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Authors
Vacondio, Federica
Silva, Claudia
Lodola, Alessio
et al.

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Structure-property relationships of a class of carbamate-based Fatty Acid Amide Hydrolase (FAAH) inhibitors: chemical and biological stability

Dr. Federica Vacondio[a], **Prof. Claudia Silva**[a], **Dr. Alessio Lodola**[a], **Dr. Alessandro Fioni**[a], **Prof. Silvia Rivara**[a], **Dr. Andrea Duranti**[b], **Dr. Andrea Tontini**[b], **Dr. Silvano Sanchini**[b], **Dr. Jason Clapper**[c], **Prof. Daniele Piomelli**[c],[d], **Prof. Marco Mor**[a], and **Prof. Giorgio Tarzia**[b]

Marco Mor: marco.mor@unipr.it

[a]Dipartimento Farmaceutico, Università degli Studi di Parma, Viale G. P. Usberti 27/A, Campus Universitario, I-43100 Parma, Italy, Fax: (+39) 0521 905006

[b]Istituto di Chimica Farmaceutica e Tossicologica, Università degli Studi di Urbino “Carlo Bo”, Piazza del Rinascimento 6, I-61029 Urbino, Italy

[c]Department of Pharmacology, University of California, Irvine, 360 MSRII, CA 92697-4625, USA

[d]Department of Drug Discovery and Development, Italian Institute of Technology, via Morego 30, I-16163 Genova, Italy

Abstract

Cyclohexylcarbamic acid aryl esters are a class of Fatty Acid Amide Hydrolase (FAAH) inhibitors, which includes the reference compound URB597. The reactivity of their carbamate fragment is involved in pharmacological activity and may affect pharmacokinetic and toxicological properties. We conducted in vitro stability experiments in chemical and biological environments to investigate the structure-stability relationships in this class of compounds. The results show that electrophilicity of the carbamate influences its chemical stability, as suggested by the relation between the rate constant of alkaline hydrolysis (log k_{pH9}) and the energy of lowest unoccupied molecular orbital (LUMO). Introduction of small, electron donor substituents at conjugated positions of the *O*-aryl moiety increased overall hydrolytic stability of the carbamate group without affecting FAAH inhibitory potency, whereas peripheral nonconjugated hydrophilic groups, which favor FAAH recognition, helped reducing oxidative metabolism in the liver.

Keywords

FAAH inhibitors; cyclohexylcarbamic acid aryl esters; structure-activity relationships; stability; liquid chromatography

Introduction

Carbamates are widely employed as pharmacological tools and therapeutic agents[1,2] to inhibit different enzymes, such as acetyl- and butyrylcholinesterases,[3,4,5] cholesterol esterase,[6,7] elastase,[8] chymotrypsin[9] and fatty-acid amide hydrolase (FAAH).[10] The carbamate group, once positioned in close proximity to the catalytic residue of a serine hydrolase, can undergo a nucleophilic attack leading to enzyme carbamoylation and

Correspondence to: Marco Mor, marco.mor@unipr.it.

deactivation. This inhibition mechanism has also been shown for the FAAH inhibitor cyclohexylcarbamic acid 3′-carbamoylbiphenyl-3-yl ester (URB597).[11,12]

Recognition at a catalytic site of a carbamate-based inhibitor depends on its overall size, shape, lipophilicity and electronic complementarity with the binding cavity, whereas its intrinsic reactivity strongly influences the rate of the reaction with the catalytic residue. This general behavior applies both to the desired targets of carbamate-based inhibitors and to other off-target proteins involved in side effects or metabolic degradation. As a consequence, modulation of carbamate reactivity should be considered during pharmacodynamic and pharmacokinetic optimization of such compounds. To this aim, in vitro model systems, measuring chemical and enzymatic stability data, can be efficiently employed to analyze structure-property relationships (SPR).

The present work reports quantitative structure-property relationships (QSPR) for a series of cyclohexylcarbamic acid aryl esters acting as FAAH inhibitors, focused on their stability in chemical and biological environments. The serine hydrolase FAAH (EC number 3.5.1.4) catalyzes the intracellular hydrolysis of a family of endogenous lipid mediators,[13,14,15] whose main representatives are the endocannabinoid arachidonoylethanolamide (anandamide),[16] the satiety factor oleoylethanolamide (OEA)[17,18] and the antiinflammatory factor palmitoylethanolamide (PEA).[19] FAAH works by a catalytic mechanism[20,21] involving a Ser-Ser-Lys triad and presents characteristic pH dependence and substrate selectivity.[22] Different classes of FAAH inhibitors have been reported,[23] among which are fluorophosphonates,[24] (thio)hydantoins,[25] ureas,[26] α-ketoheterocycles,[27] sulfonamides[28] and carbamates.[29,30,31,32,33] Irreversible FAAH inhibitors, such as URB597, represent new and attractive drug candidates characterized by anxiolytic- and antidepressant-like,[10,34,35] analgesic[36,37,38] and anti-hypertensive[39] activities.

The parent compound, which belongs to the class of cyclohexylcarbamic acid biphenyl-3-yl esters (**1**, URB524, Table 1A), inhibits FAAH activity in rat brain membranes with a half-maximal inhibitory concentration (IC_{50}) of 63 nM.[29] Quantitative structure-activity relationship (SAR) studies showed that recognition at the FAAH binding site can be improved by the introduction of polar groups at the distal ring of the biphenyl moiety,[30] as exemplified by the potent FAAH inhibitor URB597 (**9**, Table 1A, IC_{50} = 4.6 nM), which displays remarkable selectivity[10,40] and a good safety profile.[41]

The introduction of substituents on the proximal phenyl ring has shown that, while some polar groups (such as hydroxyl, hydroxymethyl and amino) at the para position are well tolerated, electron-withdrawing groups at positions conjugated with the carbamate group yield less potent compounds, suggesting that electronic effects on the carbamate group might influence the observed IC_{50} values.[31,42]

Moreover, different ratios between in vitro and in vivo potency had been observed for two *O*-phenyl carbamates with different substituents at para position,[10] suggesting that carbamate stability could also affect bioavailability.

In the present study we submitted an extended set of carbamate FAAH inhibitors to in vitro stability experiments in different chemical (pH 1.0; 7.4; 9.0) or biological (rat plasma; rat liver S_9 fraction) environments. Starting from the parent compound **1**, derivatives with substituents at the 4-, 6- or 3′-positions of the biphenyl system (**2**–**11**, Table 1A) were considered, including some of the most potent inhibitors in this series,[30] and the *m*-biphenyl moiety was replaced by *p*-biphenyl (**12**, Table 1A), 2-naphthyl (**13**, Table 1A), or flexible ω-phenylpentyl (**18**, Table 1B) moieties. Wider chemical modulations of the

carbamic group included replacement of the *N*-alkyl with a *N*-phenyl (**14**, Table 1B) and switching the O- and N-terminal groups (**19**, Table 1B). Finally, the carbamate was replaced by ester (**15**, Table 1B), thiocarbamic (**16**, Table 1B) or amidic (**17**, Table 1B) groups to give isosteres known to have marginal FAAH inhibitory potency.[29]

Chemical and biological stability data were analyzed by a SPR approach to identify the most convenient set of structural features able to combine in vitro FAAH inhibitory potency with resistance of the carbamate group to hydrolytic cleavage and oxidative metabolism.

Results and Discussion

The alkylcarbamic acid aryl esters **8**, **12**, **13**, **20** and **21** were obtained by addition of *n*-butyl- or cyclohexylisocyanate to the appropriate phenylphenol. Both the isocyanates and the phenylphenols required for the synthesis of **8**, **12** and **13** were commercially available. 6-Fluorobiphenyl-3-ol[43] and 6-methoxybipenyl-3-ol[44] were prepared by a Suzuki cross-coupling of commercially available phenylboronic acid and 3-chloro-4-fluorophenol, or 3-bromo-4-methoxyphenol,[45] respectively. The latter compound was prepared from the corresponding aldehyde. The results of chemical and enzymatic stability assays for compounds **1**–**19** are reported in Tables 1A and 1B. The IC_{50} values for all tested compounds on rat brain membrane FAAH activity[29,30,31,33] are also reported for comparison.

Chemical Stability

Stability to chemical hydrolysis of compounds **1**–**19** was evaluated measuring residual concentrations of the starting compound at different time points, in acidic solution and in buffers at physiological pH (7.4) or basic pH (9.0). All tested compounds were stable for 24 h to acid-catalyzed hydrolysis (pH = 1.0, 37 °C), with the exclusion of the ester derivative **15**, 40±2% of which was recovered after 24 h. Conversely, almost all tested compounds showed significant, but generally not complete, degradation in buffer at physiological pH after 24 h. Substituents at conjugated (**2**–**8**) positions of the biphenyl moiety of URB524 (**1**) markedly influenced both hydrolytic stability and FAAH inhibitory potency. The electron-withdrawing nitro group (**5** and **6**, Table 1A) led to a remarkable decrease in stability, which may explain the apparent low inhibitory potency of these compounds toward FAAH, due to decomposition under the assay conditions.[31] Conversely, electron donor groups at conjugated positions (**2**–**4**, **7** and **8**) increased chemical stability, up to nine-fold for the *p*-amino derivative **3**. For this compound and for the *p*-hydroxy derivative **7**, increased chemical stability was combined with maintenance of in vitro FAAH inhibitory potency. As expected, substitution at the nonconjugated meta position of the distal phenyl ring does not considerably affect chemical stability, though is critical for recognition at the FAAH catalytic site of the potent inhibitors **9**–**11**.[30]

The *p*-biphenyl isomer **12** and the 2-naphthyl derivative **13** showed chemical stability similar to that of **1**, with differences in FAAH inhibitory potency that probably reflect different efficiencies in the recognition process, due to size/shape complementarity. In the subset of compounds with wider chemical diversity (**14**–**19**, Table 1B), replacement of the carbamate group by an ester (**15**) or an amide (**17**) resulted in an increase of stability at alkaline pH, but also in a dramatic drop of FAAH inhibitory potency.[29] *N*-phenyl carbamate **14** and thiocarbamate **16** showed short half-lives at pH 7.4 (51 min and 2.6 min, respectively). Aromaticity of the carbamate O-substituent appeared critical for hydrolytic reactivity, as revealed by carbamic acid alkyl esters **18** and **19** (Table 1B), that remained virtually unmodified after 24 h at pH 9.0. Low chemical stability probably also affected the IC_{50} value measured for FAAH inhibition by the thiocarbamate **16**.

Two competing mechanisms are known to occur in alkaline hydrolysis of *O*-aryl substituted carbamates. The first ($B_{AC}2$) implies the attack of a hydroxyl anion to the carbamate carbonyl group, yielding a tetrahedral intermediate; the second is an elimination-addition mechanism (E_1cB) that involves deprotonation of the carbamate amino group and formation of an intermediate isocyanate, which in turn decomposes to the corresponding amine and carbonic anhydride.[46,47,48,49]

Hydrolysis rates at different pH values, measured for compound **13**, increase with increasing hydroxyl ion concentration (Figure 1). Whether hydroxyl ion activity is the only determinant of the apparent rate constants, as expected from a $B_{AC}2$ mechanism, a linear dependence with unit slope should be observed, while the E_1cB mechanism implies deprotonation as the limiting step, and thus a plateau when pH approaches and overcomes carbamate pK_a. Although more data are required to draw definitive conclusions, an inflection point in the pH/rate curve suggests a change in mechanism. Therefore, half-lives in aqueous buffer at pH 9.0 may result from a complex reaction mechanism, and cannot be regarded as simply deriving from the propensity of the carbamate carbonyl to be attacked by a nucleophile. They can be useful, nevertheless, as an experimental measure of the electronic effect of the *O*-aryl moiety on carbamate reactivity, provided that this effect plays the same role in both pathways. In fact, polarization of the electronic cloud on the aromatic ring can affect in similar ways both the tendency of the carbonyl to undergo a nucleophilic attack ($B_{AC}2$) and the acidity of carbamate NH (E_1cB).

To assess the role of different electronic properties on chemical hydrolysis rate, the stability data obtained at pH 9.0 were analyzed by QSPR approach. Frontier orbital energies, that had been employed to rationalize chemical and biochemical reactivity of bioactive compounds,[50,51] were calculated by the semiempirical AM1 method for minimum energy conformations of *O*-aryl carbamates (see Experimental Section) and are reported in Table 2. Furthermore, to increase the size of dataset, two additional cyclohexylcarbamic acid biphenyl-3-yl esters, having a fluorine or a methoxy group at para position on the proximal phenyl ring, were synthesized (compound **20** and **21,** respectively) and their stability in basic buffer was tested (Table 2). While the fluorine derivative **20** showed a half-life similar to that of **1** [$t_{1/2}$ = 37± 3 min, (mean±S.D)], the methoxy derivative **21** resulted slightly more stable ($t_{1/2}$ = 78± 4 min). The two nitro-substituted derivatives, **5** and **6**, resulted so unstable that it was not possible to measure their half-life times.

For the subset of substituted alkylcarbamic acid biphenyl-3-yl esters **1**–**4, 7**–**13** and **20**–**21**, a coarse correlation was found [Equation (1)] between the logarithm of alkaline hydrolysis constant (log k_{pH9}) and the LUMO energy of the carbamate, which represents its electrophilicity (higher for more negative energies):

$$\log k_{pH9} = -1.614\ (\pm 0.287)\ \mathrm{LUMO} - 2.147\ (\pm 0.063)$$
$$n=13,\ R^2=0.742,\ s=0.214,\ F=31.6,\ Q^2=0.669 \qquad \text{[Eq. (1)]}$$

This equation explains 74% of log k_{pH9} deviance, suggesting either that LUMO energy is not a suitable descriptor of experimental stability, or that other factors can affect the hydrolysis rate. Analysis of the residuals (Table 2) shows that the *p*-hydroxyl derivative **7** behaves as an outlier, being much more stable [log k_{pH9}(obs.) − log k_{pH9}(calc.) = − 0.53 log units, corresponding to a ratio of approximately 3.4 fold in $t_{1/2}$] than calculated from its LUMO energy. This could be due to inaccurate implementation of the electronic effect for a *p*-hydroxyl group in our calculations of LUMO energy, or to partial deprotonation of this free phenolic hydroxyl group, as the corresponding anion would exert a stronger electron-donating effect.

The exclusion of **7** from the dataset significantly improved the fitting of the QSPR model [Equation (2)], although the standard error of residuals was still higher than experimental uncertainty of log k_{pH9}.

$$\log k_{pH9} = -1.599\ (\pm 0.192)\ \mathrm{LUMO} - 2.102\ (\pm 0.044)$$
$$n=12,\ R^2=0.874,\ s=0.14,\ F=69.4,\ Q^2=0.795 \qquad \text{[Eq. (2)]}$$

While a wider range of electronic effect could improve the statistics of this relationships, we decided to exclude electron-withdrawing substituents because the lower stability of the corresponding carbamates reduced their importance as FAAH inhibitors and made our experimental protocol difficult to be applied in a reproducible manner. However, the similarity between the fitting parameter, R^2, and the leave-one-out Q^2 is a sign of model robustness. In fact, the LUMO energy the two nitro derivatives **5** and **6** (−1.169 and −1.118 eV, respectively) qualitatively explains their low stability, even if the log k_{pH9} values calculated by Equation (2) (−0.23 and −0.31 min^{-1}, respectively, corresponding to calculated $t_{1/2}$ of 1.18 and 1.43 min) are significantly lower than the actual ones, as no remaining carbamate was detected at the first time point (t = 20 s) for these two compounds.

Thus, while Equation (2) suggests that carbamate electrophilicity plays a major role in its hydrolysis at pH 9.0, the residuals indicate that it is hazardous to replace experimental measurements of chemical properties with calculated indexes.

Enzymatic stability

In rat plasma the concentration of the aryl carbamates showed an exponential decay, with an equivalent increase of the corresponding phenol concentration (see Supplementary Figure 1). Carbamate stability in rat plasma depends on the interaction with different, as-yet-undefined plasma hydrolases, where both carbamate reactivity and the recognition process can influence hydrolysis rates.[52] The subset of cyclohexylcarbamic acid biphenyl-3-yl esters (**1**–**4; 7**–**13**) displayed a linear correlation between hydrolysis constants at alkaline pH (log k_{pH9}) and in rat plasma (log k_{plasma}, reported in Table 3) yielding equation (3).

$$\log k_{plasma} = 1.415\ (\pm 0.171) \log k_{pH9} + 0.740\ (\pm 0.358)$$
$$n=11,\ R^2=0.883,\ s=0.234,\ F=68.1,\ Q^2=0.826 \qquad \text{[Eq. (3)]}$$

Therefore, for this set of compounds, electronic factors influencing alkaline hydrolysis also explain almost 90% of the variation in plasma stability. Recognition processes between these compounds and the catalytic sites of plasma hydrolases apparently play a minor role, likely due to the broad steric tolerance of rat plasma hydrolases[53,54] and/or to the limited structural variation within the set of compounds. An opposite conclusion was drawn for the inhibitory potency on FAAH, where recognition events, rather than reactivity, had a dominant influence on the inhibition process. Moreover, while electron-withdrawing substituents, increasing carbamate electrophilicity, also increase their tendency to be substrates of rat plasma hydrolases, a similar influence on FAAH inhibitory potency had not been observed, and electron donor groups at para position are well tolerated when the corresponding carbamates act as pseudosubstrate at the FAAH catalytic site.[31] This is probably due to the unique mechanism of FAAH-catalyzed hydrolysis, where the active serine displays an unusual high degree of nucleophilicity.[21]

It is also important to underline that plasma pseudo-half-lives reflect a variety of concurrent and heterogeneous interactions and, therefore, can be highly class-dependent; for example, the ester **15** was stable to alkaline hydrolysis, but had a very short half-life in rat plasma.

The half-lives of many carbamates with good FAAH inhibitory potency, such as URB524 (**1**) and its derivatives substituted at the distal phenyl ring, were shorter than 1 hour; this is consistent with the half-life observed in vivo for URB597 (**9**) after administration to rats.[41] Compound **8**, which showed greater in vivo/in vitro potency ratio than URB597,[10] was also significantly more stable than the latter in rat plasma. These observations support the hypothesis that certain pharmacokinetic and pharmacodynamic properties of carbamate-based FAAH inhibitors may be accounted for by their different in vitro plasma stability. The substituted *p*-amino (**3**) and *p*-hydroxyl (**7**) carbamates, endowed with good FAAH inhibitory potencies and high rat plasma stability, appear to be therefore promising candidates for in vivo studies.

All compounds of the set were susceptible to in vitro oxidative metabolism in the presence of rat liver S_9 fraction, with half-lives ranging from 1 min for the ester (**15**) and thiocarbamate (**16**) derivatives to nearly 1 h for URB597 (**9**). In the subset **1**–**14**, a progressive increase of cleavage rates was observed passing from the hydrophilic *m*-carbamoyl (**9**) to the more lipophilic *m*-hydroxymethyl (**10**), *m*-acetyl (**11**) derivatives and unsubstituted URB524 (**1**). Although no statistical correlation was found between clog *P* and oxidative metabolism (Figure 2) compound lipophilicity appeared to favor the interaction with liver S_9 metabolizing enzymes. Furthermore stereoelectronic factors appear to have a role as compound **12** resulted 3-fold more stable than its regio isomer **1**. However, even if these data may be related to the low oral bioavailability observed for URB597 (**9**),[41] their main utility was to provide indications for SPR analysis, useful for the design of new compounds with improved pharmacokinetics, rather than for the estimation of bioavailability.

Conclusions

SPR for alkylcarbamic acid aryl esters, investigated through systematic modulation of the starting structure of URB524 (**1**) and measurement of both chemical and metabolic stability under different in vitro conditions, allowed the identification of a set of structural features potentially important for in vivo potency. Thus, the introduction of a polar group at the meta position of the distal phenyl ring of **1** was not only highly favorable for FAAH recognition,[30] but also potentially useful to reduce the risk of oxidative metabolism in the liver. Moreover, the introduction of small, electron donor substituents at the para position [i.e. amino (**3**) and hydroxyl (**7**)] of the proximal phenyl ring increased the chemical and rat plasma hydrolytic stability of the carbamate group, while maintaining a good FAAH inhibitory potency in vitro. On the other hand, the results also indicated that the loss of inhibitory activity on FAAH of some carbamates (e.g. the nitro derivatives **5** and **6,** or the thiocarbamate **16**) was mainly due to the lack of chemical stability in aqueous solutions. The present investigation identifies a series of *O*-aryl carbamic derivatives that are more stable in rat plasma than the reference compound, URB597. These findings offer therefore a starting point for the development of new FAAH inhibitors endowed with longer duration of action and greater potency in vivo.

Experimental Section

Chemistry

All chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich srl, Cologno Monzese, Italy), Analyticals Carlo Erba (Carlo Erba, Rodano, Italy) and Ricci Chimica (Ricci Chimica, Ponte Valleceppi, Italy) in the highest quality commercially available. Microwave irradiation was performed on a CEM Discover® S-Class apparatus in a sealed vessel mode (fixed temperature, variable power, PowerMax). Solvents were RP grade. Chromatographic separations were performed on silica gel columns by flash chromatography (Kieselgel 60,

0.040–0.063 mm, Merck). TLC analyses were performed on silica gel on aluminum sheets (Kieselgel 60 F_{254}, Merck). Melting points were determined on a Büchi SMP-510 capillary melting point apparatus. EI-MS spectra (70 eV) were recorded with a Fisons Trio 1000 spectrometer; only molecular ions (M^+) and base peaks are given. ESI-MS spectra were recorded with a Waters Micromass ZQ spectrometer in a positive mode using a nebulizing nitrogen gas at 400 L/min and a temperature of 250 °C, cone flow 40 mL/min, capillary 3.5 Kvolts and cone voltage 60 V; only molecular ions in positive ion mode $(M+H)^+$ are given. 1H NMR and 13C NMR spectra were recorded on a Bruker AC 200 or 50, respectively, spectrometer and analyzed using the WIN-NMR software package; chemical shifts were measured by using the central peak of the solvent. IR were obtained on a Shimadzu FT-8300, or a Nicolet Atavar, spectrometer. Elemental analyses were performed on a Carlo Erba, or a ThermoQuest, analyzer.

Compounds **1**,[29] **2**–**7**,[31] **9**–**11**,[30] **14**–**15**,[33] **16**–**19**,[29] were synthesized following the quoted procedures, compounds **8**, **12**, **13, 20** and **21** as described below.

Synthesis of alkylcarbamic acid aryl esters **8**, **12**, **13, 20** and **21**: To a stirred solution of the appropriate aryl alcohol (1 mmol) in toluene (6 mL), Et_3N (0.005 g, 0.007 mL, 0.05 mmol) and RNCO (1.1 mmol) were added. The reactants were refluxed for 5 h, then a further amount of RNCO (0.109 g, 0.12 mL, 1.1 mmol of *n*-C_4H_9NCO for **8**; 0.069 g, 0.07 mL, 0.55 mmol of c-$C_6H_{11}NCO$ for **12**, **13, 20** and **21**) was added and the mixture refluxed for some additional time (19 h for **8**; 3 h for **12**, **13, 20** and **21**). The mixture was then cooled and concentrated. Purification of the residue by column chromatography (cyclohexane/EtOAc 8:2 to 7:3 for **8**; 8:2 for **12** and **13;** cyclohexane/CH_2Cl_2 1:1 for **20**; 2:8 for **21**) and recrystallization gave **8**, **12, 13, 20** and **21**.

***n*-Butylcarbamic acid 4-benzyloxyphenyl ester (8):** white crystals (0.260 g, 87%); mp: 129–130 °C (MeOH); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.96 (t, 3H), 1.27–1.63 (m, 4H), 3.27 (q, 2H), 4.97 (m, 1H), 5.05 (s, 2H), 6.92–7.07 (m, 4H), 7.30–7.46 (m, 5H) ppm; ^{13}C NMR (50 MHz, $CDCl_3$): δ = 13.8, 19.9, 31.9, 41.0, 70.4, 115.3, 122.5, 127.5, 128.0, 128.6, 137.0, 144.9, 155.0, 156.1 ppm; IR (KBr):ν = 3304, 1734, 1712 cm^{-1}; MS (EI): *m/z* 299 (M^+), 200 (100); Anal calcd for $C_{18}H_{21}NO_3$: C 72.22, H 7.07, N 4.68, found: C 72.31, H 7.17, N 4.61.

Cyclohexylcarbamic acid biphenyl-4-yl ester (12): white scales; (0.246 g, 85%); mp: 157–158 °C (EtOH); ^{1}H NMR (200 MHz, $CDCl_3$): δ =1.22–2.06 (m, 10H), 3.59 (br s, 1H), 4.95 (br d, 1H), 7.19–7.59 (m, 9H) ppm; ^{13}C NMR (50 MHz, $CDCl_3$): δ = 24.8, 25.5, 33.3, 50.2, 121.9, 127.1, 127.2, 128.0, 128.8, 138.3, 140.6, 150.6, 153.7 ppm; IR (Nujol): ν = 3308, 1744, 1706 cm^{-1}; MS (ESI): *m/z* 296.1 $(M+H)^+$; Anal calcd for $C_{19}H_{21}NO_2$: C 77.26, H 7.17, N 4.74, found: C 77.48, H 7.28, N 4.71.

Cyclohexylcarbamic acid naphthalen-2-yl ester (13): white crystals (0.256 g, 95%); mp: 156–157 °C (EtOH); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.22–2.08 (m, 10H), 3.62 (m, 1H), 4.99 (br d, 1H), 7.28–7.86 (m, 7H) ppm; ^{13}C NMR (50 MHz, $CDCl_3$): δ = 24.8, 25.5, 33.3, 50.2, 118.3, 121.5, 125.4, 126.4, 127.6, 127.7, 129.2, 131.2, 133.8, 148.8, 153.8 ppm; IR (Nujol): ν = 3289, 1695 cm^{-1}; MS (ESI): *m/z* 270.2 $(M+H)^+$; Anal calcd for $C_{17}H_{19}NO_2$: C 75.81, H 6.89, N 5.20, found: C 76.19, H 6.89, N 5.17.

Cyclohexylcarbamic acid 6-fluorobiphenyl-3-yl ester (20): white crystals (0.197 g, 63%); mp: 137–139 °C (EtOH); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.13–1.99 (m, 10H), 3.48–3.64 (m, 1H), 4.64–4.96 (br s, 1H), 7.02–7.32 (m, 8H) ppm; ^{13}C NMR (50 MHz, $CDCl_3$):δ = 24.8, 25.4, 33.3, 50.2, 116.6 (d, *J* = 25.0 Hz), 121.9 (d, *J* = 8.7 Hz), 123.7 (d, *J* = 3.8 Hz), 127.9, 128.4, 129.0 (d, J = 3.0 Hz), 129.8, 135.2 (d, *J* = 1.3 Hz), 147.0 (d, *J* = 2.9 Hz), 154.0

(d, J = 37.6 Hz), 159.3 ppm; IR (Nujol): ν = 3300, 1744, 1702 cm^{-1}; MS (ESI): *m/z* 314.0 $(M+H)^+$; Anal calcd for $C_{19}H_{20}FNO_2$: C 72.83, H 6.43, N 4.47, found: C 72.89, H 6.35, N 4.51.

Cyclohexylcarbamic acid 6-methoxybiphenyl-3-yl ester (21): white crystals (0.218 g, 67%); mp: 162–163 °C (EtOH); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.17–1.79 (m, 8H), 1.98–2.04 (m, 2H), 3.54–3.58 (m, 1H), 3.79 (s, 3H), 4.91 (br s, 1H), 6.91–6.96 (m, 1H), 7.05–7.10 (m, 2H), 7.31–7.44 (m, 3H), 7.51–7.54 (m, 2H) ppm; ^{13}C NMR (50 MHz, $CDCl_3$): δ = 24.8, 25.5, 33.3, 50.1, 56.0, 111.8, 121.2, 124.0, 127.2, 128.0, 129.5, 131.3, 137.8, 144.6, 153.8, 154.1 ppm; IR (Nujol): ν = 3289, 1735, 1701 cm^{-1}; MS (ESI): *m/z* 326.0 $(M+H)^+$; Anal calcd for $C_{20}H_{23}NO_3$: C 73.82, H 7.12, N 4.30, found: C 74.06, H 7.20, N 4.33.

Synthesis of 6-fluorobiphenyl-3-ol:[43]—To a stirred solution of 3-chloro-4-fluorophenol (0.220 g, 1.5 mmol) in dioxane (1.5 mL), Cs_2CO_3 (0.586 g, 1.8 mmol), $Pd_2(dba)_3$ (0.021 g, 0.022 mmol), P(*t*-Bu)$_3$ (0.011 g, 0.013 mL. 0.054 mmol), and phenylboronic acid (0.183 g, 1.5 mmol) were added under N_2 atmosphere. The mixture was subjected to microwave irradiation at 120 °C for 1 h, cooled, added of HCl, and extracted with CH_2Cl_2. The combined organic layers were dried (Na_2SO_4) and concentrated. Purification of the residue by column chromatography ($CHCl_3$) gave the desired product as a colorless oil. Yield: 87% (0.246 g); ^{1}H NMR and IR spectra are according to the literature.[43]

Synthesis of 6-methoxybiphenyl-3-ol:[44]—To a stirred solution of 3-bromo-4-methoxyphenol (0.305 g, 1.5 mmol) in toluene (4.5 mL), $Pd(PPh_3)_4$ (0.087 g, 0.075 mmol), a solution of Na_2CO_3 (0.795 g, 7.5 mmol) in H_2O (2.25 mL), and a solution of phenylboronic acid (0.274 g, 2.25 mmol) in EtOH (2.25 mL), were added under N_2 atmosphere. The mixture was vigorously stirred under reflux for 14 h, cooled, added of H_2O, and extracted with EtOAc. The combined organic layers were dried (Na_2SO_4) and concentrated. Purification of the residue by column chromatography (cyclohexane/CH_2Cl_2 2:8) and recrystallization gave the desired product as white crystals. Yield: 89% (0.267 g); mp: 48 °C (Et_2O/petroleum ether); ^{1}H NMR and IR spectra are according to the literature.[55]

Synthesis of 3-bromo-4-methoxyphenol:[45]—To a solution of 3-bromo-4-methoxybenzaldehyde (0.645 g; 3 mmol) in CH_2Cl_2 (3 mL), 3-chlorobenzenecarboperoxoic acid (0.518 g; 3 mmol) was added. The mixture was stirred at 40 °C for 72 h, washed with a solution of saturated $Na_2S_2O_3$, a solution of saturated $NaHCO_3$, and extracted with CH_2Cl_2. The combined organic layers were dried (Na_2SO_4) and concentrated. The brown oil thus obtained was dissolved in EtOH (10 mL), then CH_3ONa (0.162 mg; 3 mmol) was added. The mixture was stirred at reflux for 14 h then cooled and concentrated. Purification of the solid residue by recrystallization gave the desired product as white needles. Yield: 61% (0.372 g); mp: 76–77 °C (Et_2O/petroleum ether) [lit.: 77–78 °C (benzene)];[45] MS (EI): *m/z* 204 (M^+), 69 (100); ^{1}H NMR and IR spectra are according to the literature.[56]

Biological Media

Rat plasma was obtained from male Wistar rats, 250–300 g in weight (Charles River Laboratories, Milan, Italy). Animals were housed, handled and cared for according to the European Community Council Directive 86 (609) EEC and the experimental protocol was carried out in compliance with Italian regulations (DL 116/92) and with local ethical committee guidelines for animal research. Pooled plasma was obtained via cardiac puncture, collected into heparinized tubes, centrifuged (1,900*g*, 4 °C, 10 min) using a ALC refrigerated centrifuge (ALC srl, Cologno Monzese, Italy) and stored at −70 °C until use.

Rat liver S_9 fractions were obtained from the same rats, transcardially perfused with 60 mL ice-cold KCl 0.15 M. Liver was removed, weighted, sliced into small pieces, and homogenized on ice with ice-cold 0.01 M PBS buffer solution, pH 7.4 (20% w/v). S_9 fraction was obtained by centrifugation (9,000*g*, 4 °C, 30 min) and stored at −70 °C until use. Protein content was quantified via the colorimetric Bradford method, employing Bovine Serum Albumin (BSA) as internal standard.[57]

In vitro chemical stability

It was investigated under acidic (0.1 M HCl, pH 1.0), physiological (0.01 M Phosphate Buffered Saline, pH 7.4) and alkaline (0.01 M borate buffer, pH 9.0) pH conditions, at fixed ionic strength (μ = 0.15 M). Stock solutions of compounds were prepared in DMSO and each sample was incubated at a final concentration of 1–5 μM in pre-thermostated buffered solution; final DMSO concentration in the samples was kept at 1%. The samples were maintained at 37 °C in a temperature-controlled shaking water bath (60 rpm). At various time points, 100 μL aliquots were removed and injected into the High Performance Liquid Chromatography (HPLC) system for analysis. Apparent half-lives ($t_{1/2}$) for the disappearance of carbamate drugs were calculated from the pseudo first-order rate constants obtained by linear regression of the log drug concentration versus time plots and are reported in Tables 1A and 1B as means with their standard deviations (n =3).

In vitro enzymatic stability

Rat plasma was quickly thawed and diluted to 80% (v/v) with PBS, pH 7.4, to stabilize the pH of the solution, which was checked during the experiment. 400 μL of pooled rat plasma were incubated with 95 μL of PBS buffer, pH 7.4 and 5 μL of 100 μM compound stock solution in DMSO (final DMSO concentration in samples: 1%; final compound concentration: 1 μM). The coincubation with the esterase inhibitor paraoxon (final concentration: 1 mM) significantly reduced the observed hydrolysis of **1** in rat plasma ($t_{1/2,\text{inhib}}$ = 143 min). The appearance rate of the hydrolysis product *m*-biphenol from compound **1** was measured; its formation kinetics paralleled the carbamate consumption kinetics (Supplementary Figure 1).

In rat S_9 fraction stability experiments, 50 μL aliquots of S_9 fraction were quickly thawed and incubated for 5 min at 37 °C with the NADPH-regenerating system (2 mM $NADP^+$, 10 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 5 mM $MgCl_2$) in PBS, pH 7.4. At the end of the incubation period, 5 μL of a 100 μM compound stock solution in DMSO were added (final DMSO concentration in samples: 1%; final compound concentration: 1 μM). Final protein concentration in liver S_9 fraction was measured according to Bradford with BSA as a standard.[57] Original samples of centrifuged tissue were diluted in order to have a final protein concentration of 2 mg mL^{-1}.

Samples (plasma and S_9 fraction stability studies) were maintained at 37 °C in a temperature-controlled shaking water bath (60 rpm) throughout the experiments. At regular time points, 50 μL aliquots were withdrawn, additioned with two volumes of acetonitrile, centrifuged at 8,000*g* for 5 min, and analyzed by RP-HPLC. Apparent half-lives ($t_{1/2}$) for the disappearance of test compounds were calculated from the pseudo first-order rate constants obtained by linear regression of the log drug concentration versus time plots. Apparent half-lives ($t_{1/2}$) reported in Tables 1A and 1B are the means of three experiments with their standard deviations.

Analytical Method

The disappearance of test compounds was monitored by RP-HPLC employing a Gilson gradient system (Gilson Inc., Middleton, WI, USA) consisting of Gilson 305 pumps, a 20

μL Rheodyne sample injector (Rheodyne LLC, Rohnert Park, CA, USA) and a Gilson UV 115 detector, equipped with a reversed-phase C_{18} column (LC-18-DB, 5 μm, 150 × 4.6 mm i.d.; Supelco, Bellefonte, PA, USA) and a stand-alone integrator (Hewlett-Packard, USA). Mobile phases consisted of various percentages of acetonitrile: 10 mM phosphate buffer, pH 7.0 (70:30 to 50:50, v/v). Each compound was monitored at its relative absorbance maximum. A mobile phase flow rate of 1 mL min^{-1} was employed. Compound **18**, whose lower extinction coefficient did not allow quantification by the LC/UV system in the chosen concentration range, was monitored by employing an API 150EX single quadruple LC/MS system (Applied Biosystem/MDS Sciex, Foster City, CA, USA) constituted by an Agilent 1100 binary HPLC system interfaced with an APCI (Atmospheric Pressure Chemical Ionization) Heated Nebulizer Ion Source. Compound-dependent parameters were optimized by flow injection analysis and ramping. Final settings were: Declustering potential: 10.7 V; Focusing potential: 135 V, Entrance potential: 3.5 V. Higher voltages led to compound in-source fragmentation. Temperature was set at 400 °C; flow rate was 1.0 mL min^{-1} employing 80 methanol: 20 0.1% formic acid as mobile phase. The molecular ion was detected at m/z = 290.1 $[M+H]^+$ in positive ion mode. The signal at m/z = 171.0, corresponding to the $[M+H]^+$ *m*-biphenol fragment, was also recorded.

QSPR analysis

Three-dimensional molecular models were built by applying standard tools in Sybyl 7.0,[58] running on a Silicon Graphics Octane workstation. Conformational analysis on the biphenyl derivate URB524 (**1**) was performed by systematic scanning of the rotatable bonds and energy minimization to a gradient of 0.01 kcal mol^{-1} $Å^{-1}$, applying the MMFF94s force field.[59] The global minimum of **1** was employed to select the most similar minimum-energy conformation for the other compounds (with the exception of compound **8**), and the resulting structures were used as starting inputs for semiempirical calculations. Conformational analysis was also performed for compound **8** with the same protocol described for **1**. Its global minimum was thus used for further quantum calculations.

The energies of the lowest occupied molecular orbital (LUMO) were computed at AM1 level[60] and used as quantum chemical descriptors in the QSPR models. The dependent variable was log k_{pH9}, i.e. the logarithm of the apparent first-order kinetic constant observed at pH 9.0, calculated as $\ln 2/t_{1/2}$[min]. clog *P* values were calculated employing the CLOGP™ software (CLOGP 4.9, Daylight Chemical Information Systems Inc., Aliso Viejo, CA, USA, available at http://www.daylight.com/daycgi/clogp).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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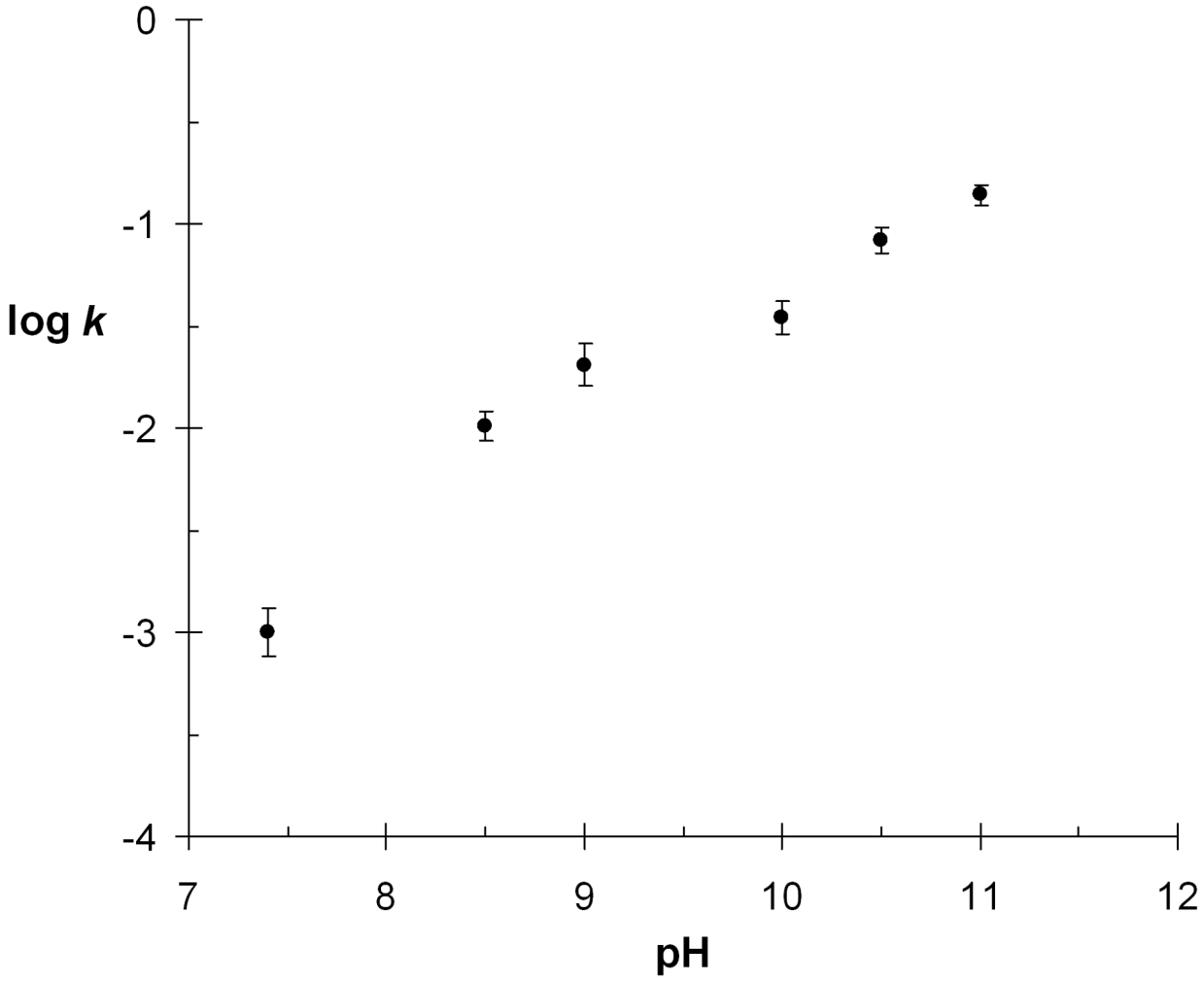

Figure 1.
Hydrolysis rates (log *k*) at different pH values for compound **13** (n = 3; vertical bars represent standard deviations).

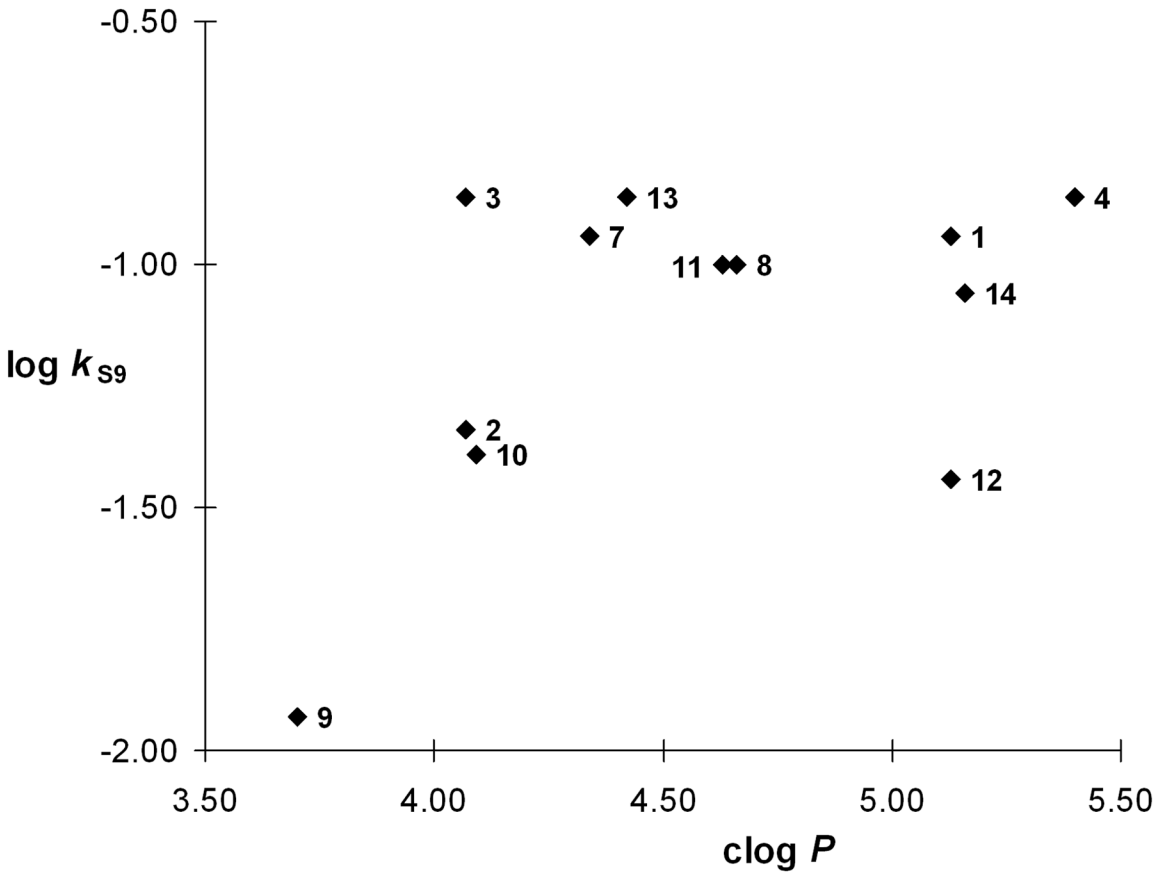

Figure 2.
Plot of lipophilicity (clog *P*) versus hydrolysis constants in rat liver S_9 fraction (log k_{S9}) for compounds **1**–**14**.

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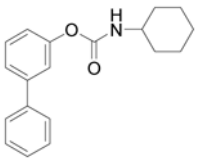
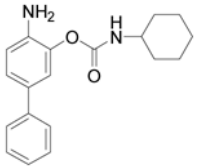
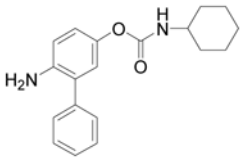
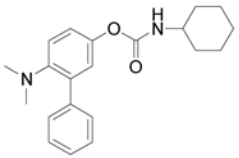
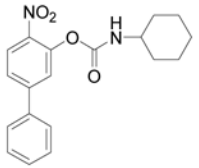
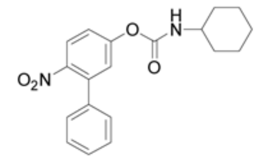
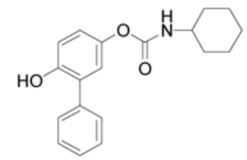
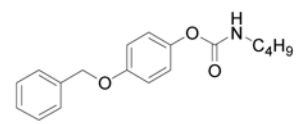
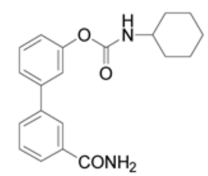
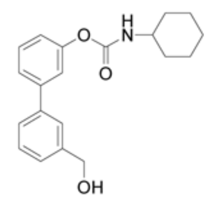
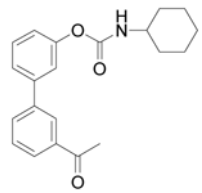
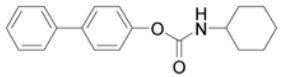
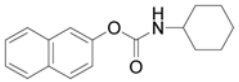
Table 1A

Alkylcarbamic acid aryl esters: chemical and enzymatic stability (half-lives in minutes or percentage of parent compound recovered after 24 hours) and FAAH inhibitory potency.

Compd.	Formula	pH 7.4[a]	pH 9.0[b]	80% RP[b,c]	liver S_9[b]	IC_{50} [nM][d]
1 URB524		41%±2	41±2	43±7	6±2	63±9[10]
2		57%±2	84±9	90±4	15±1	4830±474[31]
3		89%±1	432±33	784±56	5±1	52±4[31]
4		36%±1	106±5	499±33	5±1	1592±76[31]
5		1.8±0.2[b]	N.D.[e]	N.D.[e]	N.D.[e]	> 30,000[31]

Compd.	Formula	pH 7.4[a]	pH 9.0[b]	80% RP[b,c]	liver S_9[b]	IC_{50} [nM][d]
6		1.7±0.1[b]	N.D.[e]	N.D.[e]	N.D.[e]	> 30,000[31]
7		78%±5	255±20	950±54	6±1	45±17[31]
8		76%±12	290±20	333±7	7±1	390±66
9 **URB597**		40%±1	33±3	33±1	59±6	4.6±1.6[10]
10		43%±7	40±4	45±3	17±2	8.7±0.9[30]

Compd.	Formula	pH 7.4[a]	pH 9.0[b]	80% RP[b,c]	liver S_9[b]	IC_{50} [nM][d]
11		37%±3	32±3	22±3	7±2	9.1±0.5[30]
12		37%±2	35±1	22±1	19±4	2297±226[29]
13		34%±1	34±3	26±1	5±1	324±31[10]

[a] Percentage left at 24 h, 37 °C, unless differently indicated.

[b] $t_{1/2}$ in min calculated from pseudo-first-order rate constants. Mean±S.D. n = 3.

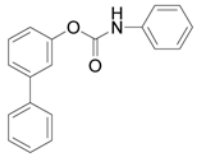
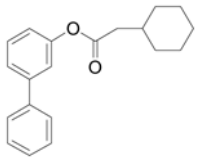
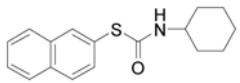
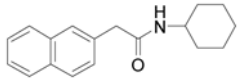
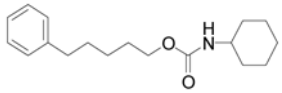
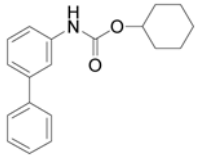
[c] RP = Rat plasma.

[d] Inhibitory potencies on FAAH in vitro activity.

[e] No remaining compound was detected at the first time point (t = 20 s).

Table 1B

Miscellaneous structures: chemical and enzymatic stability (half-lives in minutes or percentage of parent compound recovered after 24 hours) and FAAH inhibitory potencies.

Compd	Formula	pH 7.4[a]	pH 9.0[b]	80% RP[b,c]	liver S_9[b]	IC_{50} [nM][d]
14		51±4[b]	2.8±0.2	5.0±0.3	8±1	3,942±1,409[33]
15		56%±2	1003±57	0.6±0.1	1.0±0.2	2,551±1,074[33]
16		2.6±0.3[b]	N.D.[e]	N.D.[e]	0.7±0.1	15,580±650[29]
17		100%±2	100%±3[a]	73%±3[a]	3.0±0.8	> 30,000[29]
18		97%±1	95%±2[a]	79%±5[a]	2.0±0.1	> 30,000[10]
19		87%±7	84%±5[a]	85%±8[a]	15±1	> 30,000[29]

[a]Percentage left at 24 h, 37 °C, unless differently indicated.

[b]$t_{1/2}$ in min calculated from pseudo-first-order rate constants. Mean±S.D. n =3.

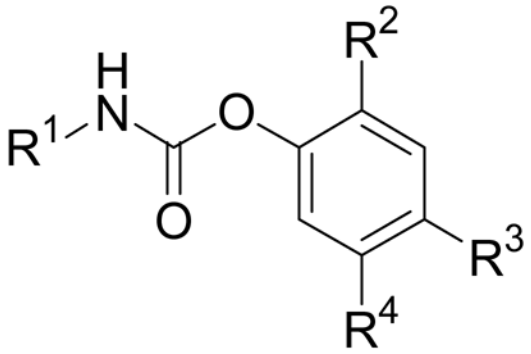
[c] RP = Rat plasma.

[d] Inhibitory potencies on FAAH in vitro activity.

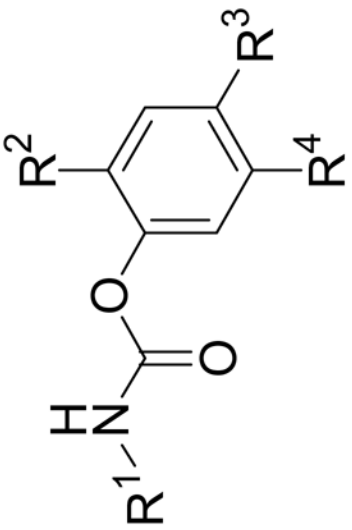
[e] No remaining compound was detected at the first time point (t = 20 s).

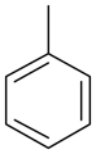
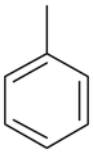
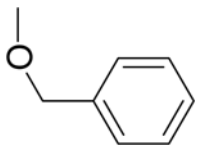
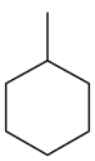
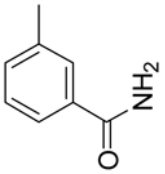
Table 2

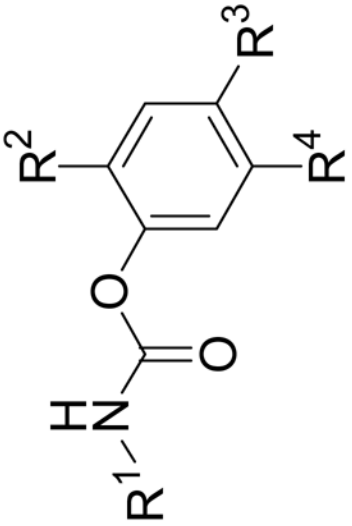
Data employed to model chemical stability of aryl carbamates

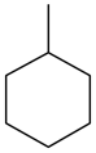
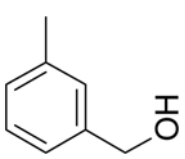
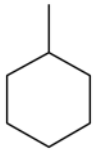
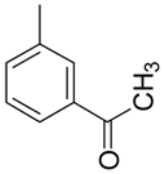
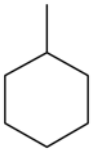
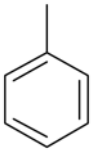
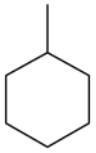


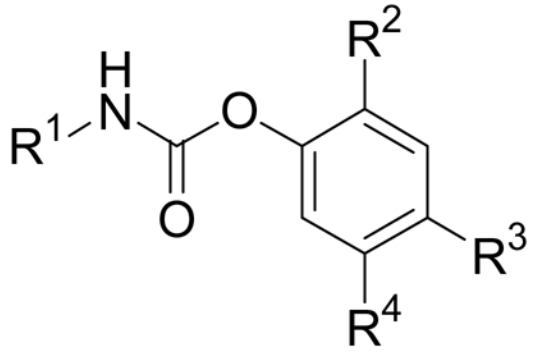
Compd	R^1	R^2	R^3	R^4	log k_{pH9}	LUMO[a]	log k_{pH9} calc. [b]	Resid.[c]
1		H	H		−1.77	−0.0795	−2.02	0.25
2		NH_2	H		−2.08	0.0623	−2.25	0.17
3		H	NH_2		−2.79	0.2870	−2.61	−0.18
4		H	$N(CH_3)_2$		−2.18	0.0708	−2.26	0.08
5		NO_2	H			−1.1686		

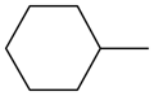
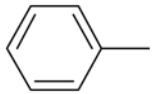
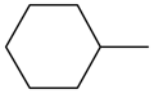
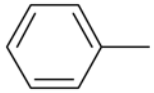


Compd	R^1	R^2	R^3	R^4	log k_{pH9}	LUMO[a]	log k_{pH9} calc. [b]	Resid.[c]
6		H	NO_2			−1.1183		
7		H	OH		−2.57	−0.0637	−2.04	−0.53
8		H		H	−2.62	0.2795	−2.60	−0.02
9		H	H		−1.68	−0.2983	−1.67	−0.01



Compd	R^1	R^2	R^3	R^4	log k_{pH9}	LUMO[a]	log k_{pH9} calc. [b]	Resid.[c]
10		H	H		−1.76	−0.1492	−1.91	0.15
11		H	H		−1.66	−0.4013	−1.50	−0.16
12		H		H	−1.70	−0.1509	−1.90	0.20
13		H			−1.69	−0.2895	−1.68	−0.01



Compd	R[1]	R[2]	R[3]	R[4]	log k_{pH9}	LUMO[a]	log k_{pH9} calc. [b]	Resid.[c]
20		H	F		−1.73	−0.2782	−1.70	−0.03
21		H	OCH_3		−2.05	−0.001	−2.15	0.10

[a] Molecular orbital energies, eV.

[b] Calculated from Equation (1).

[b] Calculated as log k_{pH9} - log k_{pH9} calc.

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Table 3

Data employed to model stability of aryl carbamates in biological media.

Compd	log k_{plasma}	log k_{S9}	clog P[a]
1	−1.79	−0.94	5.13
2	−2.11	−1.34	4.07
3	−3.05	−0.86	4.07
4	−2.86	−0.86	5.40
5			5.17
6			5.17
7	−3.14	−0.94	4.34
8	−2.68	−1.00	4.66
9	−1.68	−1.93	3.70
10	−1.81	−1.39	4.09
11	−1.50	−1.00	4.63
12	−1.50	−1.44	5.13
13	−1.57	−0.86	4.42
14		−1.06	5.16

[a] clog *P* values were calculated by the CLOGP software.