Title
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Dear Sir,

Correlation between energetics of collisionally activated decompositions, interaction energy and biological potency of carbamate FAAH inhibitors

Mass spectrometry (MS) might usefully be employed to study the mechanism of action, the structure–activity relationships (SARs) or the side effects of pharmacologically active compounds that undergo bioactivation to electrophilic intermediates or nucleophilic attack prior to the development of their effects, although the literature mainly report examples in which MS was employed to characterize reactive metabolites.[1] Quite early we used MS techniques to establish quantitative SARs for some potential anticancer agents (aryl and heteroaryl triazenes),[2–4] and more recently for novel inhibitors of the fatty acid amide hydrolase (FAAH) enzyme.[5] The latter compounds are characterized by an *N*-alkylcarbamic acid *O*-aryl ester structure, and they can be exemplified by the irreversible and systemically active inhibitor cyclohexylcarbamic acid 3′-carbamoylbiphenyl-3-yl ester (URB597).[6]

The chemical rationalization of the mode of action of these FAAH inhibitors and their SARs were approached by modeling studies[7–9] and also by MS techniques.[5] Irreversible enzyme inactivation is envisaged to occur through two distinct and consecutive processes (Scheme 1), i.e. formation of a noncovalent complex (recognition step), and nucleophile attack to the carbamate by Ser241[10,11] leading to its carbamoylation and to irreversible inactivation of the enzyme (inactivation step). The recognition step is related to stereoelectronic complementarity between the inhibitor and the active site of the enzyme and it was rationalized by molecular modeling studies,[8,9] whereas the inactivation reaction may be related to the propensity of the C(O)–O bond to be cleaved and it may be described by suitable indicators of bond reactivity. In this particular case, the second step was studied by breakdown curves,[12] relative to ESI-generated protonated molecules, which, taking place under collisional conditions during resonance excitation in an ion trap, exclusively gave fragments related to the C(O)–O bond cleavage.[5] The crossing points (CPs) (corresponding to the collision energy necessary to fragment 50% of the precursor ion population) between the decreasing $[MH]^+$ ion abundance and the increasing ion fragment abundance **w** (Scheme 2) were related to the energetics of decomposition, and showed linear correlation ($r^2 = 0.797$) with the IC_{50} (half maximal inhibitory concentration of FAAH hydrolysis of [^{3}H]AEA in rat cortical membranes) values for the examined compounds.[5] This supported the hypothesis that the ease of C(O)–O bond cleavage was relevant to explain FAAH inhibition. The results thus obtained justified the proposal of a binding mode to FAAH,[5,8,9] later supported by other authors,[13] alternative to that initially hypothesized.[7]

In the present investigation we applied the previously described approach to a series of biphenyl-3-yl carbamate analogs of URB524 (**19**, Table 1) with electron-withdrawing or electron-donating substituents on the distal (**1–10**, Table 1)[8] and proximal (**11–18**, Table 1)[9] phenyl rings. The present work was designed to investigate whether the previously found correlation between experimental CPs and IC_{50} values was still valid, since no correlation had been observed between IC_{50} values and substituent electronic effects described by tabulated σ–parameters for a series of derivatives *ortho*- and *para*-substituted at the proximal phenyl ring.[9]

ESI experiments were performed using a LCQ Deca instrument (Thermo, San José, CA, USA) operating in positive ion mode. Compounds **1–19** were dissolved in CH_3OH and their 10^{-6} M solutions were directly infused into the ESI source. The spray voltage, capillary voltage and entrance capillary temperature were 4 kV, 8 V and 220 °C, respectively. MS/MS experiments were obtained by resonance excitation[14] of the preselected ion, and the breakdown curves of $[MH]^+$ species were obtained by varying the supplementary r.f. voltage in the range 15–40% of its maximum value (5 V peak-to-peak). Helium pressure inside the trap was kept constant (2.8×10^{-5} Torr directly read by ion gauge, in absence of the N_2 stream). The isolation width was set at 2 mass units and the scan rate was 0.5 s^{-1}.

All the examined compounds showed, in ESI conditions, the production of abundant $[MH]^+$, similar to what had been previously observed.[5] In all cases, the $[MH]^+$ ion was selected and MS/MS experiments were performed by increasing the supplementary r.f. voltage. The breakdown curves obtained under these conditions showed a decrease in $[MH]^+$ ion abundance and an increase in fragment ion(s) abundance **w**. It must be noted that in ion trap experiments the energy deposition during the ion activation phase is a step-by-step phenomenon[15] and consequently the MS/MS data so obtained are related to the fragmentation pathways exhibiting the lowest critical energy, in contrast to what was observed in high-energy collisions or in MS/MS experiments performed by a triple quadrupole.

For all the compounds, with the exception of **7** and **17**, exclusively one collision-induced fragmentation was observed, originating from the cleavage of the C(O)–O bond (Scheme 2). The related breakdown curves effectively highlight differences in the decomposition energy

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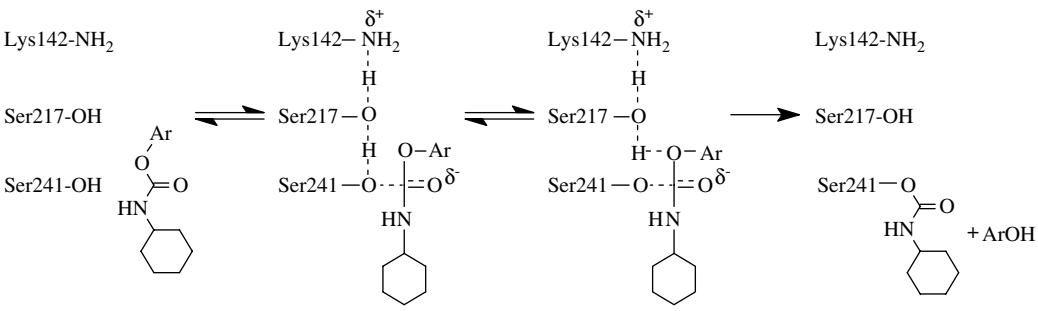
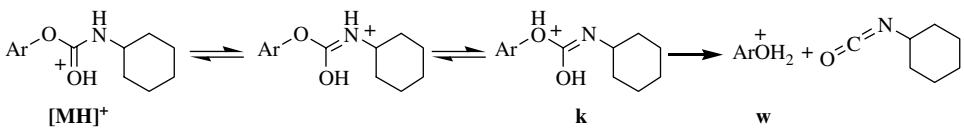
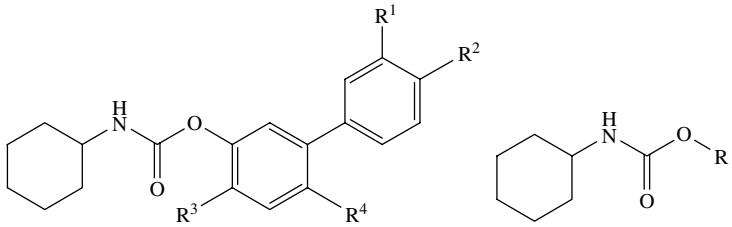


Scheme 1. Mechanism of FAAH inhibition.



Scheme 2. Cleavage of protonated FAAH inhibitors.

Table 1. Structures of compounds **1–26**, crossing point (CP) values related to the MH^+ breakdown curves and pIC_{50} values



1-19 **20-26**

Compound	R^1	R^2	R^3	R^4	CP (V)	Log P	IE[a] (kcal/mol)	pIC_{50}
1	H	CH_3	H	H	0.92	4.54	−47.58	6.81
2	H	NH_2	H	H	0.96	3.78	−48.52	6.44
3	CH_3	H	H	H	0.94	4.54	−49.12	7.21
4	$C(O)NH_2$	H	H	H	0.96	3.54	−75.61	8.34
5	NO_2	H	H	H	0.96	4.31	−54.62	7.30
6	$C(O)CH_3$	H	H	H	1.07	4.15	−62.97	8.04
7	CH_2OH	H	H	H	1.20	3.74	−61.37	8.06
8	OH	H	H	H	0.98	3.78	−47.46	8.06
9	NH_2	H	H	H	1.00	3.78	−47.07	7.19
10	H	$C(O)NH_2$	H	H	1.40	3.54	−52.95	5.23
11	H	H	NO_2	H	1.26	4.31	−55.27	<4.50
12	H	H	NH_2	H	0.99	3.78	−54.22	5.32
13	H	H	H	NO_2	1.32	4.31	−48.91	<4.50
14	H	H	H	NH_2	1.10	3.78	−48.98	7.28
15	H	H	H	CH_3	0.99	4.54	−48.74	6.75
16	H	H	H	OH	0.96	3.78	−47.12	7.35
17	H	H	H	CH_2OH	0.94	3.74	−53.68	7.34
18	H	H	H	$N(CH_3)_2$	1.19	4.23	−42.68	5.80
19[5]	H	H	H	H	0.96	4.32	−44.08	7.20
			R					
20[5]	Phenyl				1.10	3.08	−26.13	5.42
21[5]	*m*-Tolyl				0.98	3.35	−32.57	6.09
22[5]	8-Bromonaphthalen-2-yl				0.94	4.55	−39.01	6.76
23[5]	6-Ethylnaphthalen-2-yl				1.09	4.40	−49.51	5.45
24[5]	(*E*)-4-Styrylphenyl				1.11	4.69	−47.53	5.48
25[5]	(*Z*)-4-Styrylphenyl				1.03	4.69	−40.82	6.58
26[5]	3-Pentylphenyl				0.96	4.56	−42.58	6.73

[a] Interaction energy.

of protonated molecules. In particular, considering the CP between the plots of precursor and fragment ion abundance, it is possible to obtain a parameter closely related to the critical energy of the decomposition processes. The CP values obtained for compounds **1–19** are reported in Table 1. Compound **19**[5] was added as a reference compound, in view of the close similarity between its structure and that of compounds **1–18**. In the case of **7** and **17,** the CP values must be critically considered because multiple decomposition channels are activated for these compounds (Fig. 1(a), (b)) preventing an accurate evaluation of the CP values related to the C(O)–O bond cleavage. CPs of compounds **1–19** were plotted against pIC_{50} values, where pIC_{50} represents the negative logarithm of IC_{50} for the compounds under study. While in the previous paper on unsubstituted carbamic acid *O*-aryl esters a linear relationship had been obtained:[5]

$$pIC_{50} = -8.89CP + 15.29; n = 8; r^2 = 0.80; s = 0.34; F = 23.5 \quad (1)$$

here no correlation was found between CP and pIC_{50} values of compounds **1–19,**

$$pIC_{50} = -5.03(\pm 1.54)CP + 12.10(\pm 1.65);$$
$$n = 19; r^2 = 0.39; s = 0.95; F = 10.6 \quad (2)$$

(with pIC_{50} set to 4.5 for compounds **11** and **13**), the lack of correlation also resulted in a very low predictive power, as estimated by leave-one-out cross-validation[8] ($q^2 = 0.24$; Standard Deviation of Error of Prediction (SDEP) =1.00). The statistics slightly improved by omitting compounds **7** and **17**:

$$pIC_{50} = -5.87(\pm 1.45)CP + 12.87(\pm 1.55); n = 17; r^2$$
$$= 0.52; s = 0.85; F = 16.4; q^2 = 0.40; \text{SDEP} = 0.90 \quad (3)$$

on consideration of their above-discussed behavior under collisional conditions.

An additional exclusion was operated considering that the nitrogen atoms in compounds **2**, **9**, **12**, **14**, **18** are thermodynamically privileged protonation sites and therefore in ESI conditions act with an electron-withdrawing effect, opposite to that in the buffered (pH = 7.5) solution in which the determination of the pIC_{50} values was conducted. The exclusion of compounds **2**, **7**, **9**, **12**, **14**, **17**, **18** gave an acceptable correlation:

$$pIC_{50} = -6.57(\pm 1.41)CP + 13.73(\pm 1.51); n = 12; r^2 = 0.69\ s = 0.78;$$
$$F = 21.8; q^2 = 0.52; \text{SDEP} = 0.88 \quad (4)$$

similar to that reported Eqn. (1).

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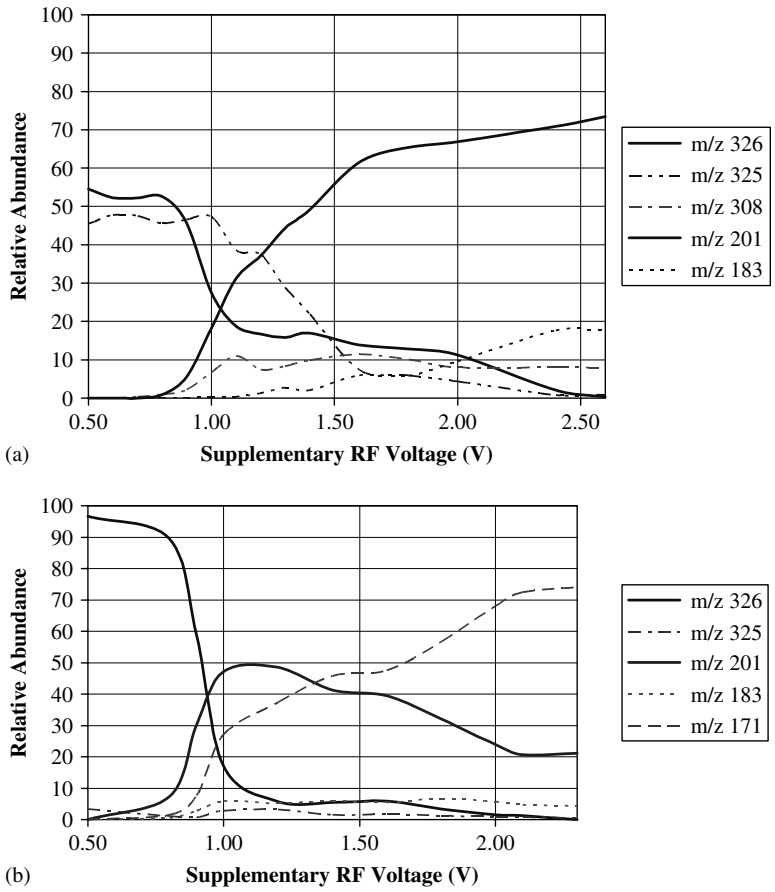

Figure 1. Breakdown curves related to $[MH]^+$ ions of compound **7** (a) and **17** (b), showing the occurrence of different fragmentation patterns also in low-collision-energy regimes.

In order to have an overview of the class, we examined the larger group of compounds composed of the subset **1–19** and that resulting from the FAAH inhibitors previously investigated and reported in Table 1 as compounds **20–26**.[5] Compounds **2**, **7**, **9**, **12**, **14**, **17**, **18** were not considered for the reasons discussed above; however, in spite of this selection, the obtained relation:

$$pIC_{50} = -6.33(\pm 1.32)CP + 13.16(\pm 1.40); n = 19; r^2 = 0.58;$$
$$s = 0.77; F = 23.0; q^2 = 0.44; \mathrm{SDEP} = 0.84 \quad (5)$$

gave a poor correlation mainly owing to outlier compounds **4, 6, 8**. These are the most active in the enlarged series and their structures contain groups able to establish hydrogen bonds within the catalytic site, probably contributing to their activity.

The ability of the descriptor CP to explain the inhibitory potency of the carbamate series **1–19, 20–26** was next evaluated in conjunction with a classical lipophilicity descriptor (estimated Log P, calculated by the Moriguchi approach)[16] and with the calculated interaction energy ((IE), representing the sum of the electrostatic and Van der Waals contributions) between the enzyme and the inhibitor, which may account for processes involved in the recognition step. IEs were calculated after manual docking of the ligands into the FAAH binding site with the *O*-aromatic moiety towards the cytosolic outlet[5] (DOCK command in Sybyl7.2[17] with the MMFF94s force field,[18] following the protocol described in Refs 8, 9). Log P and IE descriptors alone or in combination did not yield statistically significant models, neither for subset **1–19** nor for the larger set of compounds incorporating the previously published carbamate inhibitors **20–26**. However, when IE is used in combination with CP, a significant improvement in the statistics of the model is obtained. The overall set **1–26** (omitting **2**, **7**, **9**, **12**, **14**, **17**, **18** as discussed above) gave :

$$pIC_{50} = -6.71(\pm 1.02)CP - 0.048(\pm 0.013)IE + 11.26(\pm 1.19); n = 19;$$
$$R^2 = 0.77; s = 0.59; F = 26.0; q^2 = 0.65; \mathrm{SDEP} = 0.67 \quad (6)$$

This model proposes that both reactivity of the carbamate scaffold and molecular recognition are crucial for enzyme inactivation, suggesting that an optimal accommodation of the reactant at the active site is required to prompt the nucleophile attack by the active Ser241. The above correlations indicate that CP values and molecular docking provide complementary information about inhibitor potency for this class of FAAH inhibitors. They also warn that care must be exercised to account for the different behavior of some groups under experimentally different conditions, as exemplified by the electron-withdrawing/donating properties of the $-NH_2$ group in MS conditions and in buffered solution. Overall, the results of this study support the usefulness of MS in the rationalization of SARs in some special cases, when reactivity factors are important contributors to the biological activity and classical electronic parameters fail. In these cases, MS parameters may be used in substitution of classical indicators, provided intrinsic limitation and peculiarities of the technique are taken in due consideration.

Yours,

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