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Monoamine oxidase A inhibitory potency and flavin perturbation are influenced by different aspects of pirlindole inhibitor structure

Robert M.G. Hynson[a], Johan Wouters[b], Rona R. Ramsay[a,*]

[a]*Centre for Biomolecular Sciences, University of St. Andrews, North Haugh, St. Andrews, Fife KY16 9ST, UK*
[b]*Institut de Recherches Microbiologiques Wiame, 1 Av. E.Gryzon, 1070 Brussels, Belgium*

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Abstract

Reversible inhibitors of monoamine oxidase A (MAO A) are used as antidepressants. The influence of inhibitors such as pirlindole (pyrazinocarbazole) on the redox co-factor (flavin adenine dinucleotide, FAD) is a key factor in the inhibition. The kinetic, spectral, and thermodynamic changes induced by four closely related pirlindole analogues have been determined to investigate their interaction with the FAD in the active site of MAO A. For a model of flavin–inhibitor stacking, more favourable association would be expected between lumiflavin and the flatter analogues with a double bond at N3, and indeed lower K_i values were found. However, the spectral changes induced by inhibitor binding to MAO A were 45% less for inhibitors with a double bond. Both in the absence and presence of the double bond, compounds with cyclohexyl at C8 induced 85% larger decrease in absorbance at 500 nm than did those with a methyl substituent. In contrast, the K_i values for the cyclohexyl compounds were lower, indicating greater affinity despite the lower perturbation of the flavin spectrum. All inhibitors stabilised the semiquinone of the FAD when MAO A was titrated with dithionite and prevented further reduction. These results indicate that the active site of MAO A is far more sensitive to structural variation than would be predicted by the simple flavin stacking model. Further, the independent changes in inhibitory potency and flavin perturbation preclude direct interaction with the flavin as a mode of binding and indicate that inhibitor–protein interactions must be important for inhibition.

Keywords: Flavin; Reduction; Structure–activity relationship; Difference spectra; Monoamine oxidase; Molecular orbital calculations

1. Introduction

MAO (EC 1.4.3.4) A and B catalyse the oxidative deamination of neuroactive amines in the central nervous system. Inhibition of MAO A increases catecholamine levels in the brain, making MAO A a target for antidepressant therapy. Potent reversible inhibitors selective for MAO A developed as antidepressants include moclobemide, befloxatone and pirlindole.

From the many structure–activity studies conducted over the last few decades, it is clear that to be a good MAO inhibitor, the compound should be lipophilic, have an aromatic ring and contain a nitrogen or sometimes a sulphur or oxygen atom close to the phenyl ring [1]. Differences between inhibitors selective for MAO A or B are subtle but structure–activity studies using inhibitors consistently show that the binding pocket in MAO A is slightly larger (reviewed in Refs. [1,2]). For substrate binding, increasing the size of the substituent on the *para* position of the aromatic ring of benzylamine or phenylethylamine improved the affinity for MAO A, the latter study showing increased affinity with increased van der Waals volume of the substrate [3,4].

Recent quantitative structure–activity relationship (QSAR) studies have sought to define the requirements for the optimum binding to the active site. Using the rigid, planar framework offered by indoles and pyrazinocarbazoles as a molecular ruler, Medvedev *et al.* defined the limiting dimensions for effective inhibition of MAO A as 13 Å × 7 Å × 4.4 Å [5]. In another study, an extensive series of coumarin derivatives were aligned to analyse the localised parameters influencing affinity within the active site [6]. The authors concluded that only the electrostatic

* Corresponding author. Tel.: +44-1334-463411; fax: +44-1334-463400.
E-mail address: rrr@st-and.ac.uk (R.R. Ramsay).

Abbreviations: SAR, structure–activity relationship; QSAR, quantitative structure–activity relationship; MAO A, monoamine oxidase A; FAD, flavin adenine dinucleotide; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital.

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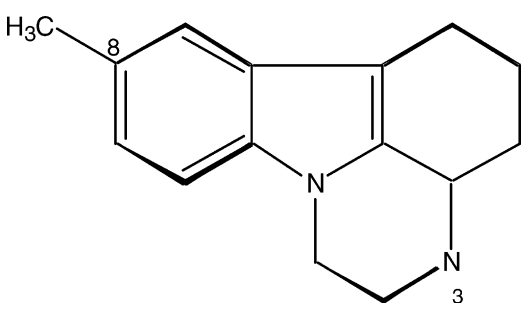
parameter influenced the interaction of these inhibitors with MAO A. In agreement with this, Cassels and co-workers [7], in a study of highest occupied molecular orbital (HOMO) energies and the charge distribution for each atom of the aromatic ring in phenylisopropylamine analogues, found that an electron-rich aromatic ring and higher HOMO levels increased inhibition.

In solution, association of riboflavin and compounds such as harmine has been observed and shown to be energetically favourable [8]. The electrostatic field surrounding the molecules plays a role in the recognition and positioning event and the topologies of complementary molecular orbitals will influence the charge transfer interaction that stabilises such stacking. Hypothetical models of the possible interactions of MAO inhibitors with the flavin moiety of the FAD co-factor of the enzyme have been constructed after calculation of the frontier orbital topologies and molecular electrostatic potential maps [9,10]. A study of flavin stacking with aromatic ligands in immobilised chemical models demonstrated the alteration of the redox properties of the flavin in response to electron donation from the ligand partner [11].

For purified MAO A, we have demonstrated that inhibitor binding induces changes in the spectrum of the enzyme [12], suggesting that stacking of the inhibitor with the flavin might occur in the enzyme. However, the crystal structure of MAO B showed that the ligand binding site is oriented between two tyrosines perpendicular to the flavin [13], so it is probable that the spectral changes result from alterations in the electronic environment of the flavin as an indirect result of inhibitor binding to the active site. Nevertheless, the spectral changes are specific for a given inhibitor [12,14]. Although the difference spectra for binding to MAO A of D-amphetamine, harmine, tetrindole, and befloxatone were qualitatively similar, the fine detail suggested that the electronic environment of the flavin was sensitive to the structural differences between the inhibitors [14].

Here, we investigate the kinetic and spectral properties of the interaction between MAO A and four inhibitors with very minimal chemical changes to probe their effect on enzyme properties and assess their interaction with the flavin. The parent nucleus of pirlindole (Scheme 1) is altered by the substituent on the aromatic ring to increase size and hydrophobicity and by the introduction of a double bond at N3 which alters the electronic distribution and planarity.



Scheme 1. Structure of pirlindole showing the atom numbering.

2. Materials and methods

2.1. Materials

Kynuramine, D-amphetamine and *n*-octyl-β-D-glucopyranoside were purchased from Sigma Chemical Company. The pirlindole analogues were the generous gift of Professor V.S. Velezheva, Nesmeyanov Institute, Russian Academy of Sciences, Russia.

Monoamine oxidase A (human liver form) was purified after expression in yeast [15,16]. The enzyme was stored at −20° in a solution of 50 mM potassium phosphate, pH 7.2, 0.8% *n*-octyl-β-D-glucopyranoside, 1.5 mM dithiothreitol, 0.5 mM D-amphetamine, and 50% glycerol. Before use, dithiothreitol, D-amphetamine, and glycerol were removed by gel filtration in a spin column.

2.2. MAO A assay and spectral titrations

Initial rates of oxidation were measured at 30° in 50 mM potassium phosphate, pH 7.2, 0.05% Triton X-100. K_i values were determined using a fixed substrate concentration range (0.1–0.9 mM) at six inhibitor concentrations over a 10-fold range. Where the K_i value was very low, the enzyme concentration was decreased to 3 nM.

All spectra were recorded on a Shimadzu UV-2101PC spectrophotometer in an anaerobic cuvette containing MAO A in 50 mM potassium phosphate, pH 7.2, containing 0.02% Brij-35, 30 mM glucose, 1 U/ml glucose oxidase, and 24 U/ml catalase [14,17,18]. Inhibitor-induced spectral changes of MAO A are known to be fast [12] so spectra were recorded shortly after mixing and repositioning the cuvette when the absorbance had stabilised (approximately 2–3 min). For dithionite titrations, the mediator, methyl viologen (1 μM) was also present. Spectra were recorded 15 min after additions to allow redox equilibration.

2.3. Molecular modelling

Frontier orbital and molecular electrostatic potential calculations were made at the Restricted Hartree-Fock (RHF) level of electronic theory using the Gaussian 94 package on an IBM SP2 computer system. Calculations were performed using the 3-21G basis set in the LCAO expansion of the molecular orbitals. Structures and isocontours were viewed and manipulated in version 2 of the GopenMol programme (http://www.csc.fi/~laaksone/gopenmol/) [19].

For the modelling, the decision to ignore solvent effects for the inhibitor–lumiflavin stacking model was taken because the active site of MAO is known to be relatively hydrophobic. Any water present in the enzyme–inhibitor

complex would not behave as a continuum with solvent so that simple aqueous calculations (such as a water box) would not be appropriate. All calculations were done using the neutral (deprotonated) form of the inhibitor because pH studies have shown that it is the deprotonated form of substrates that bind to MAO [20].

3. Results

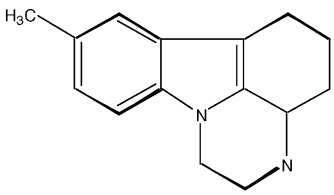
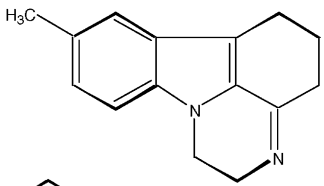
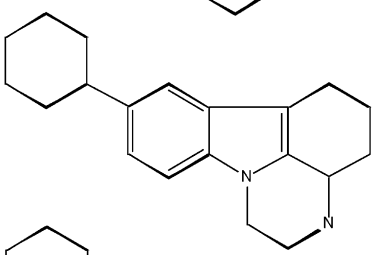
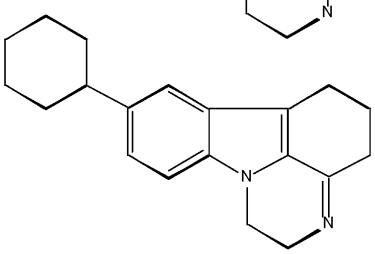
3.1. Influence of the structural alterations on the K_i values

The influence of the dimensions of the rigid, planar pyrazinocarbazole nucleus on the observed IC_{50} values for the inhibition of MAO A has been used to establish an active site mould for MAO A which has dimensions 13 Å × 7 Å × 4.4 Å [21]. To probe the influence on the flavin of inhibitors in the active site, we have used pirlindole analogues that show very different inhibitory properties despite having only minor chemical differences.

The K_i values for the four compounds in this study were determined kinetically (Table 1). The K_i is lower in each pair for the larger substituent at C8 (compare **3** with **1** and **4** with **2**) and for the unsaturated compound (compare **2** with **1** and **4** with **3**). The chemical changes increase the hydrophobicity in each pair as indicated by database CLogP values (https://www.hit2lead.com). In each pair, the K_i decreased with increased hydrophobicity, as might be expected for affinity to the hydrophobic active site cavity of MAO [1,13], but correlation of K_i with the overall hydrophobicity was not strict: **3** has a higher K_i (poorer binding) than **2** despite greater *C* log *P* (4.92 for **3** vs. 3.65 for **2**). Other factors must also contribute to the affinity.

The pirlindole analogue, tetrindole, which has a cyclohexyl group on C8 (8-cyclohexyl-pyrazinocarbazole, structure **3** in Table 1), fits the maximum size of the QSAR mould proposed by Medvedev and co-workers [21], so its position in the active site will be limited by steric hindrance. The analogue, mazindole, which has a methyl at C8 instead of the cyclohexyl ring (8-methyl-pyrazinocarbazole, structure **1** in Table 1) is smaller and so may have

Table 1
Structures of the inhibitors and their effects on MAO A

	Structure	K_i (μM)		Extinction coefficient at 500 nm (M^{-1} cm^{-1})
		Initial	Preincubated[a]	
1		0.264	0.039	1089
2		0.0147	0.0043	587
3		0.05	0.0084	1988
4		0.009	0.0028	1086

[a] Inhibitor and enzyme were incubated at 30° for 5 min before the addition of substrate.

some room for positional adjustment. Both compounds are effective inhibitors in the sub-micromolar range (Table 1). The K_i value for **3** is 5-fold less than that for **1** indicating the significant contribution of the cyclohexyl group to binding. After 5 min pre-incubation of the enzyme with the inhibitors, the K_i values are approximately 6-fold lower than without preincubation and now in the nanomolar range but the differential between **1** and **3** is maintained, indicating that the increased efficacy as a result of pre-incubation is independent of the structure of the ligand. Despite the time-dependent increase in affinity, the inhibition remains fully reversible. A similar time-dependent increase in affinity was observed for β-carboline inhibitors [12], so the effect may be due to a conformational change in the protein after ligand binding.

When a double bond is introduced at N3, giving a slightly flatter ring and altered electronic distribution (see below), the initial K_i values decreased by 17-fold for the methyl derivative **2** (compared to **1**) and 5-fold for the cyclohexyl derivative **4** (compared to **2**) (Table 1). After 5 min incubation of the enzyme with the double bond inhibitors, the K_i values are approximately 3-fold lower than for the saturated compounds (Table 1). After pre-incubation, compounds **2** and **4** are extremely effective inhibitors of MAO A with efficacy in the nanomolar range. As for **1** and **3**, the differential between **2** and **4** is the same after 5 min preincubation as it was without preincubation.

3.2. Structure influences the spectral changes induced by inhibitor binding

The binding of inhibitors to MAO A induces spectral changes specific to the inhibitor bound [12,14]. The four pirlindole inhibitors used here demonstrate the sensitivity of the MAO A spectrum to the structure of the bound inhibitor (Fig. 1). All the inhibitors induce a decrease in the absorbance at 500 nm but the decreases below 500 nm are highly individual. With inhibitor **3** (Fig. 1C), a broad negative peak is seen between 400 and 525 nm whereas with **1** (Fig. 1A) the decrease in absorbance runs from 450 to 520 nm. When the double bond is introduced, the two minima are more clearly defined as seen in the difference spectra with **2** and **4** (Fig. 1B and D). For the methyl derivatives (**1** and **2**), which are presumed to have a looser fit in the active site, the relative values for the minima at

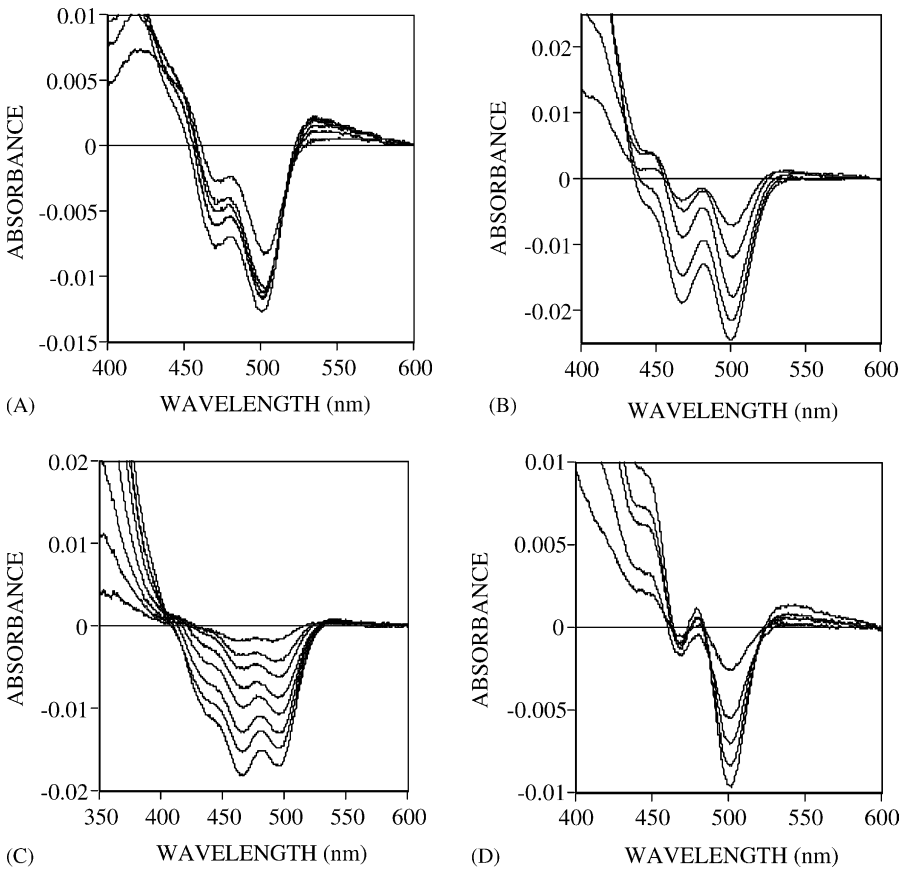


Fig. 1. Difference spectra for the changes induced by inhibitor binding to MAO A. Aliquots of inhibitor were added to MAO A under anaerobic conditions. Spectra were recorded 3 min after mixing. The original spectrum in the absence of inhibitor was subtracted from that of the enzyme–inhibitor complex to obtain the difference spectra shown. (A) MAO A (12.2 μM) + **1** (29.5–342.4 μM); (B) MAO A (14.3 μM) + **2** (4.9–83.3 μM); (C) MAO A (10.2 μM) + **3** (6.0–152.7 μM); (D) MAO A (11.0 μM) + **4** (2.5–15.3 μM). Each addition of inhibitor induced a greater difference from the zero line until saturation which is indicated in the last line after which no further change was observed.

500 and 460 nm do not change (compare Fig. 1A and B). For the larger, unsaturated cyclohexyl derivative (**4**), there is virtually no absorbance change at 460 nm on binding (Fig. 1D), in contrast to the dominant 460 nm minimum with the parent inhibitor **3** (Fig. 1C).

The extinction coefficients for the decrease in absorbance at 500 nm were used to quantify the differences (Table 1). The coefficients for the cyclohexyl inhibitors **3** and **4** are 82% higher than those for the methyl derivatives **1** and **2**, correlating with greater affinity (observed as lower K_i) for the cyclohexyl compounds. With either C8 substituent, the introduction of a double bond results in a smaller change at 500 nm. The extinction coefficient for the decrease at 500 nm induced in the MAO A spectrum by the unsaturated compound is about 55% of that for the dihydro compound despite the lower K_i (Table 1).

3.3. Influence of inhibitor on redox properties of MAO A

Changes in the flavin spectrum reflect alterations in the environment of the flavin and the environment of the flavin strongly influences its redox properties. In a model system, stacking interactions have been shown to alter the redox properties of flavin [11]. The complex of MAO A with D-amphetamine gave the semiquinone form after reduction by dithionite but could not be further reduced [18]. Also, the reduced enzyme–amphetamine complex was not reoxidised in the presence of oxygen [22].

Reductive titrations of the pirlindole–MAO A complexes are shown in Fig. 2. As with amphetamine [18], these complexes gave completely different results from the unliganded enzyme. The semiquinone spectrum was produced after addition of two electron equivalents of dithionite in the titration of the MAO A with inhibitor **1** or **3** (Fig. 2). No further reduction could be achieved even after overnight incubation in the presence of a 50-fold excess of dithionite over enzyme. The spectral changes for reduction are the same for all the inhibitors (including **2** and **4**, data not shown) and very similar to those for the MAO A–amphetamine complex [14] indicating that the alteration of the flavin redox properties is dependent on the presence of the inhibitor but not on its structure.

3.4. Molecular modelling

In previous work, interactions between planar heterocycles and lumiflavin have been modelled based on quantum mechanical calculations [9]. These results implied that MAO inhibitors such as harmine stack well with flavin. Inhibitors **1**–**4** were modelled in the same way to determine whether the factors that influence the stabilisation of stacking in the model system could explain the differences in K_i values and spectral changes that we observed in MAO A. The factors investigated were the electrostatic field that plays a role in recognition and molecular orbital topology that contributes to charge transfer.

Isocontour maps (Fig. 3) were prepared in version 2 of the GopenMol programme [19] using the outputs of the Gaussian 94 calculations. The frontier orbital topologies for lumiflavin (lowest unoccupied molecular orbital (LUMO), shown in blue) and for the inhibitors **1** and **2** (HOMO, shown in green) are shown in Fig. 3A (top row). The patterns of iso-electron density of the HOMO for the two inhibitors (and also for **3** and **4**, not shown) are very similar. This indicates that the solution model for flavin–inhibitor stacking stabilised by charge transfer will not differentiate these four inhibitors and so cannot explain the different spectral changes induced in MAO A by each of those inhibitors. Similarly, the distribution of the molecular electrostatic potential (MEP) (negative areas shown in red in Fig. 3B) is only slightly affected by the introduction of the double bond. Clearly the molecular orbital calculations can not distinguish between these two molecules to an extent that would alter the thermodynamic advantage of stacking in the model system.

The bottom row of Fig. 3 shows the difference in shape between the parent compound **1**, for which the crystal

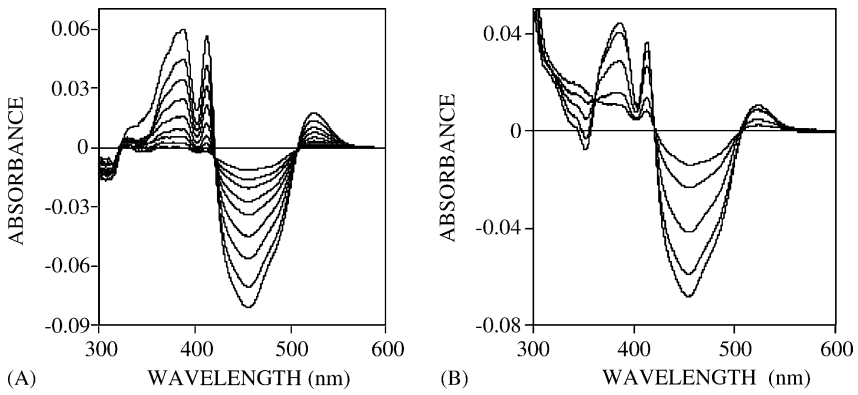


Fig. 2. Difference spectra for the reduction of inhibitor–MAO A complexes. Spectra were recorded 15 min after each addition of dithionite as follows: (A) MAO A (12.2 μM) + **1** (1892 μM) + dithionite (1.29–12.7 μM); (B) MAO A (10.2 μM) + **3** (180 μM) + dithionite (9.33–27.7 μM). The absorbance decreases at 456 nm with each addition and increases at 410 and 385 nm.

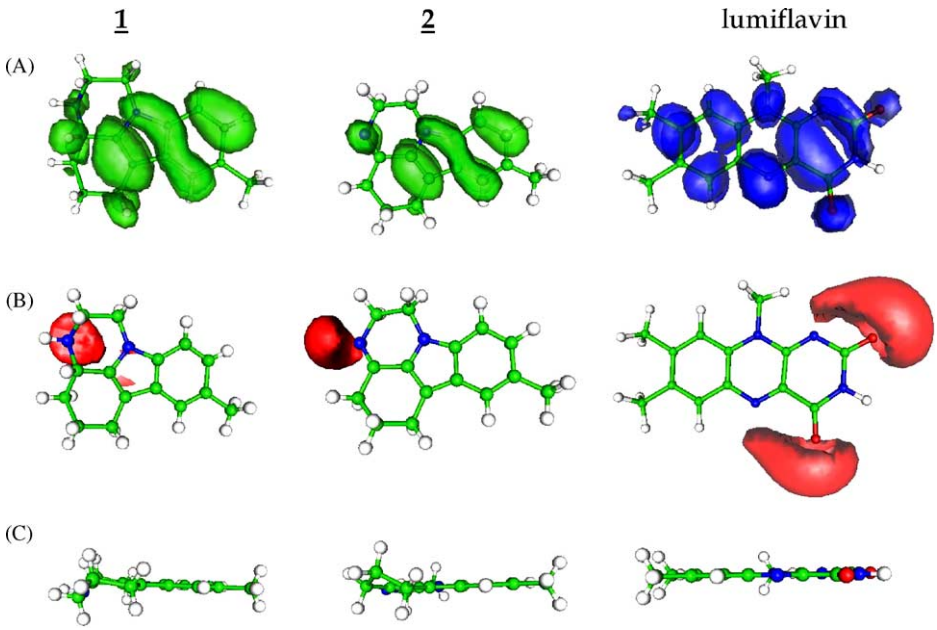


Fig. 3. Molecular modelling of the frontier orbital topology, molecular electrostatic potential, and shape of the pirlindole analogues. Top row: frontier orbital topology for the HOMO of inhibitors **1** and **2** (green) and for the LUMO of lumiflavin (blue). Middle row: MEP of the inhibitors **1** and **2**, and of lumiflavin, showing negative areas in red. Bottom row: side view of the energy minimised structures for inhibitors **1** and **2** and for lumiflavin. The atom colours are green for carbon, white for hydrogen, blue for nitrogen, and red for oxygen.

structure is known[1], and the analogue **2** with the double bond. This is the only difference revealed by the theoretical work that might be sufficiently different for these similar compounds to influence their stacking with lumiflavin. The steric difference offers the possibility of better stacking in the model system between the more planar **2** and the very flat lumiflavin ring (shown in Fig. 3, bottom panel, right), consistent with the lower K_i value found with the enzyme (Table 1). However, better stacking would also predict a stronger effect on the flavin spectrum but this is not found for the enzyme–inhibitor interaction (Table 1).

The conformation of the cyclohexyl moiety in compounds **3** and **4** may also play a role in the binding. The conformations presented in Fig. 3 correspond to the energy minimised structures. No calculations were done to analyse other possible conformations of the cyclohexyl ring as this was outside the scope of the present study which focuses on the steric and electrostatic properties of the pirlindole nucleus, the part that would stack with lumiflavin in the model. The computed steric (HOMO, Fig. 3A) and electrostatic (MEP, Fig. 3B) properties should not be influenced by the conformation of the cyclohexyl ring. The importance of the cyclohexyl group for interaction with a hydrophobic pocket in the enzyme is apparent from the much improved inhibition compared with the methyl analogues and this point should be studied further. Detailed analysis of the conformations of C8 substituents could be performed in conjunction with structural analysis of the binding site of the protein and docking simulations.

[1] J. Wouters, unpublished.

4. Discussion

SAR and QSAR studies have focused on identifying the size and functional groups for optimum binding to the active site of either MAO A or B [1,2,5,23]. The outcome of these studies gives a picture of a large, relatively hydrophobic binding site with a few polar interactions and steric limitations specific to either A or B. The recent publication of the structure of MAO B confirms this picture [13]. In the structure of MAO B covalently inactivated by pargyline, the active site is a flat cavity, about 20 Å long, oriented at approximately right angles to the pyrimidine ring of the flavin. How reversible inhibitors fit this site is still unknown.

For MAO A, the reversible pirlindole inhibitors have been well characterised by SAR and used to establish dimensions of the active site cavity in MAO A [21,24]. The results are consistent with other SAR studies on MAO A and B and compatible with the structure for MAO B [13]. In the present work, very minor alterations in the pirlindole structure have been used to reveal details of the inhibitor interaction with the active site cavity and how that interaction alters the properties of the flavin in MAO A. The results also test whether flavin–inhibitor stacking can be used as a model for the interaction of these inhibitors with MAO A.

The factors altered in the structurally homologous inhibitors used here are steric (both size and shape) and electrostatic (electron density and molecular electrostatic potential). Both the introduction of a double bond and the increase in the size of the C8 substituent increased the overall hydrophobicity, but no general trend was observed for the four compounds used here. The larger cyclohexyl

compounds (**3** and **4**), both with a length of 12.86 Å [25], are at the limit of the permitted size and so can be envisaged as having no room to move in the active site cavity when modifications result in unfavourable steric or electronic constraints. The smaller methyl substituent at C8 decreased the overall length to 9.75 Å [25]. The 5-fold lower K_i for **3** compared to **1** (Table 1) could result from the larger van der Waals or hydrophobic interaction between the protein and the cyclohexyl group at C8 compared to the methyl group. At least part of the increase in affinity could be due to greater hydrophobicity, but the greater (9-fold) decrease in K_i for a smaller increase in hydrophobicity that accompanies the introduction of the double bond, suggests that additional factors much be considered.

When the electrostatic factors were examined by molecular orbital calculations, the two parent compounds (**1** and **3**) were found to have almost identical frontier orbital topologies and molecular electrostatic potential maps. The 5-fold lower K_i for **3** compared to **1** thus does not arise from electrostatic interactions or charge transfer binding. However, the difference in K_i values is accompanied by a difference in extinction coefficient for the decrease in absorbance at 500 nm after inhibitor binding. The tighter binding of **3** induces a larger change (1988 M^{-1} cm^{-1}) than does **1** (1089 M^{-1} cm^{-1}) (Table 1). Given the similarity of the molecular orbital and electrostatic parameters (Fig. 3), this almost 2-fold difference in the spectral response of the flavin must arise not from alterations in a putative stacking with the flavin but from inhibitor-induced change in the protein that alters the environment of the flavin in MAO A. Introduction of a double bond between N3 and C2 does slightly alter the electronic distribution of the electrons near the nitrogen N3 of **1** and **2** (Fig. 3, middle row). Although not sufficient to alter predicted stacking in the model, it could be indicative of different geometries for potential hydrogen bonds between this atom and active site groups in the protein.

Despite the more effective inhibition of MAO A by the unsaturated member of each pair, the extinction coefficients at 500 nm for the spectral changes are lower than for the saturated analogues (Table 1). The decrease in extinction coefficient is the same in each pair (45% lower for the unsaturated derivative) whereas the K_i value for the analogue with methyl at C8 drops over 10-fold (compare **1** and **2**) but with cyclohexyl at C8, the K_i is decreased only 5-fold (compare **2** and **4**). The lack of correlation suggests that direct inhibitor–flavin interaction that should induce a spectral change related to the efficacy of the inhibition is not the explanation for the inhibitory efficacy.

Considering now the last of the differences between each pair of inhibitors, namely shape. The introduction of a double bond alters the planarity of the nitrogen-containing ring. In compounds **1** and **3**, the N3 is clearly displaced out of the plane of the indole part of the molecule in the energy-minimised structure (Fig. 3C, bottom row; [10]). When a double bond is introduced at N3, conjugation to the indole ring system moves N3 into the same plane, resulting in a much flatter molecule. In its energy-minimised model, fully oxidised lumiflavin (Fig. 3C, bottom row, right) is completely planar, consistent with crystallographic studies [26,27]. In theory, a flatter inhibitor molecule should have improved contacts with flavin in the model system. Although, the shape of the oxidised flavin in the active site of MAO A is not yet known, the discussion above has already concluded that stacking with the flavin in MAO A is not consistent with the lack of correlation between the changes in the K_i values and the changes in the spectra. With MAO A, the flatter molecule of each pair gave a lower K_i value (Table 1), indicating that shape is important for binding. Whether this is a requirement for optimal fit in the active site cavity rather than for flavin stacking can not be determined. However, the flatter inhibitors (**2** or **4**) did induce less absorbance change in MAO A, suggesting that the improved inhibitor–enzyme interaction caused less perturbation of the flavin moiety, rather than more perturbation as predicted by the model. If it is the fit of the inhibitor to a flat cavity in the protein (as seen in the crystal structure of MAO B [13]) that determines binding, then the data can be rationalised. The more planar inhibitors (**2** and **4**) might permit optimal Van der Waals interactions in the active site consistent with their low K_i values, and, similarly, the greater van der Waals volume and hydrophobicity of the cyclohexyl group in **4** over the methyl group in **2** is compatible with the lower K_i for **4**. The spectral differences shown here suggest that shape is important in determining flavin perturbation in MAO A. The less planar compounds (**1** and **3**) might induce strain in the active site that could result in alterations in the flavin environment.

To summarise, the data show that the steric factor altered by changing CH_3 to cyclohexyl (increased van der Waals interaction) decreased K_i and increased the effect on flavin; but when the shape of the central four-ring nucleus is flattened by the introduction of the double bond, the K_i and spectral change both decrease (Table 1). Molecular orbital calculations for a flavin–inhibitor stacking model predict that affinity and electronic interaction are directly related and would not vary much within this set of inhibitors (Fig. 3). This model, therefore, does not reflect the situation in the active site. However, shape and, thus, steric effects in relation in the active site, are critical for optimum inhibitory effect. This agrees with other SAR studies, for example, with substrates [4]. It is possible that the spectral perturbations reported here and previously for other inhibitors [12,14] arise from conformational changes in the active site as a result of inhibitor binding rather than from direct stacking as predicted by the models based on the events in solution.

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