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Potent and selective biphenyl azole inhibitors of adipocyte fatty acid binding protein (aFABP)

Richard Sulsky,[a,*] David R. Magnin,[a] Yanting Huang,[a] Ligaya Simpkins,[a] Prakash Taunk,[a] Manorama Patel,[a] Yeheng Zhu,[a] Terry R. Stouch,[b] Donna Bassolino-Klimas,[b] Rex Parker,[c] Thomas Harrity,[c] Robert Stoffel,[c] David S. Taylor,[c] Thomas B. Lavoie,[d] Kevin Kish,[d] Bruce L. Jacobson,[e] Steven Sheriff,[d] Leonard P. Adam,[c,*] William R. Ewing[a] and Jeffrey A. Robl[a]

[a]*Department of Metabolic Disease Chemistry, Bristol Myers-Squibb Pharmaceutical Research Institute, PO Box 5400, Princeton, NJ 08543-5400, USA*
[b]*Department of Computationally Assisted Drug Discovery, Bristol Myers-Squibb Pharmaceutical Research Institute, PO Box 5400, Princeton, NJ 08543-5400, USA*
[c]*Department of Metabolic Disease Biology, Bristol Myers-Squibb Pharmaceutical Research Institute, PO Box 5400, Princeton, NJ 08543-5400, USA*
[d]*Department of Macromolecular Structure, Bristol Myers-Squibb Pharmaceutical Research Institute, PO Box 5400, Princeton, NJ 08543-5400, USA*
[e]*St. Cloud State University, Department of Biological Sciences, 262 Wick Science Building 720, 4th Avenue South, St. Cloud, MN 56301-4498, USA*

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Abstract—Herein we report the first disclosure of biphenyl azoles that are nanomolar binders of adipocyte fatty acid binding protein (aFABP or aP2) with up to thousand-fold selectivity against muscle fatty acid binding protein and epidermal fatty acid binding protein. In addition a new radio-ligand to determine binding against the three fatty acid binding proteins was also synthesized.

Adipocyte fatty acid binding protein (aFABP, aP2) is a 14.6 kDa cytosolic protein located in adipocytes and macrophages, and assists in the intracellular transport of fatty acids.[1a,b–d] It is one of a class of fatty acid binding proteins (FABPs) that are found predominately in the liver, heart, intestine, and connective tissues. Hotamisligal et al. have reported that aFABP-deficient mice, when placed on a high fat diet (40% of caloric intake as fat), were significantly protected from hyperinsulinemia and insulin resistance compared to the wild type.[2] Additional genetic experiments have been reported in which aFABP null mice have been crossed with *ob*/*ob* and in another instance apoE$^{-/-}$ mice. The aFABP-deficient *ob*/*ob* mice were more insulin-sensitive when compared to *ob*/*ob* controls as demonstrated by measuring circulating glucose and insulin levels.[3] While apoE$^{-/-}$ animals developed severe atherosclerosis on a high fat diet, the apoE$^{-/-}$/aFABP$^{-/-}$ progeny have lesions that are much smaller and less complex, suggesting a role for aP2 in atherosclerosis.[4] Based on these genetic knock-out models, we pursued the development of inhibitors of aFABP for their therapeutic potential in the treatment of diabetes, obesity, and atherosclerosis.

Structurally, aFABP is a 10-stranded, 136-amino acid, 2-sheeted anti-parallel β-barrel with two short helixes forming a 'cap'. The protein contains essentially one large cavity with a volume of approximately 950 cubic angstroms. From crystallographic data of ligands bound to the protein, the interior amino acids that are known to specifically bind to endogenous fatty acids include

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* Corresponding author. Tel.: +609 818 4743; e-mail: sulskyr@bms.com

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Arg126 and Tyr128 (Fig. 2), which usually ligate an acidic moiety directly, and Arg106, which often ligates via a water bridge. A number of hydrophobic residues also line the pocket. An unusual feature of this pocket is that, dependent on substrate, it typically binds nine or ten water molecules tightly. The bound water molecules form part of the surface against which ligands contact.[5]

The ubiquity of endogenous fatty acids in living systems and the high intracellular concentration of aFABP (of the order of micromolar)[1a,b–d] implied that we needed an inhibitor of significantly greater intrinsic potency than endogenous fatty acids. In addition, muscle FABP (mFABP), which is expressed in both heart and skeletal muscle, has been correlated to fatty acid oxidation capacity in these tissues, and is necessary for their proper function.[6] Thus selectivity versus mFABP is thought to be critical for an aFABP inhibitor as a therapeutic agent. Epidermal FABP (eFABP) was also used as a measure of selectivity in our assay protocol. eFABP is widely distributed in many tissue and organs including adipose, macrophages, stomach, heart, brain, liver, spleen, muscle, lung, intestine, bone marrow, testis, retina, and mammary and endothelial cells.[1d] Because of the high intracellular concentration of aFABP, its high affinity for endogenous ligands, and need for selectivity versus other FABPs, our program objectives were to discover potent aFABP inhibitors with selectivity over mFABP and eFABP.

Directed screening (via DOCK software)[7] of our compound library based on known endogenous fatty-acid substrates of aFABP using a homology model of the human adipocyte protein generated from the mouse adipocyte protein crystal structure[5] (PDB code 1LIE, 91% identity, 95% similarity) and the LOOK program,[8] led to the identification of **1a**, a 58 nM inhibitor of aFABP that is essentially equipotent against the mFABP (Fig. 1 and Table 1). Similarity searches using **1a** as a template resulted in the identification of **1b** (Fig. 1) as a potent inhibitor of aFABP with 170-fold selectivity over mFABP (Table 1). The profile of compound **1b** is interesting in that it binds with significantly greater affinity to aFABP and has better isoform selectivity as compared to known endogenous substrates such as palmitic or oleic acid (Table 1) and the initial screening hit **1a**.

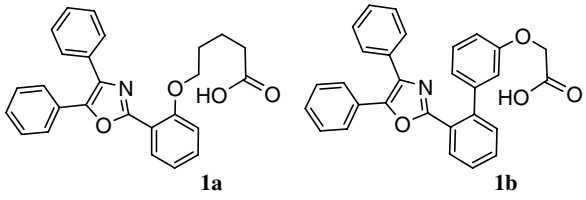


Figure 1. Virtual screening leads for aFABP binding.

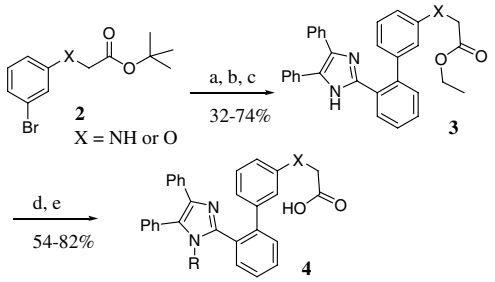
The weaker binding affinity of **1b** compared to **1a** for mFABP suggested to us that the biphenylazole chemotype could provide a useful platform for obtaining selectivity for aFABP over other FABP isoforms. Ligand **1b** contains a highly substituted oxazole ring and a distal carboxylic acid bridged together by a biphenyl core. This manuscript highlights the in vitro structure–activity relationships (SAR) of several alternate heterocyclic ring replacements for the oxazole in **1b** and the resulting FABP selectivity profiles. Compounds were assayed against aFABP, eFAPB, and mFABP to determine inhibition constants and selectivity. The 1,8-ANS (8-anilino-1-naphthalene-sulfonic acid) displacement assay developed by Kurian et al. was used to determine binding affinity (Table 1).[9b]

Our initial approach to explore the SAR of **1b** was to replace the oxazole with an imidazole. The imidazole allows for additional functionalization via *N*-alkylation to provide a new set of ligands. The facile method for the synthesis of substituted imidazoles is shown in Scheme 1. Imidazoles **4a** and **4b** were less active vs aFABP when compared to **1b**, but maintained selectivity against m- and eFABP. When N-substituents such as methyl (**4c** and **4d**) or ethyl (**4e** and **4f**) were added to the heterocyclic core, improved binding to aFABP was achieved, with K_i values in the range of 2 nM (the lower

Table 1. K_i (nM) Binding constants[a] of Oxazoles (**1**) and Imidazoles (**4**) with aFABP, eFABP, and mFABP in the 1,8-ANS assay compared to endogenous fatty acids

Compound	X	R	aFABP K_i (nM)	mFABP K_i (nM)	eFABP K_i (nM)
Palmitic acid	—	—	336 (±164)	NT[b]	802 (±336)
Oleic acid	—	—	185 (±35)	NT	248 (±12)
1a	—	—	58 (±4)	32 (±4)	>2000
1b	—	—	6 (±1)	>1000	>2000
4a	O	H	9.3 (±0.6)	>1000	>2000
4b	NH	H	18 (±6)	>1000	>2000
4c	O	Me	<2[c]	>1000	830 (±100)
4d	NH	Me	2.7 (±0.5)	>1000	>2000
4e	O	Et	<2[c]	>1000	830 (±70)
4f	NH	Et	3.4 (±0.3)	>1000	>2000
4g	NH	Isobutyl	130 (±6)	>1000	>2000
4h	NH	CH_2COOH	>2000	>1000	>2000
4i	O	FCH_2CH_2	<2[c]	>1000	>2000

[a] Values are means of three experiments, standard deviation is given in parentheses.
[b] NT, not tested.
[c] Resolution of the assay does not allow for K_i quantification below 2 nM.

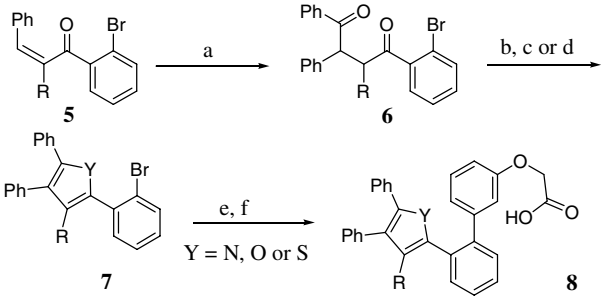


Scheme 1. Reagents and conditions: (a) $Pd(PPh_3)_4$, 2-formylphenylboronic acid, Na_2Co_3, EtOH/toluene; (b) NH_4OAc, Benzyl, HOAc, reflux; (c) H_2SO_4, EtOH, rt; (d) alkyl halide, $NaHCO_3$, DMF; (e) NaOH, H_2O, then H_3O^+.

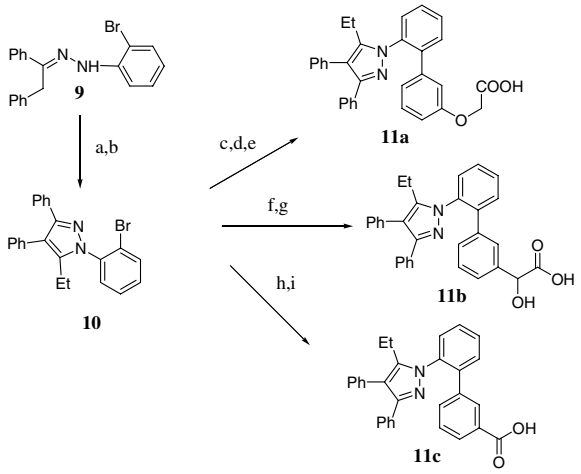
detection limit for the ANS assay).[9] Yet when the larger *iso*-butyl (**4g**) group was appended to the imidazole, a >50-fold loss in binding to aFABP was observed. Polar substituents (e.g., **4h**, R = CH_2COOH) markedly decreased activity. The most potent compounds (**4c**, **4e**, and **4i**) achieved high selectivity ratios over eFABP and mFABP (Table 1).

With the understanding that the central oxazole in **1b** could be replaced with an imidazole and maintain good binding affinity, our attention turned to examining additional heterocycles, with a view to varying both the electron density of the core and subtly influencing the spatial relationship of the peripheral substituents. We prepared electron-rich heterocycles from the common intermediate diketone **6**.[10] Furans were generated by dehydration of **6** with boron trifluoride, pyrroles by weak acid catalysis in the presence of amines, and thiophenes by reaction of **6** with Lawesson's reagent (Scheme 2).[11]

The comparatively electron-poor pyrazole **11a** was prepared in 5 steps from hydrazone **9** (Scheme 3).[12] The biological results for compounds of chemotypes **8** and **11a** (Table 2) suggest a similar SAR to the imidazoles. For these chemotypes, the ethylated compounds **8c** and **11a** were more active than the corresponding unsubstituted analogs (**8a** and **8b**). Both oxazoles and pyrazoles maintained selectivity for aFABP over mFABP and eFABP.



Scheme 2. Reagents and conditions: (a) PhCHO, N-3, 4-dimethyl-5-(2-hydroxyethyl)thiazolium iodide, Et_3N, 90%; (b) BF_3–Et_2O, Y=O, 58–78%; (c) NH_4OAc, or RNH_2, HOAc Y=N–R′, 47–68%. (d) Lawesson's reagent, Y=S, 85–100%; (e) i—$Pd(PPh_3)_4$, 4-methoxyphenylboronic acid, 63–84%; ii—BBr_3, CH_2Cl_2, −78 °C 66–81%; (f) i—NaH, ethyl bromoacetate, THF, 44–69%; ii—NaOH, H_2O, then 1 N HCl, 95%.



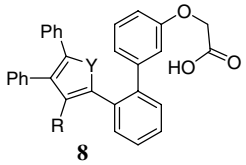
Scheme 3. Reagents and conditions: (a) NaH, Propionic anhydride/NMP, 72%; (b) NaH/DMF, 82%; (c) $Pd(PPh_3)_4$, 3-methoxyphenylboronic acid, 90%; (d) BBr_3/CH_2Cl_2, 90%; (e) i—$BrCH_2CO_2Et$, NaH/DMF, 88%; ii—NaOH, H_2O, H_3O^+, 88%; (f) $Pd(PPh_3)_4$, 3-formylphenylboronic acid, 35%; (g) i—TMSCN, Et_3N; ii—HCl (concd), 100 °C, 42%; (h) $Pd(PPh_3)_4$, 3-carbomethoxyphenylboronic acid, 30%; (i) LiOH, 90%.

Additional analogs of **11a** were prepared (Scheme 3) to probe activity relative to the position of the carboxylic acid group. As shown in Table 2, the α-hydroxy acid **11b** was found to be 10-fold less active for aFABP when compared to **11a**. The benzoic acid **11c** showed an even greater loss in aFABP binding. This SAR suggests that the position of the carboxylic acid group for the chemotype is critical for aFABP binding. Interestingly, these modifications had negligible impact on the binding affinity to m- and eFABPs.

Subsequent X-ray crystallographic analysis of **11a** bound to aFABP (Fig. 2) suggested that the ethyl attached to the heterocyclic ring comes in close proximity to Ser53. The residue Ser53 appears to play a role in the intrinsic binding affinity of the azoles to aFABP. Substitution with increasingly large groups (lone pair, H, Me, Et) may enhance binding to aFABP through improved van der Waals interactions with Ser53. Groups significantly larger (e.g., **4g**, isobutyl) or more polar (e.g., **4h** and **4i**) than ethyl lead to reduced binding affinities.

Several of our compounds shown in Tables 1 and 2 tested the limits of the fluorescence-based assay that we had relied upon for much of our work. Resolution of K_i values in this 1,8-ANS assay is limited to the range of 2–2000 nM.[9a,b] This arises from the relatively low fluorescent sensitivity of ANS, necessitating a high protein level in the assay. As the lower limit of K_i measurement is directly dependent on the protein concentration, this in itself limits the assay sensitivity. In addition, the 1,8-ANS assay is subject to compound interference by light absorption, because ANS fluoresces at 460 nm. Because of these complications, a radioligand binding assay was developed. The non-tritiated derivative of

Table 2. Fatty acid protein binding constants of Furans, Pyrroles, Thiophenes, and Pyrazoles in the 1,8-ANS assay



8

Compound	Y	R	aFABP K_i (nM)	mFABP K_i (nM)	eFABP K_i (nM)
8a	O	H	36 (±3)	>1000	>2000
8b	NH	H	12 (±1)	>1000	>2000
8c	O	Et	3.5 (±0.7)	220 (±20)	290 (±60)
11a	(Scheme 3)		<2[b]	250 (±15)	350 (±3)
11b	(Scheme 3)		28 (±5)	360 (±50)	550 (±70)
11c	(Scheme 3)		650 (±60)	110 (±5)	240 (±10)

[a]Values are means of three experiments, standard deviation is given in parentheses.
[b] Resolution of the assay does not allow for K_i quantification below 2 nM.

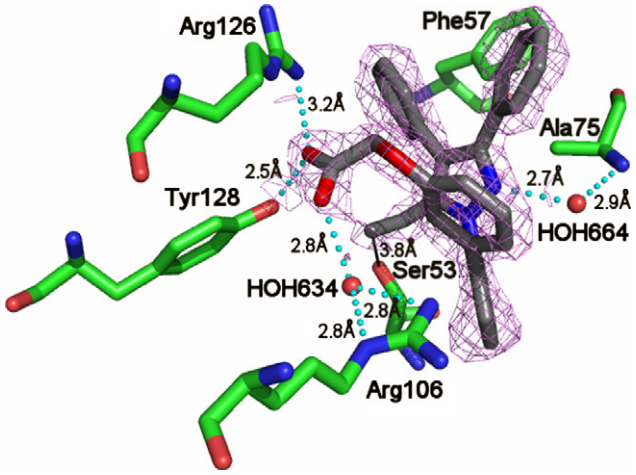
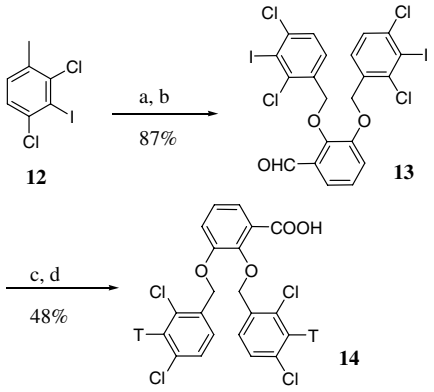


Figure 2. X-ray crystal structure of **11a** bound to the active site of aFABP.[13a] The X-ray model of **11a** bound to the binding site of aFABP with the initial 2Fo-Fc electron density map contoured at 1σ. Hydrogen bonds are shown as small spheres. For clarity only a limited number of surrounding residues are included in the figure. In particular, Ser53, which is a Thr in m- and eFABP, is proximal (3.8 Å) to the ethyl substituent of the pyrrozole ring. Figure prepared with PyMol.[13b]

benzoate **14** was identified as a potent aFABP inhibitor from other SAR studies.[14] We selected compound **14** as the radioligand since the non-labeled compound demonstrates potent binding in the 1,8-ANS assay for several FABPs including aFABP (K_i = 2 nM), eFABP (K_i = 2 nM), and mFABP (K_i = 4 nM), and is therefore of potential use as a probe for binding to each of these proteins. Compound **14** was generated by the method outlined in Scheme 4 in four steps starting from the commercially available iodide **12**. The aFABP K_d of **14** was determined by Scatchard analysis and found to be 21 nM (±2).[15]

Selected compounds were assayed against aFABP and the results were compared to the original 1,8-ANS assay data (Table 3). Within the set of compounds tested, K_i values were greater when determined with the radioligand binding assay than with the 1,8-ANS assay. Given



Scheme 4. Reagents: (a) NBS, AIBN; (b) K_2CO_3, ethanol, 2,3-dihydroxybenzaldehyde; (c) H_2NSO_3H, $NaClO_2$, THF–water; (d) T_2, ethanol, KOAc, 10% Pd/C.

that K_i values quantified in the fluorescent assay are potentially in error by a factor of 3 to 4 and the significant differences in assay format,[9a,b] variation in calculated K_i values between the two assays was expected. In addition, X-ray crystallographic analysis shows that ANS and analogs of **14** bind in distinctly different positions. These differences in binding orientation suggest another possible reason to expect differences in K_i.

Table 3. Binding constants of selected compounds in the radioligand assay and comparison to 1,8-ANS displacement assay

Compound	1,8-ANS assay K_i (nM)	^{3}H 14 assay K_i (nM)[b]
1b	6 (±1)	95 (1)
4b	18 (±6)	300 (1)
4c	<2[a]	13 (1)
4d	2.7 (±0.5)	48 (1)
4e	<2[a]	3.3 (2)
4f	3.4 (±0.3)	13 (2)
4i	<2[a]	8.7 (1)
11	<2[a]	7.4 (1)
8c	3.5 (±0.7)	32 (1)
ANS	K_d = 150	1000

[a] Resolution of the assay does not allow for K_i quantification below 2 nM.
[b] Values in parentheses refer to number of experiments.

Because the K_i values in the ANS fluorescent assay are highly variable in the low nanomolar range, the radioligand binding assay offers a significant advantage in sensitivity for tight binding ligands.

In conclusion, we have identified a novel structural class of compounds that bind to aFABP with significantly greater affinity and FABP isoform selectivity than known endogenous fatty acid substrates. In particular we have identified several compounds which are potent (nM) and selective inhibitors of aFABP. These ligands can serve as useful probes for further investigation of the potential utility of aFABP inhibitors for the treatment of diabetes, obesity, and atherosclerosis. Other series of aFABP inhibitors have been reported in the literature by Biovitrum.[16a,b] In comparison, the inhibitors described in this manuscript are of greater potency. We have also described a new and useful radioligand binding assay for the determination of binding constants for aFABP (and potentially m- and eFABP) with a greater dynamic range than the standard fluorescence assay and capable of distinguishing the SAR of our most active compounds.

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