

RESEARCH ARTICLE

Potential Therapeutic Value of a Novel FAAH Inhibitor for the Treatment of Anxiety

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Abstract

Anxiety disorders are among the most prevalent psychiatric diseases with high personal costs and a remarkable socio-economic burden. However, current treatment of anxiety is far from satisfactory. Novel pharmacological targets have emerged in the recent years, and attention has focused on the endocannabinoid (eCB) system, given the increasing evidence that supports its central role in emotion, coping with stress and anxiety. In the management of anxiety disorders, drug development strategies have left apart the direct activation of type-1 cannabinoid receptors to indirectly enhance eCB signalling through the inhibition of eCB deactivation, that is, the inhibition of the fatty acid amide hydrolase (FAAH) enzyme. In the present study, we provide evidence for the anxiolytic-like properties of a novel, potent and selective reversible inhibitor of FAAH, ST4070, orally administered to rodents. ST4070 (3 to 30 mg/kg *per os*) administered to CD1 male mice induced an increase of time spent in the exploration of the open arms of the elevated-plus maze. A partial reduction of anxiety-related behaviour by ST4070 was also obtained in *Wistar* male rats, which moderately intensified the time spent in the illuminated compartment of the light-dark box. ST4070 clearly inhibited FAAH activity and augmented the levels of two of its substrates, *N*-arachidonylethanolamine (anandamide) and *N*-palmitoylethanolamine, in anxiety-relevant brain regions. Altogether, ST4070 offers a promising anxiolytic-like profile in preclinical studies, although further studies are warranted to clearly demonstrate its efficacy in the clinic management of anxiety disorders.

Introduction

Anxiety disorders are among the most prevalent psychiatric diseases with high personal costs and a remarkable socio-economic burden. However, current treatment is far from satisfactory [see [1,2] for recent updates on this topic]. In recent years, the endocannabinoid (eCB) system

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has received special attention as a potential pharmacological target for the management of anxiety disorders, given the increasing body of evidence (from both human and animal studies) that supports its central role in emotional control [3,4,5]. The eCB system mainly consists in metabotropic membrane receptors, their endogenous ligands (eCBs), and the enzymes responsible for their synthesis and degradation. Endocannabinoids, mainly N-arachidonylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), are synthesized 'on demand' by the cleavage of membrane phospholipid precursors. The eCB signalling ends by the enzymatic activity of specific enzymes, fatty acid amide hydrolase (FAAH) in charge of AEA hydrolysis and monoacylglycerol lipase (MAGL) responsible for the hydrolysis and inactivation of 2-AG [see [6,7] for more detailed information]. The extensive research on the eCB system has provided multiple targets for the pharmacological manipulation of this neuromodulatory system. Despite the direct activation or blockade of the specific metabotropic receptors was the initial approach, the indirect enhancement of the eCB signalling has become more popular given its spatiotemporal specificity as well as the reduced adverse effects observed [8,9]. In recent years, several FAAH inhibitors have been developed, i.e. URB597, AACOCF3 or PF-3845 [10,11], and have been associated with anxiolytic, antidepressant and analgesic properties in rodents [12,13,14]. More recently, a new family of oxime carbamates has been identified as potent inhibitors of FAAH [15,16], and here we sought to interrogate the putative anxiolytic-like profile of the enol-carbamate 1-biphenyl-4-ylethenyl piperidine-1-carboxylate, ST4070. To this aim, rodents orally administered ST4070 were evaluated in behavioural paradigms widely used for preclinical screening of anxiolytic drugs. FAAH activity and eCB content in specific brain regions related to emotionality, motivation and behavioural planning (hippocampus, striatum and frontal cortex) [17] were also assessed, in order to better understand the neurobiological mechanisms through which ST4070 acts to modulate anxiety.

Material and Methods

1. Animals

This study was carried out in strict accordance with the guidelines of the European Communities (2010/63/UE) regulating animal research. The protocol was approved by the Italian Ministry of Health's dedicated Committee (N° SSA/252/14).

Male CD1 mice and *Wistar* rats were purchased from Charles River (Milan, Italy). Mice (n = 60), around 35 g at arrival, were housed four per cage in Macrolon II cages (26.7 cm x 20.7 cm x 14 cm height), with stainless steel feed racks and sterilized, dust-free bedding cobs in the *Sigma-Tau* animal facilities. The room was maintained at constant temperature ($22 \pm 2^\circ\text{C}$) and relative humidity ($55 \pm 10\%$). A circadian 12-hour cycle of artificial light was maintained (lights on at 7 a.m.). Rats (n = 50), around 250 g at arrival, were paired-housed in Macrolon III plexiglas cages (42 x 26.5 x 18.5 cm height) at the *Istituto Superiore di Sanità* animal facilities. The room was maintained at constant temperature ($22 \pm 2^\circ\text{C}$) and relative humidity ($60 \pm 10\%$). A circadian 12-hour cycle of artificial light was maintained (lights on at 8 a.m.). All cages were placed in racks that allowed the animals to see, hear, and smell other animals. All animals had food pellet diet (Mucedola, Italy) and tap water *ad libitum*.

2. Drugs and chemicals

The fatty acid amide hydrolase (FAAH) inhibitor 1-biphenyl-4-ylethenyl piperidine-1-carboxylate (ST4070) was kindly provided by Sigma-Tau (Italy) [15,16]. ST4070 was dispersed daily in a solution containing 0.5% Carboxymethylcellulose sodium salt (CMC, medium viscosity- Sigma, Milan, Italy) and 0.1% Tween 80 (Merck, Darmstadt, Germany) in distilled

water. Diazepam (FIS, Vicenza, Italy) was dispersed daily in a solution containing 3% Tween-80 in sterile water, and was used as a reference compound.

3. Evaluation of anxiety-related behaviours

Anxiety-related behaviours were measured in two different anxiety tests based on unconditioned responses—the elevated plus maze, EPM [18] and the light-dark box, LD box [19]; evaluation in these tests requires no training and usually has a high eco/ethological validity (see [20,21] for review). Moreover, both tests are extensively employed nowadays for the preclinical screening of anxiolytic drugs in rodents. In this study we specifically used the EPM for the evaluation of mice [22] and the LD box for rats [23].

3.1. Elevated-plus maze in mice. The apparatus made of grey Plexiglas consisted of two open and two closed arms linked by a common central platform. The maze was elevated 40 cm above floor level and dimly lighted. Animals were individually placed on the central platform of the maze facing an open arm. A standard 5-min test was employed [18]. The amount of time spent by each animal in either open or closed arm was recorded by an any-maze video tracking system (Ugo Basile, Milan, Italy), and so was the number of entries and the total distance walked by each animal into either arm.

3.2. Dark-light test in rats. The apparatus made in opaque Plexiglas consisted of two compartments, of which one was brightly lighted, and was placed in a sound-attenuating chamber. The box compartments (45 cm x 30 cm x 35 cm) were distinguished only by wall colour and illumination. The dark compartment had black walls, whereas the lit compartment had white walls. A guillotine door separated the two compartments. The lit compartment (450 lux) was illuminated by a desk lamp placed over the compartment itself. The location of each rat was monitored with photocells and scored by software using a computer that was interfaced with the boxes. The photocells were located a few cm from the floor along the walls of the box. Crossing from one compartment to the other was scored whenever the rat emerged far enough from one side to interrupt the first photocell beam, while no longer interrupting the photocell beams in the original compartment. Time spent in the lit compartment and entries made to the lit compartment were recorded and considered as the most significant parameters for the evaluation of anxiety, while beam interruptions in the dark compartment were used as a measure of activity rate [19].

4. Experimental design

Experiment 1: Effects of ST4070 administration in mice exposed to the elevated plus maze. Animals remained for at least seven days before the beginning of the experiment (period of acclimatization) in the animal facilities. Sixty min before the test mice were orally administered ST4070 at doses of 3, 10 and 30 mg/10 mL/kg (12 mice/group). Diazepam was intraperitoneally administered 30 min before the test at a dose of 1 mg/5 ml/kg (12 mice/group). Range of drug dosage and schedule of administration were based on previous experiments [15]. Animals were individually placed in the elevated plus-maze to begin the 5-min test session. Behavioural testing was performed between 09.00 and 14.00 and animals were randomly assigned to the different drug groups.

Experiment 2: Effects of ST4070 administration in rats exposed to the light-dark test. Animals remained for at least seven days before the beginning of the experiment (period of acclimatization) in the animals' facilities. Sixty min before the test rats were orally administered ST4070 at doses of 10 and 30 mg/2 mL/kg (16–18 rats/group). Range of drug dosage and schedule of administration were based on previous pilot experiments performed in our laboratory. Animals were individually placed in the illuminated compartment to begin the 10-min

test session in the LD box. Behavioural testing was performed between 09.00 and 14.00 and animals were randomly assigned to the different drug groups. Immediately after testing, animals were sacrificed by decapitation, brains were rapidly removed and discrete brain regions were dissected on ice and stored at -80°C until biochemical assays.

5. Endocannabinoid system analysis

Frontal cortex, striatum and hippocampus from 8 animals per experimental group were randomly selected to evaluate particular components of the endocannabinoid system. Since we have previously demonstrated that ST4070 shows remarkable selectivity for FAAH over other components of the endocannabinoid system [16], we focused our investigation on the evaluation of FAAH activity, as well as in the measurement of endogenous cannabinoid ligands targeted by this enzyme, namely anandamide (N-arachidonylethanolamine, AEA) and palmitoylethanolamide (PEA) and 2-arachidonoylglycerol (2-AG).

The hydrolysis of $10\ \mu\text{M}$ AEA-ethanolamine-1- $[\text{}^3\text{H}]$ (60 Ci/mmol) by FAAH was assayed in brain extracts ($50\ \mu\text{g}$ protein/test), by measuring the release of $[\text{}^3\text{H}]$ ethanolamine as reported [15]. FAAH activity was expressed as pmol product formed per min per mg protein (pmol/min per mg protein).

For the measurement of endocannabinoid levels, brain tissues were subjected to lipid extraction with chloroform/methanol (2:1, v/v), in the presence of d_8 -AEA, d_8 -2-AG and d_4 -PEA as internal standards. The organic phase was dried and then analysed by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS), using a single quadrupole API-150EX mass spectrometer (Applied Biosystem, CA, USA) in conjunction with a PerkinElmer LC system (PerkinElmer, MA, USA). Quantitative analysis was performed by selected ion recording over the respective sodiated molecular ions, as reported [24]. Chemicals were of the purest analytical grade. AEA and PEA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2-AG was from ALEXIS (San Diego, CA, USA). AEA-ethanolamine-1- $[\text{}^3\text{H}]$ (60 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). d_8 -AEA, d_8 -2-AG and d_4 -PEA were from Cayman Chemicals (Ann Arbor, MI, USA).

6. Data analysis

Data, expressed as mean \pm SEM, were controlled for normality (Kolmogorov-Smirnoff test) and homogeneity of variances (Levene test). In case normality failed, data were transformed accordingly. Differences between groups were evaluated using a one-way analysis of variance (ANOVA). Tukey honestly statistical difference was employed as the *post-hoc* comparison test and statistical significance was considered at a p-level value ≤ 0.05 . Statistical analyses were performed by using the IBM SPSS Statistics 19 software (IBM Corporation, New York, USA).

Results

Effects of ST4070 on anxiety-related responses

In the elevated plus-maze (Data in S1 Table), a significant drug effect was found for the time spent in the open arms [$F(4,55) = 3.67$, $p < 0.05$] and for the number of entries into the open arms [$F(4,55) = 4.18$, $p < 0.01$] (Fig 1, panels A and B). *Post-hoc* comparisons confirmed the expected reduction of anxiety-like behaviour induced in mice by diazepam, measured as the time spent in open arms and the frequency of entries into open arms. Similarly, the highest dose of ST4070 (30 mg/kg) also increased the time spent by animals in exploring the open arms, again suggesting a reduction in anxiety-like responses. No effects were found neither for

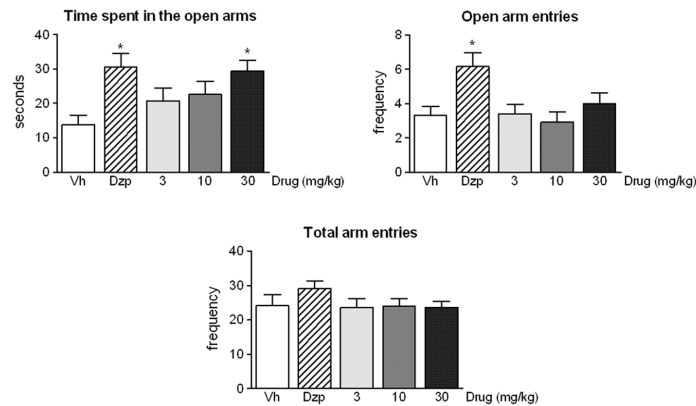


Fig 1. ST4070 effects in mice exposed to the elevated plus maze. Data are expressed as mean \pm SEM. Animals were orally administered ST4070 (3, 10 or 30 mg/kg, 60 min before testing) or diazepam (Dzp, 1 mg/kg, i.p. 30 min before testing), and were challenged in the elevated plus-maze for 5 min ($n = 12$ per experimental group). One-way ANOVA followed by Tukey *post-hoc* comparisons, * $p < 0.05$ vs. vehicle.

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diazepam nor for ST4070 on general locomotion, measured as the frequency of total arm entries [$F(4,55) = 0.97$, ns] (Fig 1, panel C).

In parallel, the highest dose of ST4070 (30 mg/kg) also seemed to increase the time spent by rats in the illuminated compartment of the LD box (Fig 2, panels A; Data in S2 Table). *Post-hoc* comparisons achieved an almost significant effect ($p = 0.07$) of the drug in the time spent by animals in exploring the light compartment, when compared to vehicle administered animals. No changes were observed in the number of transitions into the light compartment [$F(2,47) = 1.08$, ns] nor in general locomotor activity [$F(2,47) = 0.78$, ns] (Fig 2, panels B and C). It is noteworthy that the time spent in the light compartment seems to be a more sensitive parameter to the anxiolytic action of drugs than the number of transitions between lit and dark compartments of the apparatus [25,26].

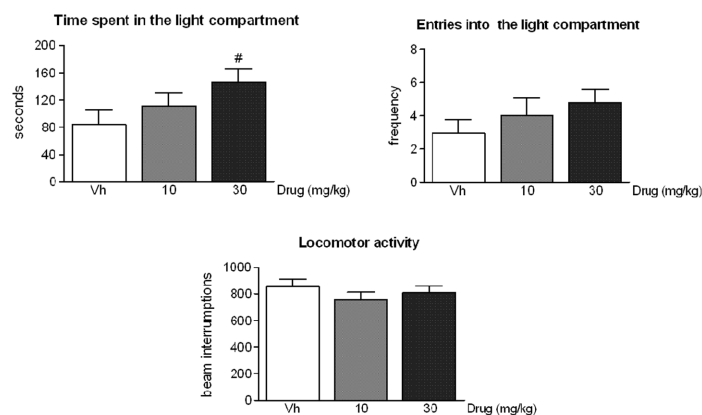


Fig 2. ST4070 effects in rats exposed to the light-dark box. Data are expressed as mean \pm SEM. Rats were orally administered ST4070 (10 or 30 mg/kg, 60 min before testing), and were challenged in the LD box for 10 min ($n = 16-18$ per experimental group). One-way ANOVA followed by Tukey *post-hoc* comparisons, # $p = 0.07$ vs. vehicle.

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Effects of ST4070 on the eCB system

Present data further support the inhibitory action of ST4070 on FAAH activity (Data in [S3 Table](#)), since the oral administration of this compound to rats remarkably reduced FAAH activity in the specific brain areas analysed [frontal cortex: $F(2,21) = 34.15$, $p < 0.001$; striatum: $F(2,21) = 39.16$, $p < 0.001$; and hippocampus: $F(2,21) = 29.29$, $p < 0.001$] ([Table 1](#)).

The inhibitory activity of ST4070 on FAAH was further supported by the evaluation of brain AEA levels ([Fig 3](#)). The highest dose of ST4070 (30 mg/kg) significantly increased AEA levels in the striatum [$F(2,17) = 3.70$, $p < 0.05$] and the frontal cortex [$F(2,18) = 3.18$, $p = 0.065$]. However, AEA levels within the hippocampus were not modified by oral administration of ST4070 at any dose [$F(2,17) = 0.05$, ns]. Although FAAH has also been reported to deactivate other eCBs, including 2-AG [[27](#)], no changes in 2-AG content were observed in the frontal cortex [$F(2,20) = 0.23$, ns], striatum [$F(2,19) = 0.84$, ns] or hippocampus [$F(2,20) = 0.50$, ns]. FAAH can also increase the levels of PEA [[28](#)]. Consistently, ST4070 significantly increased PEA levels within the striatum [$F(2,18) = 6.33$, $p < 0.01$] and the hippocampus [$F(2,19) = 5.27$, $p < 0.05$]. *Post-hoc* comparisons indicated that ST4070 at the two doses administered significantly elevated PEA levels within the striatum ($p < 0.05$), whereas in the hippocampus statistical significance was reached only at the highest dose ($p < 0.05$). In addition, a similar trend, yet not significant, could be observed for PEA content within the frontal cortex [$F(2,21) = 2.60$, ns] (Data in [S3 Table](#)).

Discussion

A link between the anxiety-related effects of FAAH inhibitors and their ability to enhance endogenous AEA signalling has been already proposed [[12,29](#)]. Here, we support this concept by using a novel FAAH inhibitor that elicits anxiolytic-like responses when administered orally.

ST4070 as a potential anxiolytic agent in animal models

Present results indicate that ST4070 modulates anxiety-like responses in both the EPM and the LD box, animal models based on rodents innate general avoidance behaviours [[30,31](#)]. Present results are in accordance with previous studies in which other FAAH inhibitors induced anxiolytic-like responses in rodents. In particular, when tested in the elevated plus-maze [[32,33,34,35](#)], zero maze [[12](#)], LD box [[11,36,37](#)], as well as in the marble burying assay [[38](#)]. Our data indicate that the highest dose (30 mg/kg) of the novel compound ST4070, orally administered to mice and rats, induced anxiolytic-like responses in the elevated plus-maze and in the LD box; thus, ST4070 arises as a novel eCB-based anxiolytic drug orally effective in rodents. More recently URB597 has been reported to shape behavioural responses to challenges [[39](#)], thus it would also be interesting to evaluate the influence of our novel compound, ST4070, on behavioural responses induced by challenges of different intensity.

ST4070 as a modulator of the eCB system

Here, we have reported that ST4070, through the inhibition of FAAH activity, elevates AEA levels in brain regions critically involved in the control of anxiety and stress response, such as frontal cortex and dorsal striatum [[17](#)]. Interestingly, it is now apparent that anxiety does not depend on specific brain areas performing unique functions, but it is rather considered an emerging property of interacting brain regions [[40](#)]. Present findings highlight the relevance of the eCB system within these two brain areas (frontal cortex and striatum) in which the control

Table 1. Fatty acid amide hydrolase (FAAH) activity.

	Frontal cortex	Striatum	Hippocampus
Vehicle	600.50 ± 29.49	308.06 ± 15.03	577.88 ± 52.38
ST4070 (10 mg/kg)	251.19 ± 61.84**	124.06 ± 30.27**	258.69 ± 34.54**
ST4070 (30 mg/kg)	145.88 ± 16.75**	65.50 ± 9.23**	169.00 ± 28.18**

Data are expressed as mean ± SEM (pmol/min per mg protein). Animals were orally administered ST4070 (or the corresponding vehicle) 60 min before the behavioural test (LD box, see text for details). Immediately after the behavioural tests, animals were sacrificed and brain regions rapidly dissected on ice (n = 8 per experimental group). One-way ANOVA followed by Tukey *post-hoc* comparisons, ** p < 0.001.

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of anxiety-related responses may probably relay on behavioural inhibition under conflict situations [40].

In a previous report ST4070 was identified as a potent reversible inhibitor of FAAH [15]. Much alike other peripherally administered FAAH inhibitors [12,13,41], oral ST4070 effectively reached its pharmacological target in the central nervous system, where it was able to produce a consistent inhibition of FAAH activity within anxiety-relevant brain regions. Despite basal FAAH activity levels differ between brain regions, ST4070 was able to inhibit FAAH activity in all the brain regions analyzed. Remarkably, the inhibitory potency of ST4070 was similar in all brain regions analyzed, being ~50% upon administration of the lowest dose (10 mg/kg), and ~75% upon administration of the highest dose (30 mg/kg). Consistently with FAAH inhibition, an increase in AEA content was observed in the striatum and the frontal cortex, although no changes in AEA levels were observed within the hippocampus. The lack of correlation between FAAH activity and AEA levels in the hippocampus seems noteworthy, and extends previous data on chronic administration of URB597 [13]. A reduction in AEA mobilization could represent a possible underlying mechanism [13]. In addition, alternative catabolic pathways for AEA [42,43] may also compensate for reduced FAAH activity in the hippocampus. ST4070 did not modify 2-AG levels, probably because enzymes other than FAAH are

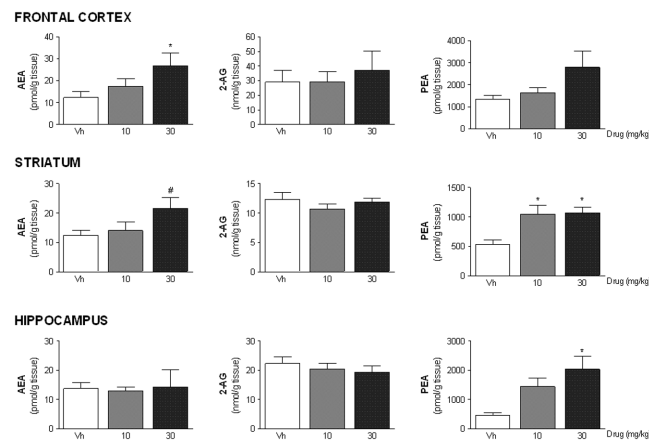


Fig 3. ST4070 effects on brain endocannabinoid content. Data are expressed as mean ± SEM. Rats were orally administered ST4070 (10 or 30 mg/kg, 60 min before testing), and were challenged in the LD box for 10 min. Immediately after testing, animals were sacrificed and brain regions rapidly dissected. Levels of anandamide (AEA), 2-arachidonoylglycerol (2-AG) and *N*-palmitoylethanolamine (PEA) were measured in specific brain regions (frontal cortex, striatum and hippocampus) (n = 8 per experimental group). One-way ANOVA followed by Tukey *post-hoc* comparisons, * p = 0.05 vs. vehicle; # p = 0.06 vs. vehicle.

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known to be the main responsible for 2-AG degradation. Indeed, MAGL [44] and alfa-beta-hydrolase domain 6 (ABHD6) [45] clearly play a prominent role in 2-AG hydrolysis within the brain.

Furthermore, our data suggest that AEA levels in the hippocampus are not involved in the anxiolytic properties of ST4070, although a contribution of FAAH inhibition in this brain region cannot be ruled out, based on recent data with URB597 [46]. Indeed the hippocampal eCB system, mainly through a role in neural plasticity, has been recognized as pivotal in emotional responses [47]. Overall, we propose that the profile of FAAH activity and AEA levels in different anxiety-relevant brain regions may change time-dependently, in order to provide an appropriate response to a certain type of conflict. For instance, an early integration of multimodal sensory information, mediated within the hippocampus, is needed prior to the behavioural inhibition directed by the striatum and the prefrontal cortex [17]. The timing of activation of the diverse neuroanatomical substrates mediating the anxiolytic-like effects of ST4070 remains to be elucidated. Incidentally, stress exposure seems to be crucial for the impact of eCB signalling on anxiety-related responses [48]. Therefore, future studies on the anxiolytic-like properties of eCB-targeted drugs should include both unstressed and stressed subjects.

Last but not least, it should also be noted that, unlike most of FAAH inhibitors with potential as therapeutics, ST4070 reversibly inhibits enzyme activity [15]. To date, only a few reversible FAAH inhibitors have been described in the literature, i.e. the α -ketoheterocycle OL-135 [49], and the covalent but slowly reversible piperazine urea JNJ-1661010 developed by Johnson & Johnson [50]. Although further research is still needed, reversible inhibitors might offer advantages over irreversible blockers as lead compounds for drug design [51], and possibly also for the management of anxiety-related disorders.

Conclusions

The new enol-carbamate ST4070 that affords potent and reversible inhibition of FAAH *in vivo*, enhances the endogenous eCB tone in specific brain regions engaged in emotional control, and induces remarkable anxiolytic-like behaviours in rodents. Although research on the neural substrates and pharmacokinetics of the anxiolytic-like effects of ST4070 in rodents is still needed, the present investigation opens new avenues to the development and further evaluation of a new family of FAAH inhibitors as drugs to be tested in clinical trials for the management of anxiety and mood disorders in humans.

Supporting Information

S1 Table. Elevated Plus Maze (EPM) in mice. (Individual data).
(XLS)

S2 Table. Light-Dark (LD) Box in rats. (Individual data).
(XLS)

S3 Table. FAAH activity and brain endocannabinoid (eCB) content. (Individual data).
(XLS)

Author Contributions

Conceived and designed the experiments: EMM AC FB GL MM. Performed the experiments: EMM CR AC. Analyzed the data: EMM CR AC. Contributed reagents/materials/analysis tools: EMM CR AC FB GL MM. Wrote the paper: EMM GL MM.

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