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N-Benzyl-indolo carboxylic acids: Design and synthesis of potent and selective adipocyte fatty-acid binding protein (A-FABP) inhibitors

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ABSTRACT

Small molecule inhibitors of adipocyte fatty-acid binding protein (A-FABP) have gained renewed interest following the recent publication of pharmacologically beneficial effects of such inhibitors. Despite the potential utility of selective A-FABP inhibitors within the fields of metabolic disease, inflammation and atherosclerosis, there are few examples of useful A-FABP inhibitors in the public domain. Herein, we describe the optimization of *N*-benzyl-tetrahydrocarbazole derivatives through the use of co-crystal structure guided medicinal chemistry efforts. This led to the identification of a potent and selective class of A-FABP inhibitors as illustrated by *N*-benzyl-hexahydrocyclohepta[*b*]indole **30**.

Fatty-acid binding proteins (FABPs) are tissue-specific, ~15 kDa cytoplasmic lipid chaperones, capable of binding and transporting endogenous fatty acids from the cell surface to the various sites of metabolism or storage.[1] Consequently, the role of FABPs is directly linked to lipid-mediated biology such as signalling pathways, trafficking and membrane synthesis.[2]

Out of the nine known isoforms, adipocyte FABP (A-FABP, FABP4 or aP2) is the predominantly present isoform in adipose tissue and also highly expressed in macrophages. This seems to translate in a prominent function in certain specific aspects of the metabolic syndrome and cardiovascular disease.[3] Disruption of A-FABP in mice prevents development of diet-induced insulin resistance and whole body, as well as macrophage-specific, deletion of A-FABP leads to protection of atherosclerosis in apolipoprotein E-deficient mice.[4,5] In line with these results, pharmacological intervention using the apparently selective A-FABP inhibitor BMS-309403 produced similar phenotypes as demonstrated in diabetic and atherosclerotic mouse models.[6] In human settings, reduced A-FABP expression in adipose tissue, as a consequence of polymorphism at the FABP4 locus, is has been linked with a lower risk for hypertriglyceridemia, type 2 diabetes and coronary heart disease.[7]

Collectively, these studies indicate that pharmacological modulation of the fatty-acid binding function of A-FABP could be of therapeutic benefit in disorders such as type 2 diabetes and atherosclerosis. Despite this potential, literature is scarce with regards to reports of small molecule inhibitors for this family of proteins.[8,9] The underlying reasons may include concerns related to the possibility to develop isoform selective compounds (see below), the lipophilic and charged nature of the endogenous ligands and how this translates to the drugability of the binding pocket. Also, the high intracellular content of A-FABP, which may limit the possibility to neutralise a sufficient amount of the target protein may pose a liability. The recent data illustrating the pharmacological impact of a potent A-FABP inhibitor on lipid-mediated biology suggests that at least the latter obstacles can be overcome.[6]

Mice with a disruption in the gene encoding for the heart or muscle variant H-FABP (or FABP3) were reported to suffer from stress-intolerability, in a few cases leading to death. Hence, in developing inhibitors for any FABP isoform, the potential need for selectivity against H-FABP should be considered.[10] The overall sequence identity of human H-FABP and A-FABP is 65%, which is the highest degree of homology among the known human FABPs.[11] Comparison of the binding sites of these two FABPs reveals only a handful of amino acid differences.

We have previously reported the structure–activity relationships (SAR) around carbazole- and indole-based inhibitors of A-FABP, leading to the discovery of submicromolar inhibitors.[9] In the present study we have continued to explore the SAR around a novel tetrahydrocarbazole series of A-FABP ligands. This has been achieved by structure-based drug design. Variation of substitution patterns as well as exploration of new benzoic acid containing scaffolds led to the identification of several ligands with nanomolar potency.

Pyrazole derivative **1** with an IC_{50} value of 1.1 μM was identified by means of high throughput screening. Although representing a sufficiently good starting point for optimization, pyrazole **1** suffered from β-oxidation as a consequence of its fatty acid-like

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character. Co-crystallization of **1** with human A-FABP revealed a binding mode which suggested that ring-closure of the aliphatic chain on the pyrazole was feasible (Fig. 1) and hence the tetrahydrocarbazole **2a** was synthesised.

The inhibitory activity of **2a** for A-FABP was confirmed (IC_{50} 0.59 μM), and this fueled a medicinal chemistry program with the aim to optimize potency and selectivity for A-FABP. A co-crystal structure of **2a** with human A-FABP was then solved (Fig. 2) to further guide the medicinal chemistry efforts.

X-ray crystallographic analysis shows that compound **2a** binds at the same site as endogenous fatty acids and other reported A-FABP inhibitors, clearly demonstrating the competitive nature of this inhibitor (Fig. 2).[12] The inhibitory activity is driven mainly by the interaction of the carboxylic acid with Y128 and R126, and several hydrophobic interactions.

Our initial objective in exploring the SAR of **2a** was to challenge the relevance of the position of the carboxylic acid. This was accomplished by the Fisher indole synthesis using 2-, 3-, and 4-benzoic acid hydrazines and cyclohexanone in refluxing acetic acid (Scheme 1). Attempts to *N*-alkylate directly were unsuccessful, since the carboxylic acid needed to be protected first as the corresponding ester. However, the alkylation proceeded smoothly using benzyl bromide and potassium hydroxide as the base in DMSO. Finally, hydrolysis of the esters with aqueous lithium hydroxide furnished the desired final compounds.

All compounds were tested for their inhibitory activity using a fluorescence polarization (FP) assay as described earlier.[13] Based on the results presented in Table 1, it is clear that the 4-position of the tetrahydrocarbazole moiety is the most favourable position for the carboxylic acid. Moving the acid to the 3-position still keeps an acceptable affinity for the fatty-acid binding site of A-FABP, but a further shift to the 2-position renders a completely inactive compound. Subsequently, the required nature of the aliphatic ring was investigated by ring expansion (cycloheptyl) or contraction (cyclopentyl), respectively. In addition, the 2,3-dimethylindole derivative **5** was prepared starting from butan-2-one utilizing the same chemistry as in Scheme 1.

As shown in Table 1, the cyclohexane and cycloheptane derivatives were equipotent, and significantly more potent then their cyclopentane and dimethyl counterparts. Thus, the 2,3,4,9-tetrahydro-1*H*-carbazole-8-carboxylic acid and 5,6,7,8,9,10-hexahydrocyclohepta[*b*]indole-4-carboxylic acid series were considered interesting enough for further modifications. Hence these scaffolds were reacted (using the conditions described above) with a variety of benzyl and alkyl halides chosen to include sterically demanding as well as electron rich or poor substituents.

Data presented in Table 2 demonstrated that the potencies of the initial unsubstituted benzyl derivatives **2a** and **2e** (IC_{50} 0.59 μM) could be improved. When introducing substituents on the benzyl ring we found that more active compounds were obtained when the substituent was in the ortho-position, hence the *ortho*-trifluoromethyl substituted **17** (IC_{50} < 0.4 μM) was more potent than its *meta*- or *para*-substituted counterparts (**18** with 1.7 μM and **19** with 2.4 μM, respectively). This trend (*ortho*-substitution > *meta* >> *para*) is general and applies to both electron withdrawing (**14**–**19**) or electron donating (**11**–**13**) groups, especially with increasing size. For the *para*-position it appears that sterically demanding groups are forbidden, regardless of the electronic properties, hence the small *para*-fluoro (**16**) or methyl (**20**) substituents gave better inhibition than the trifluoromethyl (**19**) or methoxy (**13**) groups. With respect to combinations it is clear that the 2-fluoro-6-trifluoromethyl (**21**) combination is not optimal. In fact, the IC_{50} of compound **21** was at least four times greater than either of the corresponding monosubstitutions. It is also noteworthy that

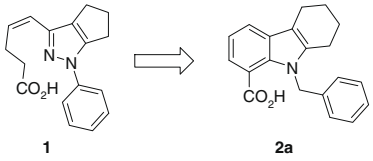


Figure 1. Structure-guided information led to the discovery of tetrahydrocarbazole derivative **2a**.

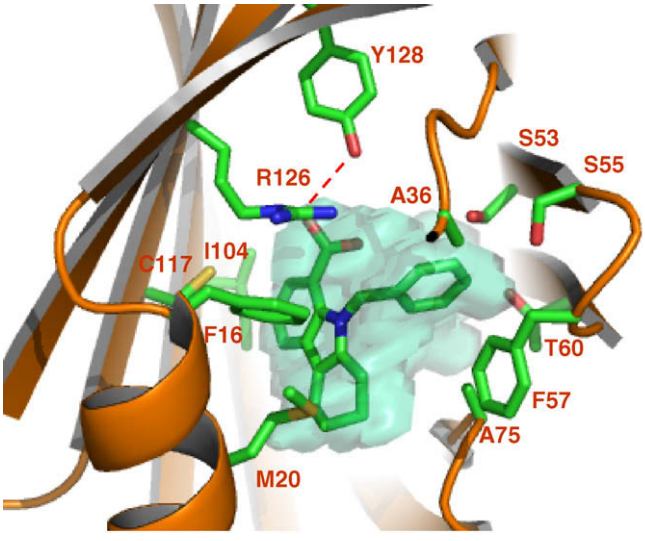
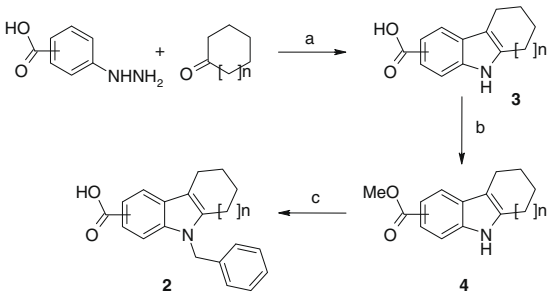
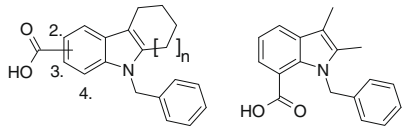


Figure 2. Co-crystal structure of **2a** in human A-FABP (PDB code: 3FR2).



Scheme 1. Reagents and conditions: (a) AcOH, reflux (17–76%); (b) MeOH, H_2SO_4, reflux (70–98%); (c) i—Benzyl bromide, KOH, DMSO, rt; ii—THF/MeOH, aq. LiOH (2 M) rt (5–80%).

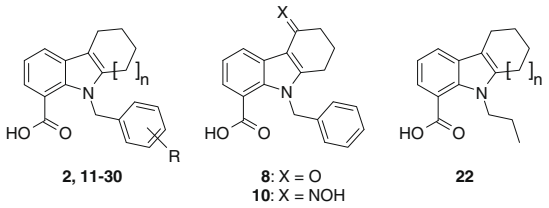
Table 1
IC_{50} (μM) values of compounds **2** and **5** for human A-FABP



Compound	COOH	n	IC_{50} (μM)[a]
2a	4	1	0.59
2b	3	1	0.75
2c	2	1	>25
2d	4	0	0.97
2e	4	2	0.59
5	—	—	3.6

[a] Values are means of triplicate fluorescence polarization experiments performed on the same dilution.

Table 2
IC_{50} (μM) values and selectivity data of compounds **2**, **8** and **10**–**30** for human A-FABP and H-FABP



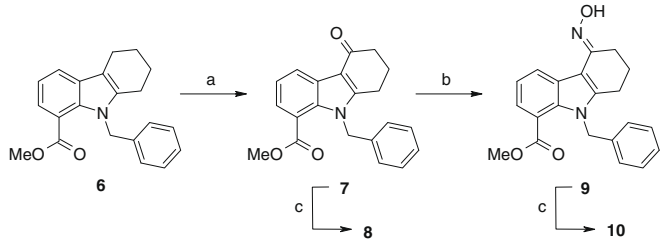
2, 11-30 **8**: X = O **10**: X = NOH **22**

Compound	*n*	R	A-FABP IC_{50}[a] (μM)	H-FABP IC_{50}[a] (μM)
2a	1	–	0.59	3.88
2e	2	–	0.59	–
8	–	–	>25	–
10	–	–	3.5	–
11	1	2-OMe	0.60	–
12	1	3-OMe	0.55	>10
13	1	4-OMe	2.68	>10
14	1	2-F	<0.4	0.68
15	1	3-F	<0.4	5.57
16	1	4-F	0.83	–
17	1	2-CF_3	<0.4	–
18	1	3-CF_3	1.66	–
19	1	4-CF_3	2.39	–
20	1	4-Me	1.09	>10
21	1	2-F, 6-CF_3	1.81	–
22a	1	–	0.73	–
22e	2	–	0.43	–
23	2	2-F	0.71	–
24	2	3-F	0.72	>10
25	2	4-F	0.78	>10
26	2	2-CF_3	0.65	<0.4
27	2	3-CF_3	1.04	>10
28	2	4-CF_3	2.03	>10
29	2	2-$CONH_2$	<0.4	–
30	2	3-$CONH_2$	0.45	>10

[a] Values are means of triplicate fluorescence polarization experiments performed on the same dilution.

the *N*-propyl derivatives **22a** and **22e** were quite active indicating that the carboxylic acid per se seems more important for binding than the lipophilic benzyl part.

Since all synthesised compounds had been highly lipophilic so far, attempts were made to develop more hydrophilic compounds in order to improve on physicochemical properties, such as solubility and serum albumin binding. We envisaged that a conserved water molecule, proximal to the 4-position of the carbazole, was a suitable H-bonding partner or could even be replaced. To achieve this, a keto- and oxim- functionality was introduced on this position by selective oxidation of the methyl ester of **2a** by DDQ (Scheme 2). The acquired product was either hydrolysed, giving ketone derivative **8** or reacted with hydroxylamine, followed by hydrolysis to give oxim analogue **10**.



Scheme 2. Reagents and conditions: (a) DDQ, THF/H_2O, 0 °C (53%); (b) $HONH_2$.HCl, NaOAc, EtOH/H_2O, reflux (71%); (c) KOH, EtOH/H_2O, reflux (61–87%).

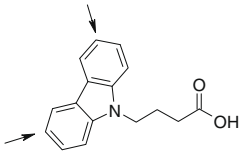
This strategy proved successful in that plasma protein binding indeed had been reduced from >99.9% for **2a** to 90% for **8**, however, this modification also rendered an inactive compound. The oxim analogue **10** showed still reasonable potency, showing the potential of this approach.

Alternatively, potential H-bonding interactions A-FABP can be anticipated via the amino acid triad made up by S53, S55 and T60. To this end, carboxamides were introduced on the *ortho*- and *meta*-position of the benzyl group of the hexahydrocyclohepta[*b*]indole-4-carboxylic acid derivatives.[14] Regioisomeric primary amides **29** and **30** were approximately equipotent in the FP assay (~ 0.4 μM). Since the protein concentration is also about 400 nM in this assay, our ability to distinguish between the most potent compounds was limited. For this reason, a more sensitive scintillation proximity assay (SPA) assay had to be developed.[11]

The non-radiolabeled derivative **31** had previously been identified as a potent inhibitor of A-FABP and H-FABP.[8] Therefore, also the selectivity for both FABPs could potentially be addressed using tritium-labeled **31** in a SPA-bead setting. The tritium-labels were introduced on the 3- and 7-position of the carbazole moiety via the reduction of the di-bromo derivative (Fig. 3).

Selected compounds were compared in this new SPA-bead assay and some of the inhibitors displayed indeed much better potencies for A-FABP (Table 3). The sensitivity improved dramatically, and could discriminate between submicromolar inhibitors, such as the *N*-propyl analogues **22a** and **22e** on the one hand, and low nanomolar inhibitors on the other hand. IC_{50} values down to 49 nM for A-FABP were observed, as demonstrated for *ortho*-CF_3 analogue **26**. Unsubstituted **2e** was two fold less potent (96 nM), and introduction of a carboxamide on the *ortho*-position resulted in a similar inhibitory activity. While **26** could not discriminate between A-FABP (49 nM) and H-FABP (34 nM), *meta*-carboxamide derivative **30** actually was very selective for A-FABP (69 nM) versus H-FABP (>10 μM) and also E-FABP (data not shown).

Again, X-ray crystallography provided an explanation for the differences observed in selectivity between **26** and **30**, because the two compounds bind differently to A-FABP (Fig. 4). The CF_3 group of **26** is accommodated in a lipophilic region, whereas the carboxamide is optimally positioned for H-bonding interactions with a serine residues S53 and S55 and backbone carbonyl of K58, causing the benzyl group to flip by 180°. Interestingly, the S53 of A-FABP resembles T53 in H-FABP and a repositioning of



31

Figure 3. Structure and ^{3}H-labeling position of **31**.

Table 3
IC_{50} (μM) values for selected compounds for human A-FABP as determined in a SPA-bead assay

Compound	*n*	R	A-FABP IC_{50}[a] (μM)	H-FABP IC_{50}[a] (μM)
2e	2	–	0.096	–
22a	1	–	0.67	–
22e	2	–	0.58	–
26	2	2-CF_3	0.049	0.034
29	2	2-$CONH_2$	0.098	–
30	2	3-$CONH_2$	0.069	> 10

[a] Values are means of triplicate SPA-bead experiments performed on the same dilution.

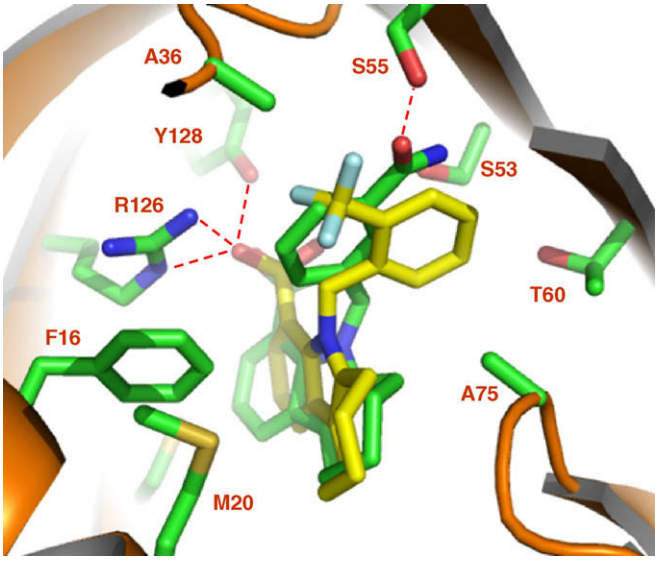


Figure 4. Overlays of co-crystal structures of **26** (in yellow) and **30** (in green) in human A-FABP (PBD codes: 3FR4 and 3FR5).

the hydroxyl group of S53 was observed while comparing co-crystal structure complexes with **30**, versus **26**. T53 in H-FABP is presumably unable to adjust or simply causes a steric clash, which most likely explains the superior selectivity of **30**. The relative higher polarity of **30** resulted in a slightly reduced plasma protein binding (99.7%) as compared to the more lipophilic congeners.

In conclusion, **2a** proved to be a viable starting point of a lead optimization program, and using a structure-based drug design approach, several improvements were effected. Expanding the right-hand ring of the tetrahydrocarbazole to a seven-membered ring gave improved affinity. Introduction of a CF_3 group in the *ortho*-position (**26**) or a carboxamide in the *meta*-position (**30**) of the *N*-benzyl substituent, gave a significant improvement leading to nanomolar inhibitors and with different selectivity profiles against H-FABP. This difference could in part be explained by solving the co-crystal structures showing two distinctly different binding modes for this novel class of hexahydrocyclohepta[*b*]indole-based A-FABP inhibitors. Inhibitors such as **30**, will further help to delineate the exact role of A-FABP in fatty-acid homeostasis in adipose tissue and macrophages.

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14. Carboxamide derivatives **29** and **30** were prepared *via* the respective 2- and 3-cyanobenzyl intermediates. Hydrolysis of the methyl ester and nitrile to the final compounds was effected in one step with KOH in refluxing EtOH/water (5/1 v/v).