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Identification and characterization of 4-aryl-3,4-dihydropyrimidin-2(1*H*)-ones as inhibitors of the fatty acid transporter FATP4

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Abstract—Several potent, cell permeable 4-aryl-dihydropyrimidinones have been identified as inhibitors of FATP4. Lipophilic ester substituents at the 5-position and substitution at the *para*-position (optimal groups being $-NO_2$ and CF_3) of the 4-aryl group led to active compounds. In two cases racemates were resolved and the *S* enantiomers shown to have higher potencies.

Blocking the absorption of fats (triglycerides) by administration of an anti-absorptive agent is of interest for the treatment of obesity.[1] Ingested dietary triglycerides are hydrolyzed by gastric and pancreatic lipases, and the resulting fatty acids are taken up by enterocytes lining the small intestine where they are re-esterified to triglycerides and then transported into the blood. The lipase inhibitor orlistat (Xenical™) blocks fat absorption by inhibiting the hydrolysis of dietary fat to fatty acids[2] with administration leading to a concomitant decrease in body weight and improvement of blood lipid profiles. A family of proteins, termed fatty acid transport proteins (FATPs), that mediate the uptake of fatty acids into cells has been described.[3,4] Previous studies[5–8] provided evidence that fatty acid transport protein 4 (FATP4) mediates the transport of fatty acids from the gut into enterocytes both in vitro and in vivo. We therefore reasoned that inhibitors of FATP4 might be expected to have benefits similar to orlistat. Since FATP4 inhibition would result in the accumulation of free fatty acids rather than triglycerides, we would also expect a different, possibly improved, side-effect profile compared to orlistat. The FATP family of proteins is most closely related in sequence to the ATP-utilizing acyl-CoA-synthetase enzymes.[9–12] While much published work on FATPs has primarily focused on net fatty acid transport activity, recent data from these laboratories[13] and published in the literature[9–12] show that FATP4 and its closest homolog FATP1 have acyl-CoA-synthetase activity. A prominent model of fatty acid transport proposes that the acyl-CoA-synthetase activity of FATPs mediates fatty acid uptake by formation of fatty acyl-CoA derivatives thereby creating a concentration gradient that drives uptake.[9] Moreover, human FATP4-mediated uptake of labeled fatty acids can be competed by long-chain fatty acids (>C-12), but not by short-chain carboxylic acids, methyl esters of fatty acids, heterocyclic isosteres of carboxylic acids with long-chain substituents,[14] or other lipophilic molecules. These data are consistent with viewing FATP4 as a long-chain acyl-CoA-synthetase.

Initially, we sought FATP4 inhibitors using an assay conducted under comparable conditions to those used for target validation.[6] Thus, a cell-based assay was run in an HTS format measuring inhibition of the uptake of a fluorescent long-chain fatty acid, 12-BODIPY-lauric

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acid, into HEK 293 cells stably transfected with human FATP4.[15] In order to identify non-specific inhibitors, cell-based counter-screens were run measuring inhibition of uptake of the same substrate into mouse FATP5 and human FATP2. Among the hits from the HTS that showed selectivity over FATP2 and FATP5, the dihydropyrimidinone **1a** (Fig. 1) was found to have an IC_{50} value of 1.2 μM. Herein we describe optimization of this compound series leading to analog **1p**.

Dihydropyrimidinones **1** can readily be prepared by the Biginelli three-component condensation reaction between a urea, an aldehyde, and a β-ketoester.[16–18] In order to probe the SAR at the 5-position alkoxy group without the need to prepare individual β-ketoesters we sought a synthetic route to carboxylic acid **2** (Scheme 1).

Condensation between urea, 4-nitrobenzaldehyde, and 2-cyanoethylacetoacetate in the presence of boron trifluoride[19] afforded **3** which, unlike other esters,[20] could be hydrolyzed by treatment with base under mild conditions to give **2**. Esterification of **2** using alcohols or phenols in the presence of EDC afforded several examples of series **1** (Table 1). To probe the effect of the oxidation

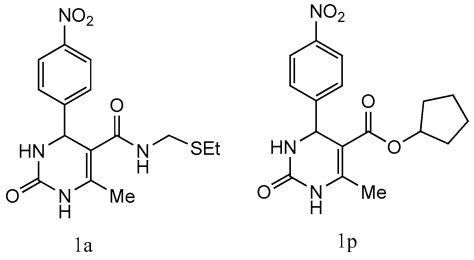
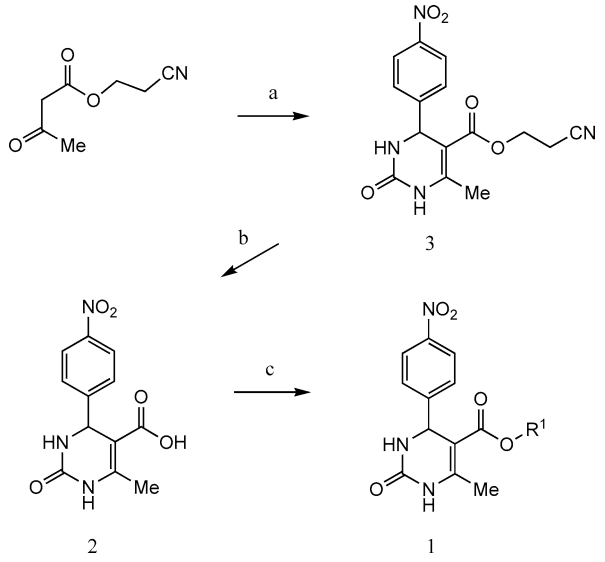
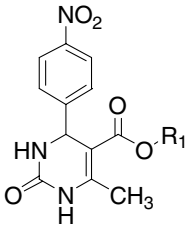


Figure 1. High-throughput screening hit **1a** and optimized FATP4 inhibitor **1p**.



Scheme 1. Reagents and conditions: (a) urea, 4-nitrobenzaldehyde, $BF_3{\cdot}OEt_2$, (1.3 equiv), CuI (0.1 equiv.), HOAc (0.1 equiv), THF, reflux, 18 h, 88%; (b) NaOH, Me_2CO, H_2O, 0–25 °C, 95%; (c) R^1OH, EDC, DMAP, DMF, 50 °C, 16 h, 25–55%.

Table 1. Inhibition of FATP4 mediated uptake of 12-BODIPY-lauric acid in HEK 293 cells by dihydropyrimidinones[a,b]



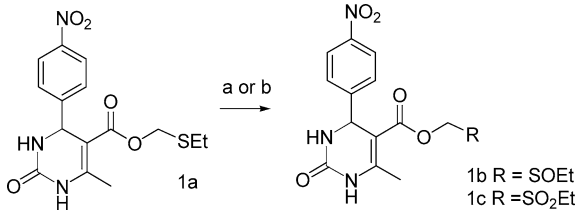
1-2

Compound	R^1	FATP4 IC_{50} (μM)
2	H	>30
1a	CH_2CH_2SEt	1.2
1b	CH_2CH_2SOEt	>30
1c	$CH_2CH_2SO_2Et$	>30
1d	CH_2CH_2OEt	>30
1e	*n*-Pentyl	2.0
1f	Et	9.5
1g	Me	>30
1h	Allyl	1.5
1i	*trans*-2-Butenyl	0.65
1j	*trans*-2-Pentenyl	0.64
1k	*trans*-2-Hexenyl	0.26
1l	*trans*-2-Heptenyl	0.09
1m	*cis*-2-Pentenyl	0.70
1n	2-Cyclohexenyl	0.18
1o	Cyclobutyl	1.2
1p	Cyclopentyl	0.25
***S*-1p**	Cyclopentyl	0.20
***R*-1p**	Cyclopentyl	25.0
1q	Cyclohexyl	0.50
1r	Cycloheptyl	1.2
1s	*trans*-4-Et-cyclohexyl	0.15
1t	*cis*-4-Et-cyclohexyl	2.4
1u	Ph	>30
1v	CH_2Ph	>30

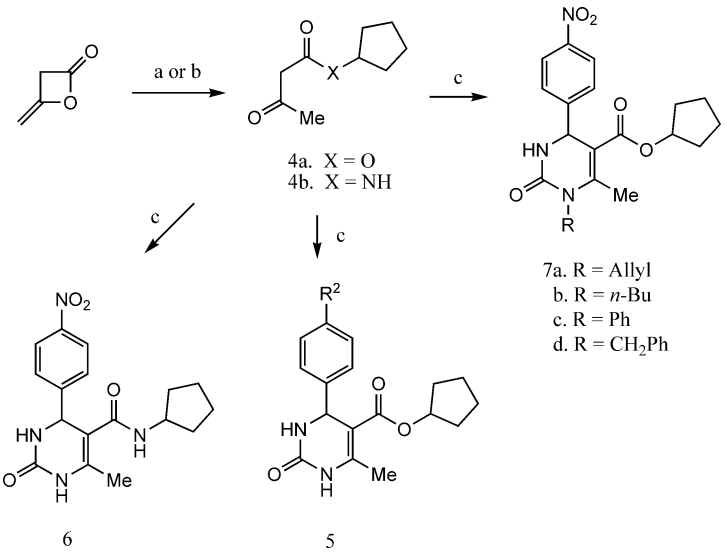
[a] For assay conditions see Ref. 15.
[b] All compounds are racemates unless indicated otherwise.

state of the sulfur atom in **1a**, we effected oxidations to the sulfoxide **1b** and sulfone **1c** as shown in Scheme 2.

Investigations of the SAR at the *para*-position of the 4-aryl group were confined to derivatives with the optimal cyclopentyl ester (vide infra) at the 5-position. Reaction of cyclopentanol with diketene afforded ketoester **4a** which was subjected to the BF_3 catalyzed[19] Biginelli condensation with urea and the appropriate aldehyde to give **5a–o**. A representative amide, **6**, was prepared



Scheme 2. Reagents and conditions: (a) *m*-CPBA, CH_2Cl_2, 25 °C, 8 h, 68%; (b) i—*m*-CPBA, CH_2Cl_2, reflux, 18 h; ii—crystallization (acetone/hexane), 34%.



Scheme 3. Reagents and conditions: (a) cyclopentanol, DIEA, CH_2Cl_2, 0–25 °C, 95%; (b) cyclopentylamine, THF, 25 °C, 30%; (c) urea, or *N*-alkyl-urea, ArCHO, $BF_3{\cdot}OEt_2$, (1.3 equiv), CuI (0.1 equiv), HOAc (0.1 equiv), THF, reflux, 18 h, 50–70%.

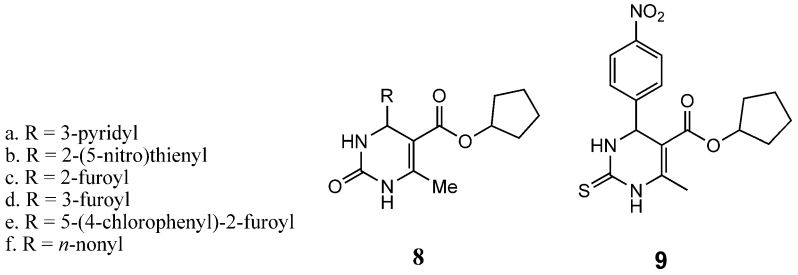


Figure 2.

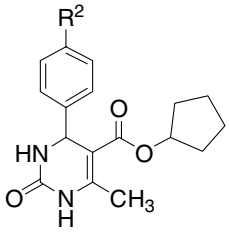
similarly from ketoamide **4b**, and 1-substituted derivatives, **7**, were synthesized from N-substituted ureas (Scheme 3).

Dihydropyrimidinones **8** with heteroaryl and alkyl groups at the 4-position shown in Figure 2 were prepared similarly from the appropriate aldehyde. A representative dihydrothiopyrimidinone **9** was obtained in 65% yield by condensing thiourea with 4-nitrobenzaldehyde and β-ketoester **4a** in ethanolic HCl.[21]

In the whole cell uptake assay measuring the inhibition of uptake of the fluorophore 12-BODIPY-lauric acid,[15] carboxylic acid **2** and amide **6** proved to be inactive. In contrast, several derivatives with lipophilic ester groups at the 5-position exhibit significant activity (Table 1). The requirement for a lipophilic substituent is further illustrated by comparison of thioether **1a** (IC_{50} = 1.2 μM) with the more polar analogs, sulfoxide **1b**, sulfone **1c**, and ether **1d** all of which were found to be inactive compounds. The *n*-pentyl analog **1e** exhibits comparable potency to **1a** but shorter chains, as in ethyl ester **1f** and methyl ester **1g**, confer little activity. Unsaturated aliphatic substituents were next investigated and it was found that allylic esters show enhanced potencies compared to saturated analogs. Potency also improved with increasing chain length (series **1h–l**). In the case of the 2-pentenyl esters the *cis* and *trans* isomers have comparable activities (compare **1j** with **1m**). Alicyclic groups were also investigated and, in general, potency enhancements were observed relative to their open chain analogs. Within this series, potency improved in the order cycloheptyl (**1r**) = cyclobutyl (**1o**) < cyclohexyl (**1q**) < cyclopentyl (**1p**). Substituted cyclohexyl derivatives were also found to be active, and, for compounds derived from 4-ethylcyclohexanol, the *trans* isomer **1s** was found to be some 20-fold more active than the *cis* isomer **1t**. In contrast, aryl (**1u**) and benzyl (**1v**) esters were found to be inactive.

With regard to substituent effects in the 4-aryl ring system, HTS data indicated that *ortho*- and *meta*-substituted compounds are considerably less potent than *para* derivatives.[22] When the optimal cyclopentyl ester group was retained, we found several compounds with approximately 1 μM activity (Table 2) where the *para*-substituents were OCF_3 (**5j**), halogen (**5d** and **5e**), and CF_3 (**5k**) but no significant potency enhancements were observed compared to the nitro compound **1p**. Replacement of the substituted phenyl group with heteroaromatics did not result in any significant activity, compounds **8a–e** (Fig. 2) all having IC_{50} values > 30 μM. Likewise, alkyl

Table 2. Inhibition of FATP4 mediated uptake of 12-BODIPY-lauric acid in HEK 293 cells by dihydropyrimidinones[a,b]



5

Compound	R^2	FATP4 IC_{50} (μM)
5a	H	>30
5b	Me	4.5
5c	F	8.0
5d	Cl	1.0
5e	Br	1.0
5f	CN	1.4
5g	OH	26
5h	OMe	5.0
5i	O-*i*-Pr	12.7
5j	OCF_3	1.1
5k	CF_3	1.0
***S*-5k**	CF_3	0.6
***R*-5k**	CF_3	>30
5l	NH_2	>30
5m	NMe_2	16
5n	SMe	8.0
5o	SO_2Me	>30

[a] For assay conditions see Ref. 15.
[b] All compounds are racemates unless indicated otherwise.

substitution at the 4-position (e.g., derivative **8f**) and N-1 substitution (derivatives **7a–d**) led to inactive compounds. Replacement of an oxygen atom by a sulfur atom in the pyrimidinone ring resulted in a loss in potency; for example, compound **9**, analogous to **1p**, has an IC_{50} value of 3.4 μM.

The cyclopentyl esters **1p** and **5k** are among the most potent analogs found in our SAR investigations. These racemic compounds were separated into their component enantiomers by chiral chromatography on a chiral-cell OD column[23–25] and their orientations tentatively assigned by comparing their relative retention times with those of closely related compounds whose absolute configurations have been established.[24,25] On this basis, we found that **S(+)-1p** was more potent than **R(−)-1p** (Table 1) with a similar trend for the enantiomeric pair **5k** (Table 2).

In order to verify that these lead compounds inhibit the uptake of native fatty acids as well as BODIPY-labeled analogs, an assay measuring uptake of radiolabeled lauric acid was configured.[26] All compounds tested ($n \sim 20$) showed similar potency and stereopreference in this assay compared to the BODIPY-lauric acid assay. Finally, none of the compounds listed in Tables 1 and 2 were found to have any activity in an uptake assay for mouse FATP5 or human FATP2 (family members with ~40% homology to FATP4).

Several members of the dihydropyrimidinone series described above were found to be orally bioavailable in DIO mice.[27] In an efficacy experiment[28] mice were placed on a high fat diet, administered racemic **1p** (100 mpk), and then given access to food. Pharmacokinetic analyses showed plasma concentrations of **1p** > 3 μM at 0.5 h (the T_{max} value) and 0.4 μM after 6 h. More importantly, high concentrations (>17-fold the IC_{50}) of **1p** were detected both within the tissues of the small intestine and in the lumen (Table 3).[29] Despite these distributions, dosing of **1p** had no significant effects on the total lipid content of either the lumen or the tissue of different parts of the small intestine. In contrast, orlistat significantly decreased tissue lipid in the duodenum and increased luminal lipid in the ileum. These data demonstrate that dosing of **1p** does not affect fat absorption in vivo. Possible reasons for the observed lack of efficacy include assay conditions, potency and mechanism of action of the inhibitors.[30]

In summary, our study represents the first report of specific small molecule inhibitors of a member of the FATP family of proteins.

Table 3. The distribution and effect on lipid levels[a] in the small intestine of dihydropyrimidinone **1p** and orlistat dosed at 100 mpk[b]

		Duodenum	Jejunum	Ileum
1p in lumen (μM)		9.3	26.7	11.0
1p in tissue (μM)		4.3	4.9	5.5
Lipid in lumen (%)	Vehicle	69.7 (±2.8)	32.1 (±2.0)	6.6 (±1.6)
	Orlistat	69.6 (±9.7)	38.2 (±1.8)	30.6 (±3.5)*
	1p	91.0 (±24.8)	23.6 (±8.4)	4.8 (±1.4)
Lipid in tissue (%)	Vehicle	9.7 (±0.1)	13.9 (±1.7)	10.9 (±1.6)
	Orlistat	5.9 (±0.4)*	9.9 (±1.4)	11.0 (±1.7)
	1p	7.9 (±1.4)	11.6 (±1.0)	11.9 (±1.0)

[a] Dried luminal content and lyophilized tissue homogenates were extracted successively with H_2O–chloroform–methanol (1:3.3:4) and chloroform–methanol (2:1) for 1 h. The extracts were dried, weighed and assigned as total lipid expressed as percentage of dried luminal material or lyophilized tissue weight.
[b] Mean ± SEM.
* $p < 0.01$.

Acknowledgments

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13. Hubbard, B. et al. manuscript in preparation. His-tagged FATP4 was purified by chromatography on a nickel column and shown to have 3-fold enhanced catalytic activity as compared to crude membrane preparations. Experiments were conducted with an homologous series of long-chain fatty acids and the formation of the CoA adduct monitored by LC-MS. Optimal activity was observed at pH 8 in the presence of Mg^{2+} and K_m values were determined as follows: lauric acid, 21 μM; CoA, 29 μM; ATP, 470 μM.
14. Several heterocyclic compounds including tetrazoles and triazoles with long alkyl chains were found to have no effect on the uptake of labeled fatty acids in the whole cell assay.
15. HEK 293 cells stably transfected with human FATP4 were incubated for 1 h with 3.5 μM, 12-BODIPY-lauric acid (Molecular Probes D-3823) in the presence of 1 mM taurocholate in 20 mM Hepes at pH 7.5 in the presence of variable concentrations of inhibitor. Following washing, intracellular fluorescence was measured on a plate reader. Assays were performed in triplicate with at least two independent determinations for each data point. IC_{50} values were determined using the XL-fit software with vector-transfected cells as 100% inhibition control.
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20. The corresponding methyl ester afforded acid **2** in only 40% yield after prolonged refluxing in methanolic sodium hydroxide. Mild hydrolysis of related esters has been reported previously Nagarathnam, D.; Wetzel, J. M.; Miao, S. W.; Marzabadi, M. R.; Chiu, G.; Wong, W. C.; Hong, X.; Fang, J.; Forray, C.; Branchek, T. A.; Heydorn, W. E.; Chang, R. S. L.; Broten, T.; Schorn, T. W.; Gluchowski, C. *J. Med. Chem.* **1998**, *41*, 5320.
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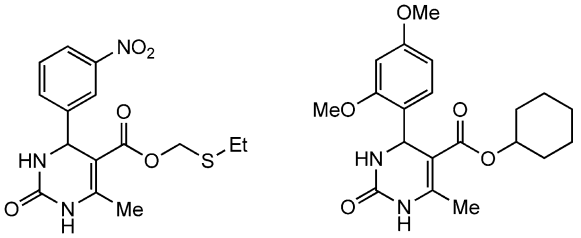
22. For example, the compounds shown below, which are analogous to potent compounds described in the text, showed no significant inhibition at 20 μM in the HTS.



23. Chiral preparative chromatography was conducted on a Chiralcel OD column (2× 25 cm) eluting with isopropanol–hexane (1:4) at a flow rate of 9 mL/min with UV detection at 270 nm. *R*- and *S*-Designations were assigned by comparison of the retention times and the sign of the optical rotation of the individual enantiomers with those published in the literature for closely related compounds.[22] Chromatography of racemate **1p** gave: *S* isomer: t_R = 12.81 min, $[\alpha]_D$ +56.0 (MeOH); *R* isomer: t_R = 13.45 min, $[\alpha]_D$ −54.5 (MeOH). Chromatography of racemate **5k** gave: *S* isomer: t_R = 10.94 min, $[\alpha]_D$ +37.2 (MeOH); *R* isomer: t_R = 13.45 min, $[\alpha]_D$ −36.9 (MeOH).
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26. HEK 293 cells stably transfected with human FATP4 were incubated in assay buffer (Hank's balanced salt solution, 10 mM Hepes, and 0.1% fatty acid free BSA, pH 7.5) containing 50 nM [1-^{14}C] lauric acid (specific activity ~50 mCi/mmol; American Radiochemicals, St. Louis, MO) and 10 μM lauric acid for 1 h at ambient temperature. Cells were then washed twice in assay buffer and lysed by incubating in 50 mM NaOH for 15 min. Cell-associated radioactivity was measured in a MicroBeta scintillation counter. Assays were performed in triplicate with at least two independent determinations for each data point. IC_{50} values were determined using the XL-fit software with vector-transfected cells as 100% inhibition control.
27. For example, plasma exposure data for compound **1p** and two analogs in DIO in mice after 30 mg/kg oral dose are as follows:

	1p	**1a**	**5k**
Plasma AUC (nM h)	4650	6525	10,700
Plasma $T_{1/2}$ (h)	3.9	4.9	5.3
Plasma C_{max} (nM)	1320	710	1300

28. C57BL/6 male mice were placed on a 45% high fat diet for 1 week. After an overnight fast, FATP4 inhibitor **1p** (racemic) or the lipase inhibitor orlistat (Xenical™), or vehicle was administered by oral gavage. Mice were then given access to food immediately after dosing. After 6 h, blood, stomach, small intestine (duodenum, jejunum, and

ileum), other tissues and their contents were collected and analyzed for lipids and **1p**.

29. In order for FATP4 inhibitors to distribute to the site of action (enterocytes) and to function at the intracellular active site, cell permeable compounds are required, (several compounds with attenuated permeabilities such as quaternary ammonium derivatives and high molecular weight symmetrical dimers were also prepared but none showed any FATP4 inhibitory activity; unpublished results). Subsequent distribution into the blood compartment may, however, be detrimental to efficacy; nonetheless, the residual high concentrations of **1p** in intestinal tissues were thought to be sufficient to demonstrate proof of concept.
30. Analysis of FATP4 knock-out animals showed that 50% inhibition of fatty acid uptake by enterocytes does not result in a detectable defect in fat absorption in vivo, suggesting a large excess capacity for fatty acid uptake in the small intestine.[7] This excess capacity will make it more difficult to detect inhibition in vivo. Furthermore, while compound **1p** was able to inhibit fatty acid uptake in a transfected cell system, we have not yet assessed its ability to inhibit uptake in primary enterocytes in vitro using conditions that mimic the environment of the small intestine. Depending on the mechanism of inhibition, the potency of **1p** may be significantly altered in the intestinal environment.