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Discovery of heterocyclic carbohydrazide derivatives as novel selective fatty acid amide hydrolase inhibitors: design, synthesis and anti-neuroinflammatory evaluation

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ABSTRACT

Fatty acid amide hydrolase (FAAH) is a promising target for the development of drugs to treat pain, inflammation, and other central nervous system disorders. Herein, a series of novel heterocyclic carbohydrazide derivatives were firstly designed by the classic scaffold-hopping strategy. Then, multi-steps synthesis and human FAAH enzyme inhibiting activity assays were conducted. Among them, compound **26** showed strong inhibition against human FAAH with IC_{50} of 2.8 μM. Corresponding docking studies revealed that the acyl hydrazide group of compound **26** well-occupied the acyl-chain binding pocket. It also exhibited high selectivity towards FAAH when comparing with CES2 and MAGL. Additionally, compound **26** effectively suppressed the LPS-induced neuroinflammation of microglial cells (BV2) via the reduction of interleukin-1β and tumor necrosis factor-α. Our results provided significative lead compounds for the further discovery of novel selective and safe FAAH inhibitors with potent anti-neuroinflammation activity.

The endocannabinoid (eCB) system plays an important neuromodulatory role in the periphery and central nervous system, which can regulate several physiological processes such as appetite, anxiety, mood and pain. It contains cannabinoid receptors (CBs, CB1 and CB2) and their main transmit{Keith, 2014 #55}ters, including endocannabinoids anandamide (AEA), 2-arachidonoylglycerol (2-AG), and the enzymes responsible for these biosynthesis and degradation (Fig. 1). AEA is formed “on-demand” during several physiological and pathophysiological conditions and also mediates analgesic and anti-inflammatory effects by activation of the cannabinoid receptors CB1 and CB2, which is rapidly hydrolyzed into arachidonic acid (AA) and ethanolamine by fatty acid amide hydrolase (FAAH). FAAH belongs to the serine hydrolase family,[3] and characterized by the unusual catalytic triad Ser217 - Lys142 - Ser241. In addition to AEA, FAAH also degrades other ethanolamides, such as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), which suppress pain and inflammation through the peroxisome proliferator-activated receptor (PPAR-α).[4]

Genetic or chemical inhibition of FAAH produces elevated levels of AEA in the brain and periphery, as well as potentially benefits in animal models of anxiety and pain[5] without undesirable side effects (such as hypothermia, catalepsy, and hyperphagia) observed with direct cannabinoid receptor agonists.[1,5,6] Hence, during recent decades, regulation of the AEA signaling through the inhibition of FAAH by the activation of the cannabinoid (CB) G-protein coupled receptors has been considered as an attractive therapeutic approach for the treatment of pain, inflammation[7] and other central nervous system disorders.[8]

In the past years, many potent FAAH inhibitors have been developed (Fig. 2).[9,10,11] The most widely investigated FAAH inhibitor is URB597, which was reported to display anxiolytic and antidepressant-like activity in various rodent models.[12] It’s worth mentioning that, several FAAH inhibitors (e.g. BIA 10–2474, JNJ-40355003, ASP 8477) have entered into clinical trials to assess their potential efficacy in patients suffering from major depressive disorder (MDD), social anxiety or post-traumatic stress disorder (PTSD).[10] In 2018, PF-04457845 was demonstrated to be effective on patients suffering from cannabis withdrawal symptoms in a phase II study.[12] Furthermore, FAAH inhibitors are useful for the treatment of Parkinson's disease via preventing or reducing the inflammatory process associated with Aβdeposition.[13–15]

However, during the phase I trial of BIA 10-2474, one death and four mild-to-severe neurological symptoms were reported.[16] Subsequent studies had confirmed that the severe adverse effects of BIA 10-2474 did not result from the inhibition of FAAH, but from the produced substantial alterations in human cortical neurons (such as CES2 and

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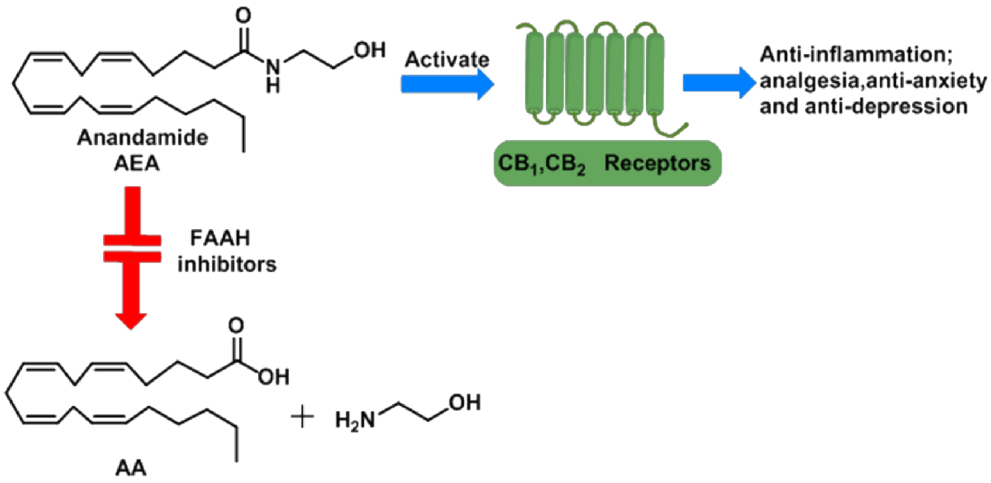


Fig. 1. FAAH inhibitors mediated biological effects.

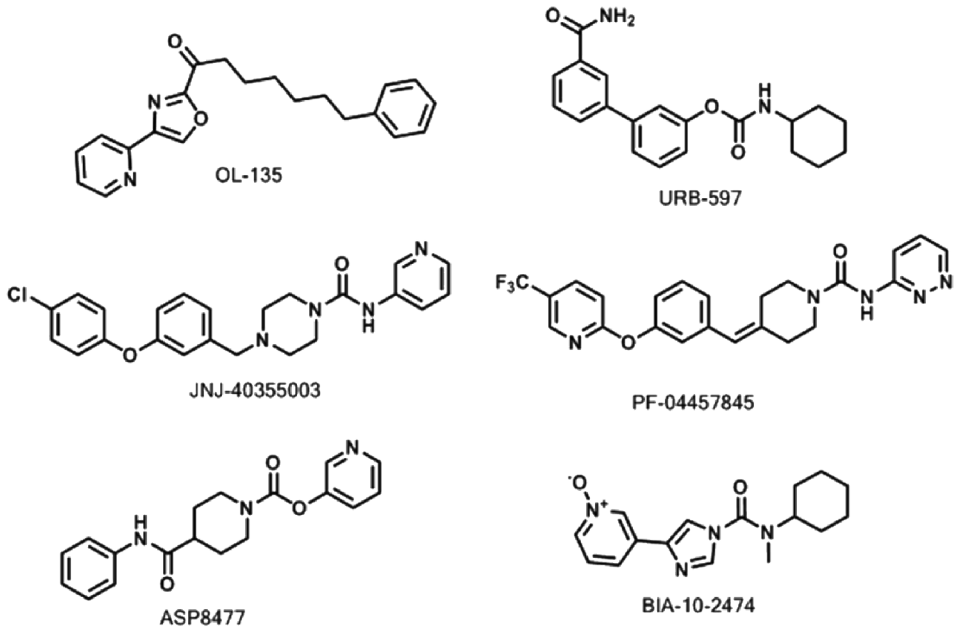


Fig. 2. Structures of some known FAAH inhibitors.

PNPLA6),[17] which resulted in the neurotoxicity due to disruption of cellular lipid networks. Thus, the development of novel FAAH inhibitors with specificity and safety is an urgent issue to combat central nervous system disorders.

FAAH inhibitors can be broken into the three modular units[18]: part tail group, an acyl-chain binding part (urea or carbamate group) and head group which acts as a leaving group upon covalent binding to Ser ^{241}-OH of FAAH.[19,20] As shown Fig. 2, most FAAH inhibitors such as PF-04457845, ASP8477 and JNJ-40355003 are all urea derivatives with a long hydrophobic chain as tail group, which had been well tolerated in phase I study. It was reported that off-target effects of BIA-10–2474 were related to the highly reactive carbonyl imidazole structural element.[2] Meanwhile, a shorter hydrophobic chain was found to occupy the hydrophobic cavity in membrane access channel.[21] Therefore, employing BIA-10-2474 as a lead structure, we planned to design and synthesis of a series of novel highly selective FAAH inhibitors (Fig. 3) with carbohydrazide cores as acyl-chain binding part (scaffold A). Moreover, imidazol head group was substituted with either heteroaromatic such as indoles, pyrazoles, oxazoles (**compounds 1–12**) as well as extended aromatic tail group such as phenyl, naphthalene, benzyloxy (**compounds 12–26**). Docking studies indicated that the carbonyl of the carbohydrazide core could form a tetrahedral intermediate with the Ser241, Ile238 and Gly239, which were essential for inhibitory activity. In addition, the introduction of different heteroaromatic carbohydrazide groups instead of the carbonyl imidazole group was supposed to optimize physicochemical parameters (such as solubility).

Our initial converted the pyridazole moiety of lead compound BIA-10-2474 to various heteroaromatic groups (**compounds 1–6**). In addition, different link chains (cyclohexyl, phenyl) were also investigated (compounds 7–12). The IC_{50} results were listed in Table 1. Interestingly, the heteroaromatic compounds such as indolyl, phenylisoxazoleyl and phenyl-1H-pyrazoleyl, benzimidazolyl exhibited moderate to good FAAH inhibiting activity, instead of the cyclohexyl with benzene as links the activity had not apparently changed (compounds 7–12). Among them, the head group substituted by benzimidazolely group resulted in the most active compound **9** (IC_{50} = 12.4 μM), which was selected as the new lead compound for further optimization.

Our optimization strategy was replacing the tail groups with various substitutes (F, Cl) to explore more potent compounds and to establish the structure–activity relationship. We synthesized the *meta*-substituted analogue of **14** (compound **13**) and *para*-substituted analogue of **15** (compound **16**). These compounds exhibited a similar trend in the

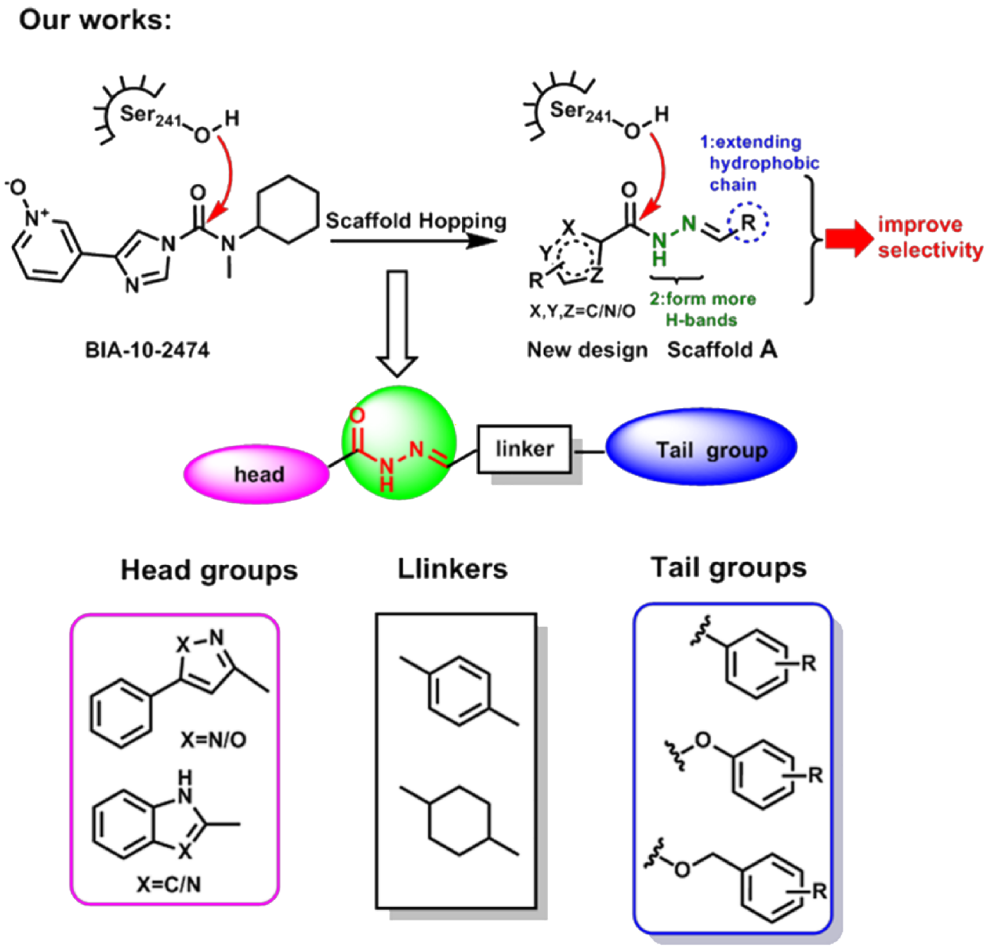


Fig. 3. Design of new FAAH inhibitors.

Table 1

Biological activity of compounds 1–12.

Compd	R^1	R^2	Inhibition of FAAH IC_{50}(μM)
1	2-indolyl	cyclohexyl	18.6
2	3-indole ethyl	cyclohexyl	> 50
3	2-benzimidazolyl	cyclohexyl	13.0
4	2-benzooxazolyl	cyclohexyl	27.1
5	5-phenyl-3-pyrazolyl	cyclohexyl	41.3
6	5-phenyl-3-isoxazolyl	cyclohexyl	38.7
7	2-indolyl	phenyl	20.6
8	3-indole ethyl	phenyl	46.6
9	2-benzimidazolyl	phenyl	12.4
10	2-benzooxazolyl	phenyl	25.0
11	5-phenyl-3-pyrazolyl	phenyl	38.6
12	5-phenyl-3-isoxazolyl	phenyl	46.1
BIA-10–2474	/	/	4.1

inhibitory activities against FAAH, since *meta*-substitution (compound **13** and **15**) decreased the inhibitory activity while *para*-substitution (compound **14** and **16**) improved it.

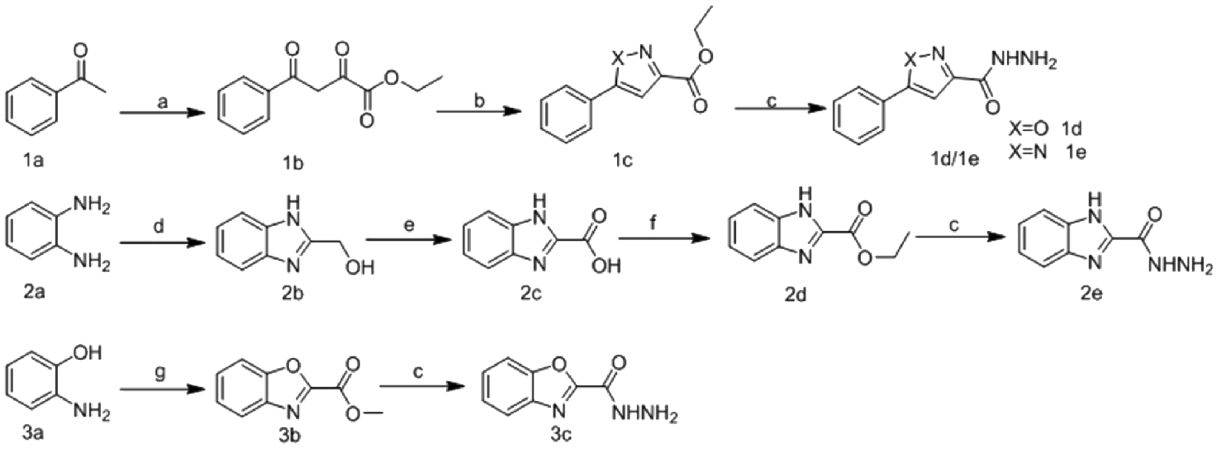
As shown in Table 2, compounds synthesized by replacing the tail groups with a variety of hydrophilic groups (OH, CN, morpholinyl) were evaluated the FAAH inhibition (e.g **18–20**). Substituents in the 4-position with hydrophilic groups (such as **18, 19, 20**) are disadvantageous to the activity, while 4-substitution with lipophilic groups (such **21, 22, 23**) tended to improve the potency. After changing different hydrophobic groups as tail groups, such as naphthalene, indole, benzyloxy and diphenyl, the compound 26 was found to be the most active one (IC_{50} = 2.8 μM) among these molecules, which is approximately 3-fold stronger than compound 9. Those results indicated that the hydrophobic tail groups (benzyloxy) with appropriate length and rigidity was quite favorable for the inhibitory activity.

Table 2

Biological activity of compounds 13–26.

Compd	R^1	Inhibition of FAAHIC50(μM)
3	cyclohexyl	13.0
9	phenyl	12.4
13	3-flurophenyl	27.2
14	4-fluorophenyl	17.9
15	3-chiorophenyl	20.1
16	4-chlorophenyl	7.3
17	4-methoxyphenyl	11.5
18	4-cyanophenyl	21.1
19	4-hydrophenyl	24.3
20	4-morpholinphenyl	29.8
21	4-biphenyl	10.3
22	4-phenoxyphenyl	7.2
23	4-benzyloxyphenyl	4.2
24	2-naphthyl	5.7
25	5-indolyl	4.0
26	4-(3-fluorobenzyloxy)phenyl	2.8
BIA-10–2474	/	4.1

The synthetic routes of the key intermediates **1d/2e/3c** were illustrated in Scheme 1. Acetophenone **1a** was used as the starting material. After being treated with diethyl oxalate in the presence of NaOEt,



Scheme 1. Synthesis of intermediates 1d/1e/2e/3c. Reagents and conditions: (a) Diethyl oxalate, NaOEt, 8 h; (b) Hydroxylamine hydrochloride/ hydrazine hydrate, EtOH, reflux, 2 h; (c) Hydrazine hydrate, EtOH, reflux, 3 h; (d) Glycolic acid, EtOH, reflux; (e) Potassium permanganate, NaOH/H_2O, reflux, 5 h; (f) Ethanol, HATU, THF, rt; (g) Ethyl bromoacetate, pyridine, reflux.

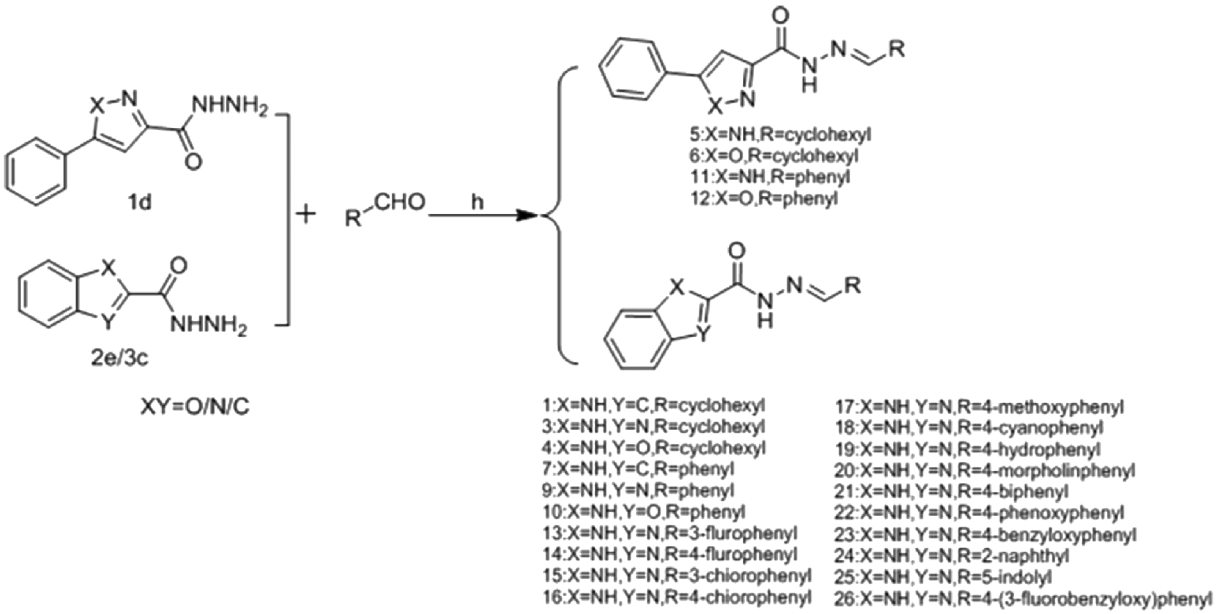
which was claisen condensation, the intermediate **1b** was obtained. Then, **1b** was allowed in a ring-closure reaction with hydroxylamine hydrochloride or hydrazine hydrate to form **1c**. Compound **1c** was further treatment with hydrazine hydrate to afford **1d**. The 1H-benzimidazole-2-carboxylic acid (**2c**) was synthesized through oxidizing 2-(hydroxymethyl) - benzimidazole in alkaline conditions with o-phenylenediamine. Synthesize **2e** was prepared according to the similar procedure described above.

The target compounds **1–26** were formed according to the reaction pathways illustrated in Scheme 2. The intermediates hydrazides (**1d/2e/3c**) were further refluxed with suitably substituted benzaldehydes in ethanol for 3 h, gave the target compounds **1–26**[27].

For further understanding of the interaction between these inhibitors and protein (FAAH) for guiding the structure–activity relationship (SAR), we carried out a docking study of compound **9**, **20** and **26** with human FAAH (**PDB: 2WJ2**) via the program covalent docking of the Schrödinger suite. The proposed binding mode of compound **26** was illustrated in Fig. 4. It was observed that the central acylhydrazine module of compound **26** fitted into the oxyanion hole, which was composed with Ser241, Ile238, Gly239 and Gly240. Moreover, the acylhydrazine groups formed hydrogen bonds with residues Ser217, Ser193 and Met191, respectively (Fig. 4). At the tail of the inhibitor compound **26**, the lipophilic *meta*-fluorobenzyloxy formed hydrophobic interactions with the surrounding amino acid residues Phe381 and Phe432. Whereas compound **9** with a short hydrophobic chain, which poorly occupied the hydrophobic cavities (Fig. S1). Moreover, the poor activity of compounds 20 may be attributed to their increased lipophilicity (the end of the inhibitor is morpholinyl), which prevented them from interacting with residues Phe381 and Phe432. These docking results were in accord with their potencies, so this binding model could explain the SAR observed above.

Previous reports have confirmed that many of the off-targets of BIA 10–2474 occurred due to lipolytic enzymes including human carboxylesterase (CES2), which may have a contribution to the neurotoxicity of BIA 10-2474.[18] Therefore, compounds **23**, **25** and **26** were tested against CES2. Meanwhile, the selectivity of these compounds to monoacylglycerol lipase, which belonged to another major endocannabinoid degrading enzyme, was investigated. As shown in Table 3, these compounds exhibited strong inhibition of FAAH without inhibition of other serine hydrolase MAGL. Furthermore, compounds **23**, **25** and **26** were found low inhibition of CES2 (IC_{50} > 100 μM). It was suggested that these novel FAAH inhibitors posed a preferable selectivity, which less likely cause off-targets in the body.

Inflammation was the main causes of neurodegeneration in the



Scheme 2. General synthesis of the target compounds. Reagents and conditions: (h) ethanol, reflux, 1–3 h.

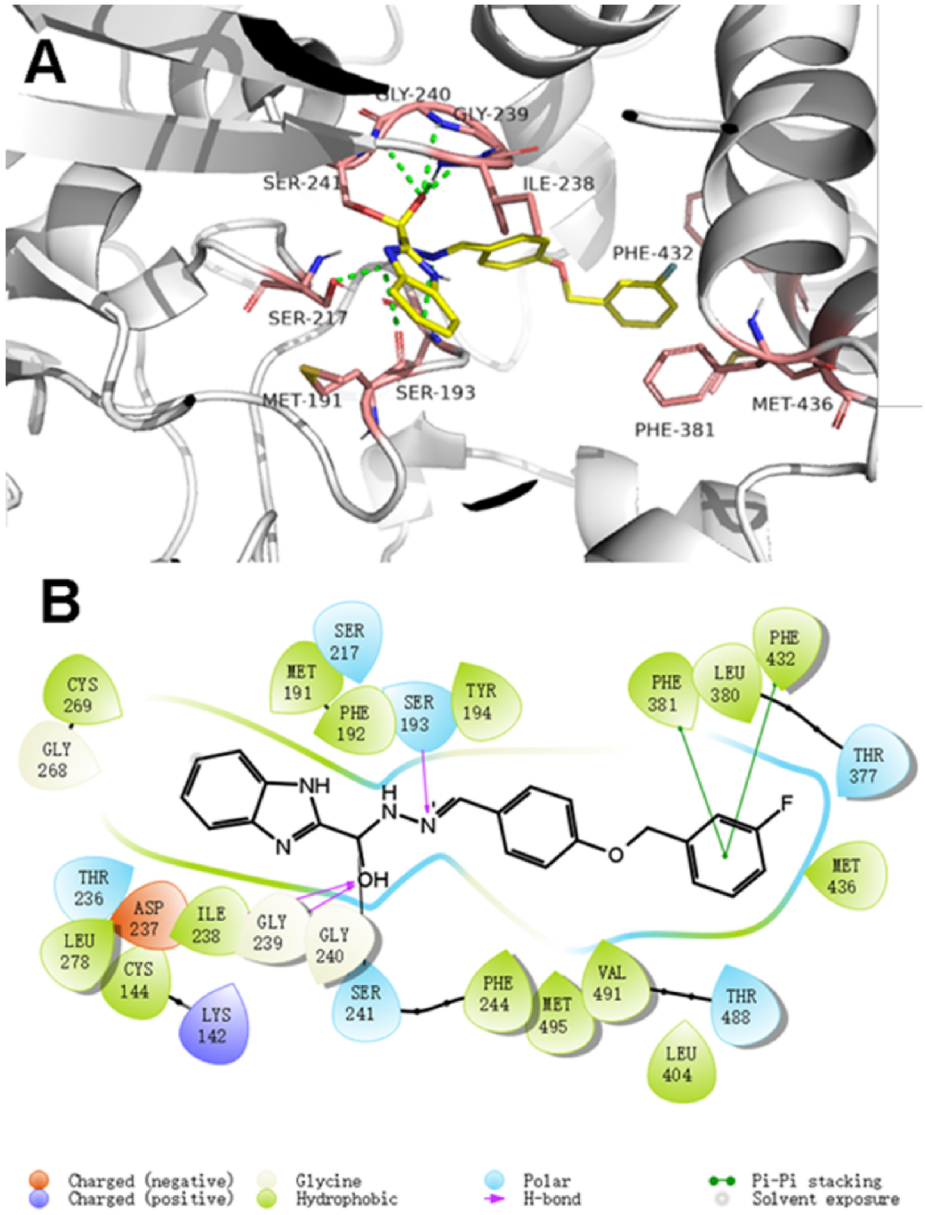


Fig. 4. Docking compounds 26 into the FAAH (PDB: 2WJ2). The protein was shown as ribbons. The key amino acids forming the pocket were represented by stick with carbon atoms colored in brown. Compound 26 was shown in stick with carbon atoms colored in yellow, oxygen atoms in red and nitrogen atoms in blue, hydrogen bond was denoted by green dash lines respectively.

Table 3
Biological activities (IC_{50} values for FAAH, MAGL and CES2) of compounds 23, 25 and 26.

Compd	FAAH IC_{50} (μM)	MAGL IC_{50} (μM)	CES2 IC_{50} (μM)
23	4.2	No active	> 100
25	4.0	No active	> 100
26	2.8	No active	> 100
BIA-10-2474	4.1	No active	0.26 μM

central nervous system (CNS).[22,23] The anti-inflammatory activity of compound **26** was evaluated on LPS-activated microglia inflammatory cell model.[27] As shown in Fig. 5, LPS induced a robust increase in transcription of IL-1β and TNF-α ($p < 0.0001$). Treatment with compound **26** with 40 μM significantly reduced the unregulated cytokine IL-1β ($p < 0.05$) and TNF-α ($p < 0.001$) in a dose-dependent manner, suggesting that compound **26** could obviously reduce the pathological inflammatory response. Therefore, this compound could be a promising lead compound for further discovery of new safe and effective FAAH inhibitors for treatment of inflammation involved the central nervous system (CNS) disease.

The toxicity risks (mutagenicity, tumorigenicity, irritation, and reproduction) of partly inhibitors, as well as ADMET prediction experiment, were predicted using Osiris Property explorer and DS4.0.[25] All active compounds reported herein were predicted to be safe and had good pharmacokinetics and pharmacodynamics properties (Fig. s4). The additional maximum tolerated dose (MTD) determination showed that compound **26** had obviously low toxicity with MTD values over 2 g/kg.

In summary, a series of novel heterocyclic carbohydrazide FAAH inhibitors were firstly reported in this paper. Amongst all the tested compounds, compound **26** (IC_{50} = 2.8 μM) was found to be the most active compound. Docking studies indicated the oxyanion hole channel was the preferential compound **26** binding sites and the newly developed compounds possed a long aryl chain in the acylhydrazide group, which had high inhibition potency on FAAH and strongly reduced their affinity to MAGL and CES2 respectively. In addition, compound **26**

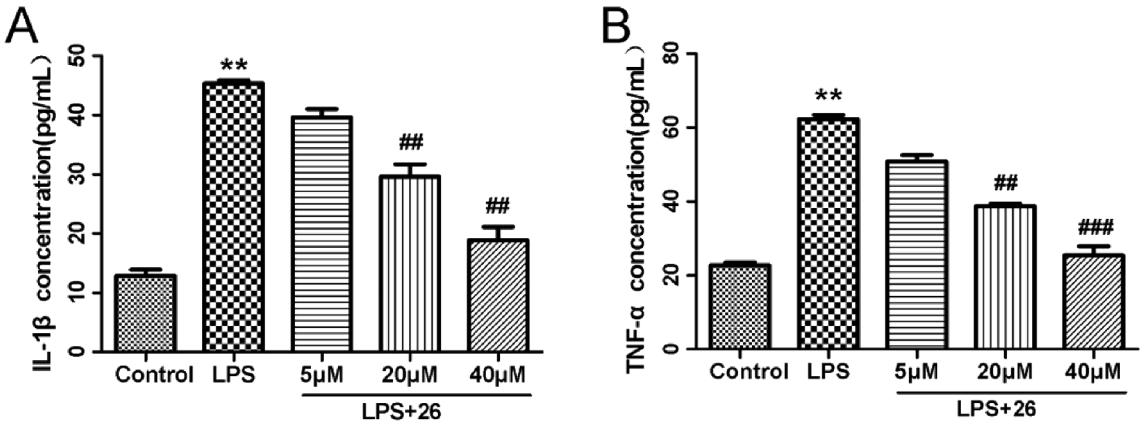


Fig. 5. FAAH inhibitor 26 reduced inflammation in cells. Normal BV2 cells were induced by LPS and then treated with different concentrations of 26. Cytokines were evaluated by ELISA. $^{**}p < 0.0001$ compared to control group, $^{\#\#}p < 0.05$ compared to LPS group, $^{\#\#\#}p < 0.001$ compared to LPS group.

effectively suppressed the LPS-induced neuroinflammation of mouse-derived microglial cells (BV2) via the reduction of IL-1β and TNF-α. These research results displayed novel heterocyclic carbohydrazide **26** is a helpful FAAH inhibitor for the potential treatment of central nervous system disorder. And further structural optimization and biological assays are in progress.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127118.

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25. (*E*)-N'-(4-((3-fluorobenzyl)oxy)benzylidene)-1H-benzo[d]imidazole-2-carbohydrazide(26) ; 1H NMR (300 MHz, DMSO-d6) δ 11.17 (s, 1H), 7.85 (s, 1H), 7.53 (t, J = 8.5 Hz, 4H), 7.21 (d, J = 7.6 Hz, 4H), 6.99 (dd, J = 8.7, 3.5 Hz, 4H), 5.08 (s, 2H). 13C NMR (400 MHz, DMSO-d6) δ 172.19, 165.81, 163.86, 161.44, 159.95, 159.75, 142.70, 140.23, 140.16, 131.00, 130.92, 128.99, 128.64, 127.74, 124.02, 115.59, 115.22, 115.02, 114.85, 114.63, 68.89. HRMS(ESI) m/z calculated for C15H11FN4O [M+1]+: 389.1257, found 389.1262.
26. BV2 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum under 37°C and 5% CO2. Cells were seeded at 2×105 cells per well in 96-well plates and incubated overnight. Following, inhibitor was added into the cell culture, and incubated for another 24 h. Each well was added LPS to a concentration of 50 ng/mL and cells were continuously maintained for another 6 h. Then TNF-α or IL-1β was measured by ELISA method.