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Heteroaryl urea inhibitors of fatty acid amide hydrolase: Structure–mutagenicity relationships for arylamine metabolites

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ABSTRACT

The structure–activity relationships for a series of heteroaryl urea inhibitors of fatty acid amide hydrolase (FAAH) are described. Members of this class of inhibitors have been shown to inactivate FAAH by covalent modification of an active site serine with subsequent release of an aromatic amine from the urea electrophile. Systematic Ames II testing guided the optimization of urea substituents by defining the structure–mutagenicity relationships for the released aromatic amine metabolites. Potent FAAH inhibitors were identified having heteroaryl amine leaving groups that were non-mutagenic in the Ames II assay.

Fatty acid amide hydrolase (FAAH) is responsible for degrading a family of fatty acid amide signaling molecules including the endogenous cannabinoid receptor (CB1/CB2) agonist anandamide.[1] Modulating the cannabinoid system by FAAH inhibition is an attractive approach for treating a variety of conditions associated with cannabinoid receptor function including pain, anxiety, and depression. Elevating levels of anandamide by inhibiting its degradation is anticipated to have an improved side effect profile relative to exogenous cannabinoid receptor agonists such as Δ^9-tetrahydrocannabinol (THC) that cause cognitive and motor impairment. Genetic knockout mice lacking FAAH have anandamide levels that are elevated 15-fold relative to wild-type animals and exhibit an analgesic phenotype but are otherwise healthy,[2] providing a rationale for the therapeutic value of FAAH inhibition.[3]

The majority of FAAH modulators that have been reported in the literature are mechanism-based inhibitors that inactivate the enzyme by forming a covalent bond with active site serine-241 (Fig. 1),[4–7] although an increasing number of recent publications have described compounds that inhibit FAAH by noncovalent mechanisms.[8–11] Covalent modification[12] is an effective approach for modulating FAAH activity because nearly complete enzyme inhibition is required before anandamide levels are significantly elevated.[13,14] Phenyl carbamate inhibitors exemplified by URB-597 (**1**) were reported to be effective in animal models of analgesia and anxiety.[15,16] However, the clinical utility of URB-597 may be limited

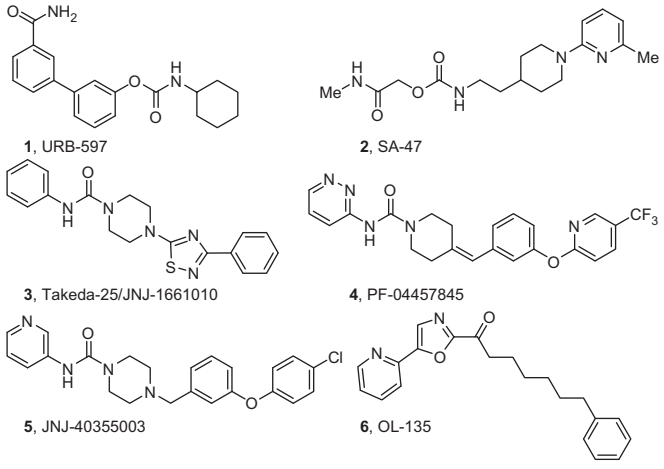

Figure 1. Representative FAAH inhibitors.

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by the high reactivity of the phenyl carbamate electrophile and the corresponding diminished selectivity with respect to other serine hydrolase enzymes.[14] Sanofi-Aventis introduced alkyl carbamate FAAH inhibitors (**2**)[17] having increased chemical stability and an improved selectivity profile relative to **1** using activity based protein profiling.[18,19] Aryl ureas such as **3** have been extensively explored in the medicinal chemistry efforts of Takeda,[20] Johnson & Johnson,[21–23] Pfizer,[24] and Astellas.[25] Heteroaryl ureas have subsequently been disclosed by Pfizer[14,26–28] (**4**) and Johnson & Johnson (**5**).[29–32] A structurally and mechanistically distinct series of ketooxazole FAAH inhibitors exemplified by OL-135 (**6**) was discovered and extensively characterized by Boger and co-workers at The Scripps Research Institute.[33–36] The structure of OL-135 contains an electrophilic ketone that inhibits FAAH by trapping the active site serine-241 in a reversible hemiketal.[37–39]

Our efforts to improve the pharmacological properties of aryl urea FAAH inhibitors such as **3** have focused on identifying heteroaryl amine replacements for the aniline fragment to reduce the potential risk associated with expelling an equivalent of aniline upon reacting with the enzyme. Acute exposure to aniline causes methemoglobinemia in humans via oxidation of hemoglobin (Fe^{2+}) to methemoglobin (Fe^{3+}), which does not effectively deliver oxygen to tissues.[40,41] There are many examples of arylamines that are known to be carcinogenic,[42] although reports on the carcinogenicity of aniline itself are conflicting.[43] Aniline replacements having a reduced toxicity risk are required for urea FAAH inhibitors with improved safety profiles relative to phenyl ureas.

It is desirable to identify risks such as carcinogenicity early in a drug discovery effort,[44] however the low throughput and high cost of animal models of carcinogenicity limit their utility for triaging compounds in the lead optimization stage. Genetic toxicology tests are a more rapid alternative that are routinely used for predicting the carcinogenicity of preclinical drug candidates. In general, a battery of tests is used to assess the different types of genotoxic effects that might be associated with human disease. The widely used bacterial reverse mutation assay, or Ames test, detects the tendency of a molecule or its metabolites to inflict genetic damage by measuring the frequency of reverse mutations in set of *Salmonella typhimurium* strains.[45] Although the predictive ability of such in vitro experiments has limitations, including an inability to detect non-mutagenic mechanisms of carcinogenicity,[43,46] the Ames test is a useful selection tool for eliminating high risk molecules before investing in more resource-intensive *in vivo* safety models. The Ames II assay[47,48] is a liquid fluctuation modification in microplate format of the traditional Ames assay with reduced compound requirements and higher throughput. TAMix, a mixture of TA7001–TA7006 strains, is used for the detection of base pair substitutions and TA98 to detect frameshift mutations.

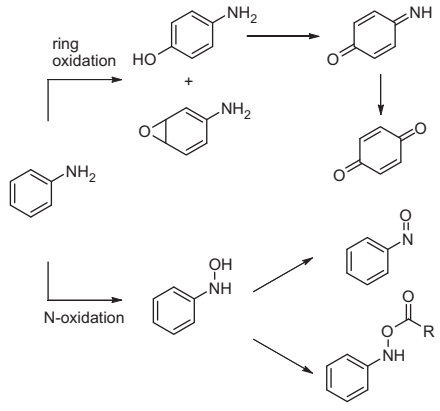


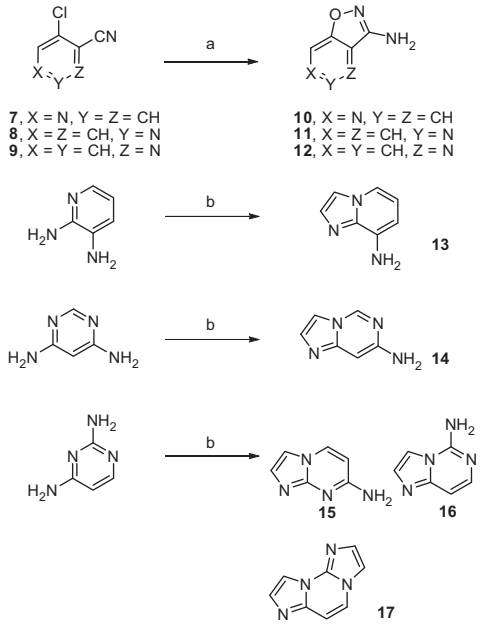
Figure 2. Oxidation of aniline to reactive intermediates.

Aromatic amines are typically not harmful themselves, but they can be susceptible to oxidation by cytochrome P450 enzymes to reactive metabolites. In the Ames II assay, the test compounds are preincubated with a rat liver extract (S9) to simulate metabolic activation by liver enzymes. Several characterized pathways for the oxidative metabolism of anilines to generate reactive species have been described (Fig. 2).[49] Carbon oxidation of the electron-rich aromatic ring can yield electrophilic epoxides or quinones that are capable of forming conjugates with biological macromolecules. Alternatively, N-oxidation produces *N*-hydroxylamines[50] that have the potential to be reactive themselves or can act as intermediates in the formation of other reactive species including O-acylated hydroxylamines[51] or nitrosobenzenes.[41] Aryl nitrosyl functionality plays a direct role in arylamine-induced methemoglobinemia by mediating the oxidation of hemoglobin, and also can cause carcinogenicity through covalent modification of DNA.

Quantitative structure–activity relationships (QSAR) of aromatic amines have been used to design computational models that predict mutagenicity and mutagenic potency.[49] An important molecular feature that contributes to the occurrence of mutagenicity in arylamines is the energy of the highest occupied molecular orbital (HOMO),[52] an indication of a molecule's propensity for N-oxidation. Steric hindrance surrounding the amino group can also block the formation of reactive metabolites by reducing access of the amine to metabolizing enzymes.[53] Mutagenic potency, measured by the frequency of revertant colonies in the Ames assay, is correlated with lipophilicity of the aniline and the number of fused rings, which can be rationalized as an increased tendency for planar, hydrophobic molecules to bind and intercalate DNA.

Herein, we report the synthesis and structure–activity relationships for urea FAAH inhibitors having modifications to the heteroaryl amine fragment, and Ames II data for the corresponding arylamine leaving groups.

The syntheses of non-commercially available heteroaryl amines **10**–**12** were completed according to Scheme 1.[30] Isoxazolo[5,4-*c*]pyridin-3-ylamine **10** was prepared by treating 3-chloroisonicotinonitrile **7** with an excess of acetohydroxamic acid under basic conditions. S_NAr addition of the hydroxamate followed by



Scheme 1. Synthesis of heteroaryl amines. Reagents and conditions: (a) acetohydroxamic acid, K_2CO_3, DMF, rt to 50 °C, 20 h, **10**, 41%; **11**, 77%; **12**, 25%; (b) chloroacetaldehyde, H_2O, $NaHCO_3$, rt, 20 h, **13**, 50%; **14**, 37%; **15**, 25%; **16**, 30%; **17**, 4%.

cyclization onto the nitrile provided the corresponding desired product in a one-pot procedure.[54,55] An analogous method was used to prepare the isomeric heterocycles **11** and **12**. Imidazo[1,2-*a*]pyridin-8-ylamine (**13**) was synthesized by treating 2,3-diaminopyridine with chloroacetaldehyde, giving the desired product after alkylation and cyclization to the fused imidazole.[56] Pyrimidine-4,6-diamine was converted to **14** using an analogous approach. Pyrimidine-2,4-diamine was capable of cyclizing as distinct isomers **15** and **16** which were both isolated in comparable yields, along with a byproduct **17** from reaction with another equivalent of chloroacetaldehyde.

A diverse set of heteroaryl amines reacted readily with phenyl chloroformate in the presence of pyridine to give the corresponding phenyl carbamates as shown for representative examples **18a–h** (Scheme 2). Some highly electron-deficient heteroaryl amines such as benzo[*d*]isoxazol-3-ylamine (**18l**) returned only starting material under these reaction conditions. The desired carbamates **18i–p** were prepared at elevated temperature using phenyl chloroformate as the limiting reagent, without additional base.

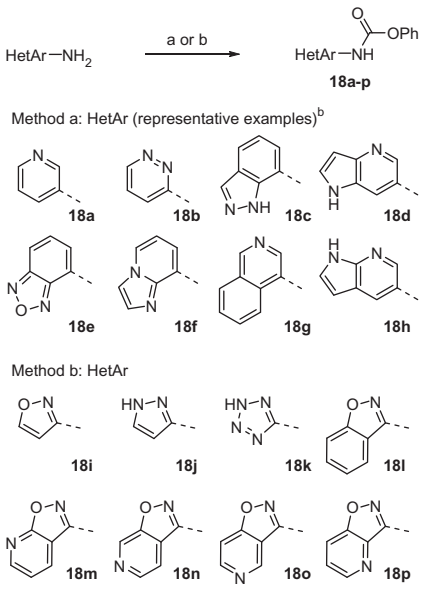
The urea FAAH inhibitors were prepared using a modular synthesis that was readily amenable to diversification of urea substituents (Scheme 3). The benzyl group was appended to *N*-Boc piperazine by reductive amination, followed by deprotection under acidic conditions to give intermediate **19**.[30] The 3-(4-chlorophenoxy)-benzyl piperazine was elaborated to ureas having diverse *N*-heteroaryl substituents by reaction with phenyl carbamates. Selected ureas were assembled in a one-pot procedure by acylating the amine with disuccinimidyl carbonate, then treating with **19** to provide the final ureas without isolation of the intermediate carbamate.

The structure–activity relationships for the heteroaryl amine fragment were defined by evaluating diverse heterocyclic substituents while maintaining the optimized 3-(4-chlorophenoxy)-benzyl-piperazine portion of the molecule constant (Table 1). All of the compounds prepared in this series were expected to be time-dependent inhibitors of FAAH based on their structural similarities to characterized covalent inhibitors **3** and **4**.

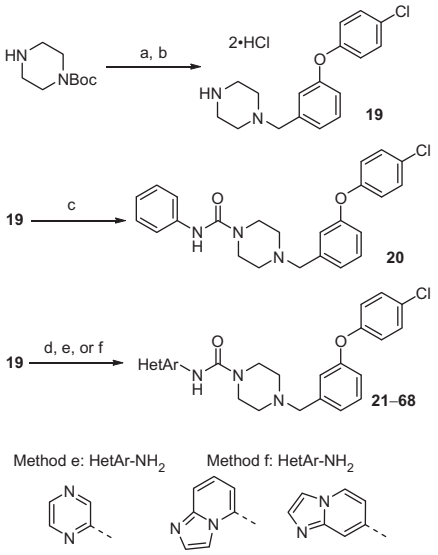
The inhibitory potencies in Table 1 are reported as apparent IC_{50} values and were run under identical incubation times.[57] A series of six-membered heteroaryl ureas were prepared, displaying low nanomolar human FAAH inhibitory potencies that were similar to aniline **20** under the assay conditions. 3-Aminopyridine (**5**)[32] was the optimal six-membered heteroaryl amine in this series. Compounds having pyridine, pyridazine, and pyrazine substituents collectively demonstrated that nitrogen atoms were tolerated in all ring positions. A diverse set of five-membered amino-heterocycles (**23–26**) were somewhat less effective urea substituents. With the exception of the tetrazole analog **24**, the compounds are generally several-fold more potent at human FAAH than rat FAAH.

The free arylamines in Table 1 were evaluated in the Ames II assay with and without (S9) metabolic activation to assess the risk of mutagenicity for the fragment that is released after reaction with FAAH. All unsubstituted five- and six-membered heteroaryl amines shown in Table 1 were Ames II negative, consistent with QSAR predictions that small, electron-deficient and relatively polar heteroaryl amines are less susceptible to metabolic activation leading to DNA damage. However, the 4-chloro-3-amino-oxazole found in **26** was unexpectedly Ames II positive, despite having an electron-deficient amine substituent. Heteroaryl amines usually require metabolic activation to cause mutagenicity in the Ames II test, but 4-chloro-3-amino-oxazole was positive with and without metabolic activation. The molecular characteristics that determine mutagenicity are complex, and exhaustive Ames II testing is required to detect outliers such as 4-chloro-3-amino-oxazole.

Fused bicyclic heteroaryl ureas were prepared to extend the structure–activity relationship data to include larger amine substituents. Several bicyclic heteroaryl ureas were potent human and rat FAAH inhibitors, including compounds having diverse spacial arrangements and functionality (Table 2). Initially, the compounds in Table 2 appeared to be a promising new direction for optimization, however the bicyclic amines were much more likely to be mutagenic than the monocyclic amines in Table 1, with six out of the seven heteroaryl amines testing positive in the Ames II assay. All six Ames II positive compounds in Table 2 were mutagenic only when treated with S9 to simulate metabolic activation. The impact of adding a fused aryl ring is apparent in the comparison between 3-aminopyridine (Ames II negative) in **5** versus the more lipophilic bicyclic analog isoquinolin-4-amine (Ames II

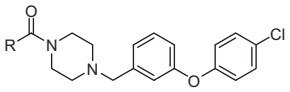


Scheme 2. Preparation of heteroaryl carbamates. Reagents and conditions: (a) PhOCOCl, pyridine, CH_3CN, rt, 2 h, 39–100%; (b) 0.33 equiv PhOCOCl, CH_3CN, 70 °C, 20–33%.



Scheme 3. Preparation of heteroaryl piperazinyl ureas. Reagents and conditions: (a) 1.5 equiv 3-(4-chlorophenoxy)benzaldehyde, 3 equiv $NaB(OAc)_3H$, DCM, rt, 23 h 70%; (b) 4 N HCl in dioxane, DCM, rt, 23 h, 50%; c) 1.1 equiv PhNCO, 4 equiv DIPEA, DCM, rt, 23 h, 77%; (d) **18**, DIPEA, CH_3CN, rt, 24 h, 50–95%; (e) HetAr-NH_2, disuccinimidyl carbonate, rt, 16 h then **19**, DIPEA, 63%; (f) HetAr-NH_2, disuccinimidyl carbonate, DMAP, rt, 16 h then **19**, DIPEA, 9–53%.

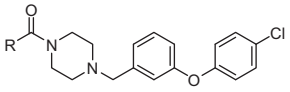
Table 1
Monocyclic heteroaryl ureas

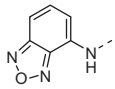
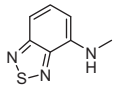
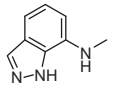
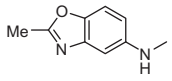
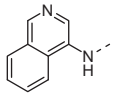
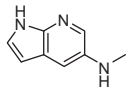
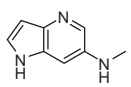


	R	Apparent hFAAH IC_{50}[a] (nM)	Apparent rFAAH IC_{50}[a] (nM)	HetAR-NH_2 Ames II result
20		6 ± 4	300 ± 86	Neg
21		8.7 ± 6.3	150 ± 64	Neg
22		7.7 ± 2.3	93 ± 22	Neg
5		1.4 ± 0.4	33 ± 9	Neg
23		32 ± 5	150 ± 80	Neg
24		103 ± 32	46 ± 9.6	Neg
25		28 ± 7	720 ± 330	Neg
26		5.2 ± 1.1	20 ± 4.4	Pos

[a] Values reported as the mean ± SEM of at least three independent determinations in duplicate. IC_{50} values were measured with a 60 min preincubation.

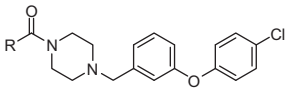
Table 2
Fused bicyclic heteroaryl amines

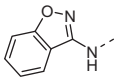
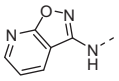
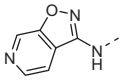
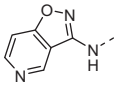
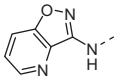
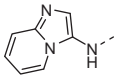
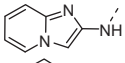
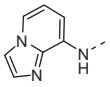
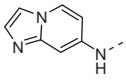
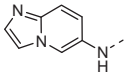
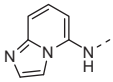
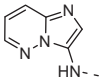
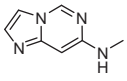
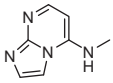
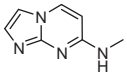


	R	Apparent hFAAH IC_{50}[a] (nM)	Apparent rFAAH IC_{50}[a] (nM)	HetAR-NH_2 Ames II result
27		28 ± 13	120 ± 50	Pos
28		5.8 ± 1.9	25 ± 6	Neg
29		1300 ± 680	6000 ± 2500	Pos
30		7.7 ± 4.2	200 ± 140	Pos
31		6.7 ± 2.3	8.3 ± 4.1	Pos
32		3.4 ± 1.7	21 ± 7	Pos
33		69 ± 49	1100 ± 240	Pos

[a] Values reported as the mean ± SEM of at least three independent determinations in duplicate. IC_{50} values were measured with a 60 min preincubation.

Table 3
Fused bicyclic heteroaryl ureas



	R	Apparent hFAAH IC_{50}[a] (nM)	Apparent rFAAH IC_{50}[a] (nM)	HetAR-NH_2 Ames II result
34		10 ± 2	8.1 ± 2.5	Neg
35		22 ± 15	14 ± 6.7	Neg
36		0.5 ± 0.3	0.7 ± 0.5	Neg
37		180 ± 80	2200 ± 700	Neg
38		13 ± 3	22 ± 6	Neg
39		46 ± 16	120 ± 53	Neg
40		>10,000	>10,000	Neg
41		310 ± 70	3500 ± 1900	Neg
42		33 ± 16	2600 ± 1600	Pos
43		100 ± 60	3600 ± 2200	Neg
44		2.7 ± 0.4	5.7 ± 1.1	Neg
45		3.7 ± 2.6	53 ± 41	Neg
46		17 ± 2	470 ± 100	Neg
47		3.0 ± 1.2	24 ± 11	Neg
48		140 ± 70	2400 ± 158	Neg

[a] Values reported as the mean ± SEM of at least three independent determinations in duplicate. IC_{50} values were measured with a 60 min preincubation.

positive) in **31**. This observation is consistent with previous studies correlating increased lipophilicity with mutagenicity. Despite having encouraging FAAH potency, the high frequency of positive results in the Ames II assay for the bicyclic amines in Table 2 precluded these analogs from further profiling.

An additional series of bicyclic heteroaryl amines was designed to address the high frequency of mutagenicity identified in Table 2, based on the hypothesis that compounds having strongly electron-deficient heteroaryl groups would be less likely to participate in the metabolic activation that often characterizes aniline toxicity. Benzo[d]isoxazol-3-ylamine urea **34** (Table 3) was a potent FAAH inhibitor having an Ames II negative heteroaryl amine that served as a starting point for further optimization. Pyridyl analogs of **34** were prepared to reduce the lipophilicity associated with the aromatic ring. The isoxazolo[5,4-*c*]pyridin-3-ylamine urea **36** was a subnanomolar inhibitor of both human and rat FAAH, and isomers **35** and **38** were equipotent with the **34**, although the location of the nitrogen atom in isomer **37** was not well tolerated. None of the five heteroaryl amines related to benzo[*d*]isoxazol-3-ylamine scaffold were mutagenic in the Ames II assay, demonstrating that bicyclic heteroaryl amines can be useful urea substituents provided that they are strongly electron-deficient.

Having shown that heteroatoms are well tolerated in the urea substituent, imidazo[1,2-*a*]pyridin-3-ylamine **39** was prepared as

an isosteric analog of **34** having a more polar, basic imidazole in place of the oxazole ring. The orientation of the imidazo[1,2-*a*] pyridine ring system strongly influenced the FAAH IC_{50}. The highest potency was generally achieved when the amino group was located adjacent to a ring fusion (**39**, **44**) versus in a linear arrangement (**40**). Analogs having the imidazole fused to pyrimidine and pyridazine rings were prepared to modify the electronic properties of the arylamine and the fused imidazole. The urea derived from imidazo[1,2-*b*]pyridazin-3-ylamine **45** was 10-fold more potent against human FAAH than the pyridine analog **39**. Similarly, imidazopyrimidines **46–48** maintained or improved FAAH activity relative to the corresponding imidazopyridines. One of the five compounds having the fused imidazole ring system (**42**) was positive in the Ames II assay, although two closely related analogs **46** and **48** were not mutagenic. The more polar and electron-deficient fused pyrimidine ring may contribute to reduced metabolic activation of **46** and **48** relative to **42**.

The structure–activity relationships for a series of mechanism based inhibitors of fatty acid amide hydrolase were explored, focusing on modifications to the heteroaryl urea that forms a covalent bond with FAAH. The covalent mechanism of enzyme inhibition required evaluating each released heteroaryl amine metabolite separately from the parent urea for its mutagenic potential using the Ames II assay. A variety of unsubstituted five- and six-membered heteroaryl ureas were potent inhibitors of FAAH, and the corresponding heteroaryl amines were Ames II negative in all cases. Bicyclic heteroaryl amines were much more likely to be mutagenic, but several analogs related to the benzo[*d*]isoxazol-3-ylamine **34** and imidazo[1,2-*a*]pyridin-3-ylamine **39** were potent FAAH inhibitors having Ames II negative metabolites.

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