Dear Sir,

The collisional behavior of ESI-generated protonated molecules of some carbamate FAAH inhibitors isosteres and its relationships with biological activity

Recently, we reported some studies meant to rationalize the mode of action of a class of compounds acting as fatty acid amide hydrolase (FAAH) inhibitors\(^{1–3}\) and characterized by an N-alkylcarbamodic acid O-aryl ester structure.\(^{4,5}\) The enzyme inactivation is considered to take place through two distinct and consecutive processes, reported in Scheme 1, i.e., formation of a noncovalent complex (recognition step) and a nucleophilic attack by Ser245\(^{6,7}\) on the carbamate, leading to an irreversible inactivation of the enzyme by carbamoylation (inactivation step). The recognition step, related to the stereoelectronic complementarity between the inhibitors and the enzyme active site, was studied by molecular modeling\(^{8–10}\) which, however, could not completely account for the inactivation step. As a part of a wider program of application of mass spectrometry (MS) to structure–activity relationships (SARs) studies we hypothesized that the inactivation reaction could be related to the propensity of the C(O)–O bond to be cleaved. We started therefore an investigation on collisional experiments on the ESI-generated protonated molecules. Interestingly, the energetics of this process, obtained by breakdown curves,\(^{11}\) showed a linear correlation between the propensity of the C(O)–O bond to be cleaved under collisional condition and the IC\(_{50}\) (half maximal inhibitory concentration of FAAH hydrolysis of \(^{3}\)H]AEA in rat cortical membranes) values for the examined compounds.\(^{12}\) In a further study the same approach was applied to a series of biphenyl-3-ylcarbamates with electron-withdrawing or electron-donating substituents on the distal or proximal phenyl ring.\(^{13}\) The results we obtained warned us that caution must be taken when trying to extend previous results to a more complex series of structures, but in general supported the usefulness of MS in SARs at least when reactivity factors contribute to the biological activity.

During the exploration of the series of the N-alkylcarbamodic acid O-aryl esters a small number of putative bioisosteres (1–6) (Fig. 1) were synthesized to establish the role of the carbamate function on the observed FAAH inhibitory behavior.\(^{14}\) The fact that the compounds 1–6 did not inhibit FAAH\(^{15}\) led us to investigate their MS behavior to verify whether the compound 1–6 were dissolved in CH\(_3\)OH and their concentration of FAAH hydrolysis of \(^{3}\)H]AEA in rat cortical membranes) and the enzyme active site, was studied by molecular modeling,\(^{1,5,8–10}\) related to the stereoelectronic complementarity between the inhibitors with the previously studied active analyzed in ESI conditions and the related \([M + H]^{+}\) species of compound 1 leads to the product ion spectra reported in Fig. 2. The primary fragmentation processes might be rationalized by admitting an initial protonation of the nitrogen atom, but it must be taken into account that the protonation can more reasonably take place on the \(R_1\) group, which, because of the electron delocalization, represents the most basic site of the amide (or thiamide) moiety. The protonation on \(R_1\) is confirmed by the high abundance of \([M + H]^{+}\) ion for 2 and 4; in these cases the negative charge on the \(R_2\) group is reinforced by both the nitrogen-donating groups. The most abundant species, at \(m/z\) 186, is due to the cleavage of the cyclohexyl–NH bond with H-rearrangement (Scheme 2, cleavage \(\alpha\)). The ion at \(m/z\) 141 originates from the C(OH)–CH\(_3\) bond cleavage, whereas that of the NH–C(OH) bond leads both to ions at \(m/z\) 169 and \(m/z\) 100. MS\(_3\) experiments show that the ion at \(m/z\) 186 decomposes, through NH\(_3\) and CH\(_3\)NO losses, leading to ions at \(m/z\) 169 and 141, respectively. The latter species is also produced by the acylum ion at \(m/z\) 169 through a decarboxylation process.

The observed fragmentation pathways can also be explained by an isomerization of \([M + H]^{+}\) ions into a proton-bound dimer associating cyclohexyamine and naphtylketene, giving account for the formation of protonated cyclohexyamine (\(m/z\) 100) and the acylum cation at \(m/z\) 169. Analogous isomerizations can be invoked for the protonated molecules of the other compounds under investigation.

It is to be noted that all the observed fragmentation pathways lead to even electron product ions, in agreement with the even electron rule.\(^{15}\) Thus, for compound 1 the most favored decomposition route is no longer that observed in the carbamate derivatives (as in Fig. 1, cleavage \(\gamma\)), but rather that due to the cleavage of cyclohexyl–NH bond (as in Fig. 1, cleavage \(\alpha\)). This behavior might be explained by the weaker CH–NH bond strength relatively to that of the C(OH)–CH\(_2\). Of course this hypothesis would have to be confirmed by theoretical calculations, but they are not available in our lab.

In the case of \([M + H]^{+}\) of compound 2 the most abundant collisionally induced fragmentation product was that due to the C(OH)–NH bond cleavage with H2 rearrangement (Fig. 1, cleavage \(\gamma\); Fig. 3; Scheme 3.

[Table 1. Relative abundances of \([M + H]^{+}\), \([M + Na]^{+}\), and \([2M + Na]^{+}\) ions in the ESI spectra of 1–6.]

|          | \([M + H]^{+}\) | \([M + Na]^{+}\) | \([2M + Na]^{+}\) |
|----------|----------------|----------------|-----------------|
| 1        | 79             | 95             | 100             |
| 2        | 100            | 30             | 95              |
| 3        | 72             | 100            | 65              |
| 4        | 100            | 22             | 18              |
| 5        | 55             | 71             | 100             |
| 6        | 85             | 42             | 100             |

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**Figure 1. Structures of compounds 1–6.**
cleavage $\gamma$), though also cleavage $\alpha$ takes place. Also for compound 2 the formation of a proton-bound dimer allows to rationalize some of the observed decomposition pathways. Thus the proton-bound dimer constituted by cyclohexylamine/naphthyl–N=C=O justifies the formation of the cyclohexyl–NH$_3^+$ ion ($m/z$ 100), while the dimer cyclohexyl–N=C=O/naphthyl–NH$_2$ gives account for the formation of the naphthyl–NH$_3^+$ species ($m/z$ 144).

The [M + H]$^+$ of 3 showed the most abundant fragment at $m/z$ 161, corresponding to naphthyl–SH$_2^+$. The fragment $m/z$ 204 as a result of cyclohexene loss was also present, even if in low abundance (Table 2).

This seems to suggest that protonation took place on the sulfur atom. It should be considered, however, that different intramolecular proton-bridged forms can be present, as those shown in Fig. 4, which could explain the observed behavior.

Compound 4 showed the formation of naphthyl–NH$_3^+$ at $m/z$ 144 (Table 2) and a further decomposition product at $m/z$ 188, because of the cleavage of the NH–C(SH) bond (Fig. 1, cleavage $\beta$) formally corresponding to naphthyl–NH–CH=SH$^+$ ion.

An interesting decomposition was observed in the case of 5, whose collisional spectra of [M + H]$^+$ ions showed the formation of two ions.
**Figure 2.** MS² spectrum of [M + H]⁺ ions of compound 1 (m/z 268).

**Figure 3.** MS² spectrum of ion at m/z 269 related to [M + H]⁺ ions of compound 2.

**Figure 4.** Possible intramolecular H⁺ bridged structures of [MH]⁺ of 3.
Collisionally induced decomposition pattern of protonated compound 2.

Table 2. Collisionally generated fragmentation products of [MH]^+ of 1–6

|   | [M + H]^+ | [MH–C_6H_{10}]^+ | Naphthyl–R_2^+ | Naphthyl–R_2H^+ | Others |
|---|-----------|------------------|----------------|-----------------|--------|
| 1 | 268       | 186              | 169            | 141             | –      |
| 2 | 269       | 187              | –              | 144 (H rearr.)  | –      |
| 3 | 286       | 204              | –              | 161 (2H rearr.)| –      |
| 4 | 285       | –                | 188 (H rearr.)| 144 (H rearr.) | –      |
| 5 | 286       | –                | –              | 145 (H rearr.) | 161    |
| 6 | 302       | –                | –              | 161 (2H rearr.)| 142    |

at m/z 145 and m/z 161. In the case of the former ion the structure naphthyl–SH_2^+ could be proposed; this ion would originate from a Newman–Kwart rearrangement (converting phenols to thiophenols, as shown by the example reported in Scheme 4\cite{16}) induced by a protonation reaction leading to compound 3. In fact, the most abundant ion from 3 is the naphthyl–SH_2^+ one, which is also observed in the spectrum of 6, together with an ion at m/z 142, because of the cleavage of the CIS–S bond, with charge localization on the cyclohexyl-containing species.

The above results indicate that compounds 1–6 behave quite differently from what was previously observed in the case of N-alkylcarbamic acid O-aryl esters. The naphthyl–R_2H^+ ion is produced by collision of [M + H]^+ in the present case as well, but many concurrent decomposition pathways are present, mainly because of protonation sites and bond strengths different from those of the carbamates. The lack of FAAH inhibitory activity of compounds 1–6 may thus be explained also on the basis of the MS data reported here, clearly indicating a different reactivity of the putative bioisosteres in comparison with that of the parent carbamic acid ester FAAH inhibitors.

Yours,

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References

[1] A. Lodola, M. Mor, J. C. Hermann, G. Tarzia, D. Piomelli, A. J. Mulholland. QM/MM modelling of oleamide hydrolysis in fatty acid amide hydrolase (FAAH) reveals a new mechanism of nucleophile activation. Chemical Communications 2005, 4399.
[2] A. Lodola, M. Mor, J. Zurek, G. Tarzia, D. Piomelli, J. N. Harvey, A. J. Mulholland. Conformational effects in enzyme catalysis: reaction via a high energy conformation in fatty acid amide hydrolase. Biophysical Journal 2007, 92, L20.
[3] A. Lodola, M. Mor, S. Rivara, C. Christov, G. Tarzia, D. Piomelli, A. J. Mulholland. Identification of productive inhibitor binding orientation in fatty acid amide hydrolase (FAAH) by QM/MM mechanistic modelling. Chemical Communications 2008, 214.
[4] S. Kathuria, S. Gaetani, D. Fegley, F. Valino, A. Duranti, A. Tontini, M. Mor, G. Tarzia, G. La Rana, A. Calignano, A. Giustino, M. Tattoli, M. Palmery, V. Cuomo, D. Piomelli. Modulation of anxiety through blockade of anandamide hydrolysis. Nature Medicine 2003, 9, 76.
[5] G. Tarzia, A. Duranti, A. Tontini, G. Piersanti, M. Mor, S. Rivara, P. V. Plazzi, C. Park, S. Kathuria, D. Piomelli. Design, synthesis, and structure–activity relationships of alkylcarbamic acid aryl esters, a new class of fatty acid amide hydrolase inhibitors. Journal of Medicinal Chemistry 2003, 46, 2352.
[6] M. P. Patricelli, M. A. Lovato, B. F. Cravatt. Chemical and mutagenic investigations of fatty acid amide hydrolase: evidence for a family of serine hydrolases with distinct catalytic properties. Biochemistry 1999, 38, 9804.
[7] M. K. McKinney, B. F. Gravatt. Evidence for distinct roles in catalysis for residues of the serine–serine–lysine catalytic triad. *Journal of Biological Chemistry* **2003**, *278*, 37393.

[8] M. Mor, S. Rivara, A. Lodola, P. V. Plazzi, G. Tarzia, A. Duranti, A. Tontini, G. Piersanti, S. Kathuria, D. Piomelli. Cyclohexylcarbamic acid 3′- or 4′-substituted biphenyl-3-yl esters as fatty acid amide hydrolase inhibitors: synthesis, quantitative structure-activity relationships, and molecular modelling studies. *Journal of Medicinal Chemistry* **2004**, *47*, 4998.

[9] G. Tarzia, A. Duranti, G. Gatti, G. Piersanti, A. Tontini, S. Rivara, A. Lodola, P. V. Plazzi, M. Mor, S. Kathuria, D. Piomelli. Synthesis and structure–activity relationships of FAAH inhibitors: cyclohexylcarbamic acid biphenyl esters with chemical modulation at the proximal phenyl ring. *ChemMedChem* **2006**, *1*, 130.

[10] M. Mor, A. Lodola, S. Rivara, F. Vacondio, A. Duranti, A. Tontini, S. Sanchini, G. Piersanti, J. R. Clapper, A. R. King, G. Tarzia, D. Piomelli. Synthesis and QSAR of fatty acid amide hydrolase inhibitors: modulation at the N-portion of biphenyl-3-yl alkylcarbamates. *Journal of Medicinal Chemistry* **2008**, *51*, 3487.

[11] J. Gronowska, C. Paradisi, P. Traldi, U. Vettori. A study of relevant parameters in collisional-activation of ions in the ion-trap mass spectrometer. *Rapid Communications in Mass Spectrometry* **1990**, *4*, 306.

[12] E. Basso, A. Duranti, M. Mor, D. Piomelli, A. Tontini, G. Tarzia, P. Traldi. Tandem mass spectrometric data-FAAH inhibitory activity relationships of some carbamic acid O-aryl esters. *Journal of Mass Spectrometry* **2004**, *39*, 1450.

[13] G. Valitutti, A. Duranti, A. Lodola, M. Mor, G. Piersanti, D. Piomelli, S. Rivara, A. Tontini, G. Tarzia, P. Traldi. Correlation between the mass spectrometry parameter crossing point and biological potency of carbamates FAAH inhibitors. *Journal of Mass Spectrometry* **2007**, *42*, 1624.

[14] R. E. March, J. F. Todd. *Quadrupole Ion Trap Mass Spectrometry*, 2nd ed, Wiley: Hoboken **2005**, 136.

[15] M. Karni, A. Mandelbaum. The ‘even-electronic rule’. *Organic Mass Spectrometry* **1980**, *15*, 53.

[16] G. C. Lloyd-Jones, J. D. Moseley, J. S. Renny. Mechanism and application of the Newman-Kwart O → S rearrangement of O-aryl thiocarbamates. *Synthesis* **2008**, 661.