μM); however, the compounds were too insoluble for further titration. As a consequence the results could not be further confirmed. Experiments with more soluble analogues were confounded by the observations that these compounds appear to π-stack in solution and interact non-specifically with AMP in the absence of protein. In order to determine whether AMP site binding was a possibility, we docked compound **4h** into the AMP binding site of human liver F16BPase.[16] As a preliminary control experiment, we used Autodock[17] version 2.41 to dock AMP into the site. The program PS-GVB[18] was used to optimize the starting structure of AMP. Thirty-two Autodock runs of 30,000 steps were run, with the AMP oriented randomly within the AMP binding pocket; the ribose and phosphate torsions were allowed to move. Eight distinct conformational clusters were generated. The lowest energy cluster was reproduced 13 times and had an RMS error from the crystal structure of only 0.8 Å. Following this success, the compound **4h** was docked into the active site using the same methodology. In order to maintain a favorable conformation, we kept all torsions planar except the amino–phenyl torsion, which was allowed to rotate during the simulation; all rigid-body translational and rotational degrees of freedom were sampled. In this simulation, the 32 runs gave 10 distinct conformational clusters. The lowest energy conformation, shown in

Figure 7, was more than 5 kcal/mol more favorable than the others. Unlike the higher energy conformations, this conformation is consistent with many SAR features: (1) the methoxy groups are exposed to solvent; the more hydrophobic ethoxy groups are less potent; (2) the aniline nitrogen is in contact with the backbone carbonyl of Glu 12 and substitution is not allowed; (3) the quinazoline 2-position is close to Val 144 and only small substitutions would be tolerated; and (4) the aniline aromatic ring is buried deeply in the adenine binding pocket with little room for substitution. It will be noted that only two hydrogen bonds are possible with this binding model, as opposed to the 12 hydrogen bonds made by AMP with F16BPase.[19] The results of this docking experiment suggest that these compounds are capable of fitting the AMP site in a manner similar to that observed with the tyrosine kinases and that is consistent with the observed SAR. However, the docking results are not sufficient to be taken as proof that these compounds bind at the AMP site, nor do they preclude the possibility of binding elsewhere.

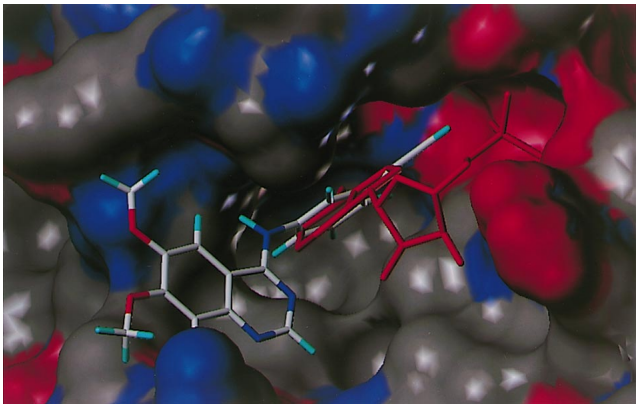


Figure 7. Model in human F16BP structure. **4h** is colored by atom type, the bound AMP is shown for reference in red.

In general, the sequences of F16BPase are conserved between mammalian species (e.g., identity of human and rabbit = 91%). Therefore, it was surprising that when these compounds were tested against F16BPases isolated from other species, large differences in activity were observed. A comparison of the enzyme inhibitory potency of selected analogues against various F16BPase isozymes is shown in Table 4. In general, the anilinoquinazolines were less potent against the porcine kidney isoform of the enzyme,[20] and entirely inactive against the rat liver[21] and rabbit liver[22] isoforms.

Conclusions

Anilinoquinazolines such as **1a**, **6**, and **7** represent the first low molecular weight (< 500) inhibitors of fructose 1,6-bisphosphatase that are not substrate mimics or AMP analogues. Given that they are small molecules with submicromolar potency and drug-like properties, they represent a new approach to inhibition of F16BPase and serve as leads for further drug design. Enzyme inhibition is achieved by binding at an as-yet unidentified allosteric site. Selectivity for F16BPase inhibition is clearly an issue with these compounds, since many (e.g., **1a**–**1j**, **4a**–**4m**, **6**, and **7**) are highly potent inhibitors of EGFR tyrosine kinase, with IC_{50} values of 5 nM or lower. Further work on these compounds directed towards improving selectivity, aqueous solubility, and potency for fructose 1,6-bisphosphatase inhibition is in progress.

Table 4. Inhibition of species isoforms of F16BPase[a]

Compound	Human liver	Rabbit liver	Rat liver	Porcine kidney
1a	1.6	>10	>10	3.9
1c	1.7	>10	>10	>10
1h	0.53	>10	>10	3.1
4a	1.3	>10	>10	>10
4b	1.0	>10	>10	>10
4h	0.90	>10	>10	1.6
6	0.29	>10	>10	0.34
7	0.25	>10	>10	0.77

[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.

Acknowledgements

The authors wish to thank Dr. George A. Karam and Dr. Walter C. Soeller for expression and purification of the recombinant human, rabbit, and rat F16BPase enzymes. The authors wish to thank Professor Evan R. Kantrowitz (Boston College) for a supply of porcine kidney F16BPase. The authors wish to thank Professor M. Raafat El-Maghrabi (SUNY Stony Brook) for kind provision of the human and rat F16BPase cDNAs.

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