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Development of selective and reversible pyrazoline based MAO-B inhibitors: Virtual screening, synthesis and biological evaluation

Nibha Mishra *, D. Sasmal

Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, Ranchi 835 215, India

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ABSTRACT

In an effort to develop selective MAO (monoamine oxidase) B inhibitors, structure based virtual screening was initiated on an in-house library. Top 10 HITS were synthesized and evaluated for MAO (A and B) inhibitory activity, both against human and rat enzymes. All the compounds were found selective, reversible and active in nM range (100 times more potent than selegeline) towards MAO-B. Outstanding co-relation between predicted and experimental K_i values were observed.

Human monoamine oxidases A and B (MAO-A and MAO-B) modulate the intracellular levels of arylalkylamines such as dopamine and serotonin by catalyzing their oxidative deamination with the concomitant production of hydrogen peroxide.[1] Impairment in the catabolism of neurotransmitters and oxidative damage are important factors in the physiology of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases.[2] For this reason, MAO-B inhibition represents one of the strategies to alleviate symptoms of patients suffering from these pathological conditions. There have been considerable efforts in the last few years to develop reversible and selective MAO inhibitors to be used as neuroprotective agent. Selective inhibitors are also required to avoid unwanted side effect so called cheese effect.[3]

Recently from our laboratory we have reported[4–6] a few pyrazolines with selective and reversible MAO inhibitory property. However, majority of those reported compounds are selective MAO-A inhibitors. So far, we were unsuccessful in developing any promising selective MAO-B inhibitors. The current study is therefore, initiated with the objective to develop potent, selective and reversible MAO-B inhibitors. Our study is designed as follows:

1. A focused combinatorial library was constructed with the available synthetically feasible fragments.
2. The generated library of compound was virtually screened.
3. Top 10 hits were identified and synthesized.
4. The synthesized compounds were evaluated for MAO (A and B) inhibitory activity using both human and rat enzymes.

A focused combinatorial library of pyrazolines was enumerated with SmiLib. The library was constructed as follows: Twenty different acetophenones and 25 different benzaldehydes were initially considered, to generate $20 \times 25 = 500$ chalcones. These chalcones were connected with as many as 36 linkers in all possible combinations to generate $500 \times 36 = 18000$ molecules. Altogether we had $18000 + 500 = 18500$ molecules (Fig. 1). The generated smiles structures of the molecules were converted to sdf through SmiLib v 2.0 program.[7] They were then processed with the LigPrep program to assign protonation states appropriate for pH 7.0. Conformer generation was carried out with the MacroModel. Potentials were computed using the OPLS2005 force field. After these ligands were prepared they were saved individually in pdb format.

In our earlier studies we employed structure based docking with AUTODOCK 4.0 for analyzing the binding mode. One of the interesting observations from those reports was, an outstanding correlation was observed between the predicted and experimental activities. More interestingly, this correlation was equally good for both the isotypes (MAO-A and MAO-B). Therefore, we have a strong basis to select the same computational method for VS purpose in the current work.

For VS we have used AUTODOCK 4.0,[8] the entire VS process was automated by an in house program recently reported by our group.[9] Docking Parameters used, are as follows: Number of Genetic Algorithm (GA) runs: 10, Population size: 150, Maximum number of evaluation: 2500000, Maximum number of generation: 27000.

After structure based virtual screening top 10 HITS with superior selectivity towards MAO-B were identified. The structures of the identified HITS are given in Table 1. Surprisingly, all the identified HITS were 3,5-diaryl pyrazolines of 9-anthracene

* Corresponding author.
E-mail address: nmishra@bitmesra.ac.in (N. Mishra).

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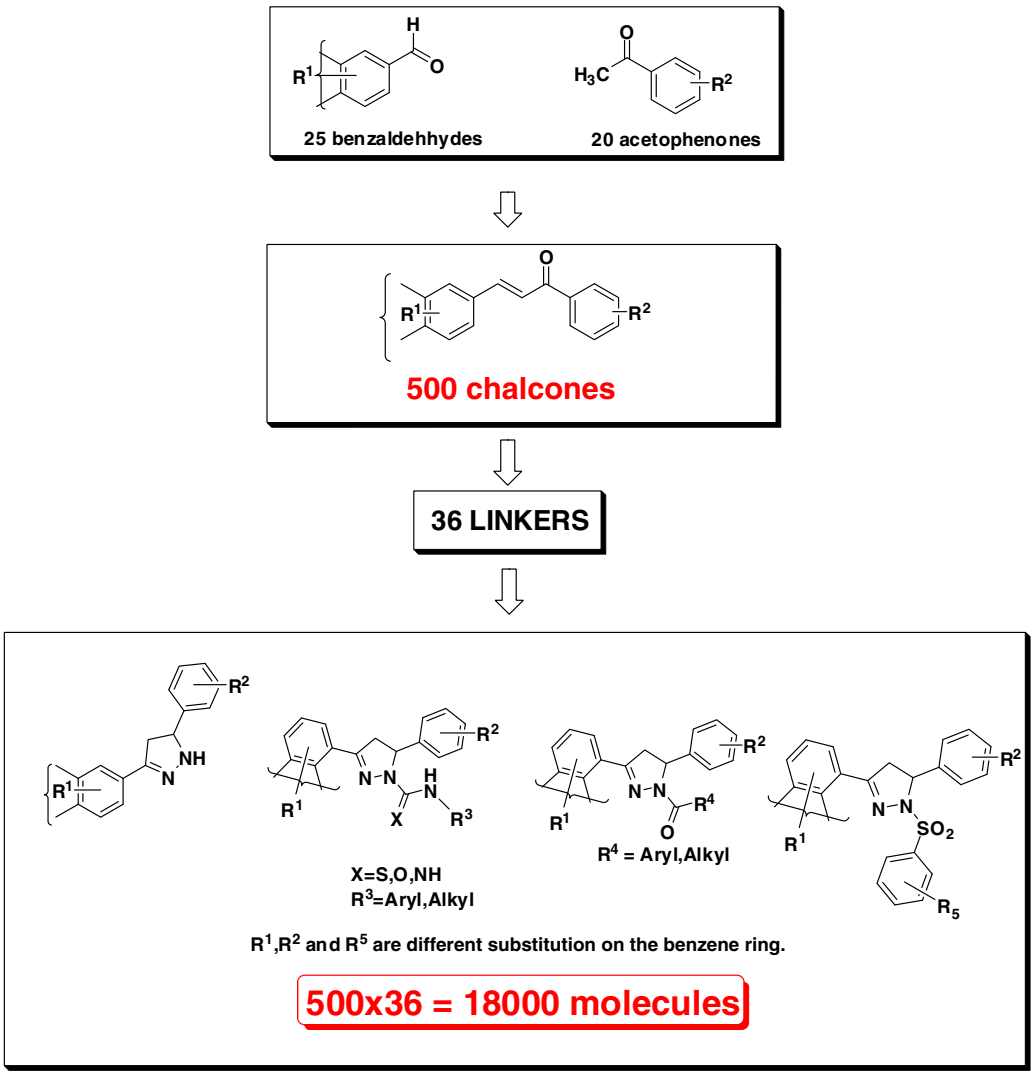


Figure 1. Construction of the in-house library for structure based virtual screening.

carboxaldehyde. In-order to gain more insight into their binding modes molecular docking results was scrupulously analyzed.

The active site architecture of MAO-B[10] is characterized by two large pockets. **Pocket1,** large aromatic cage made up of FAD, PHE 343 and four tyrosine residues TYR 188,189,435 and 398. **Pocket2,** a hydrophobic pocket characterized by LEU 171, TYR 326, PHE 168, ILE 198 and 199. All the top 10 HITS with a bulkier anthracene ring perfectly fit to **Pocket1** (Fig. 2a and b). The other aromatic ring also accommodates well in the other hydrophobic pocket, **Pocket2.** Surprisingly these top scoring molecules does not show any hydrogen bonding or π stacking interactions with the receptor. However, their docking scores are superior >−12.5 with predicted K_i in nanomolar range.

The identified HITS not only showed a superior affinity towards MAO-B, but also provided a high MAO-B/A selectivity index.

In order to understand this superior selectivity, structures of these two subtypes (MAO-A and B) along with their docked ligands were overlaid (Fig. 2c). This overlaid structure revealed a few important results. Human MAO-A and MAO-B share a high sequence similarity 72%, however, a significant structural difference is observed in the active site loop conformation, consisting of residues 210–216 in hMAO B.[10,11] This loop in the active site was found intrinsic for selectivity towards either of these isotypes. For MAO-A this loop deviates to almost 3 Å, making the active site more accommodative. For MAO-B, however, this loop is compressed, spanning more inside the active site, making it sterically impossible for any other group to accommodate itself.

The identified HITS were synthesized and characterized.[12] The compounds were synthesized according to previously reported method,[4–6] shown in Scheme 1. Briefly chalcones **1–10** were prepared through Claisen–Schmidt condensation of different acetophenone with anthracene-9-carboxaldehyde. Compounds **11–20** were synthesized by the reaction of excess hydrazine hydrate (99%) with **1–10**, respectively in ethanol. Reactions were monitored by thin-layer chromatography on silica gel plates in either iodine or UV chambers. The intermediates were characterized by elemental analysis for CHN. In the elemental analysis, the observed values were within ±0.4% of the calculated values. Final compounds were characterized by ^{1}H NMR and FAB mass spectrometry (MS). The structure, physicochemical and spectral data of these analogs were given in Table 1.

The synthesized compounds were assayed for MAO (A and B) inhibitory activity using both rat and human enzymes. For assay employing rat enzymes, MAO was purified from the rat liver[13] and for assay involving human enzymes, recombinant human MAO-A and B were procured from Sigma–Aldrich Chemical Co.

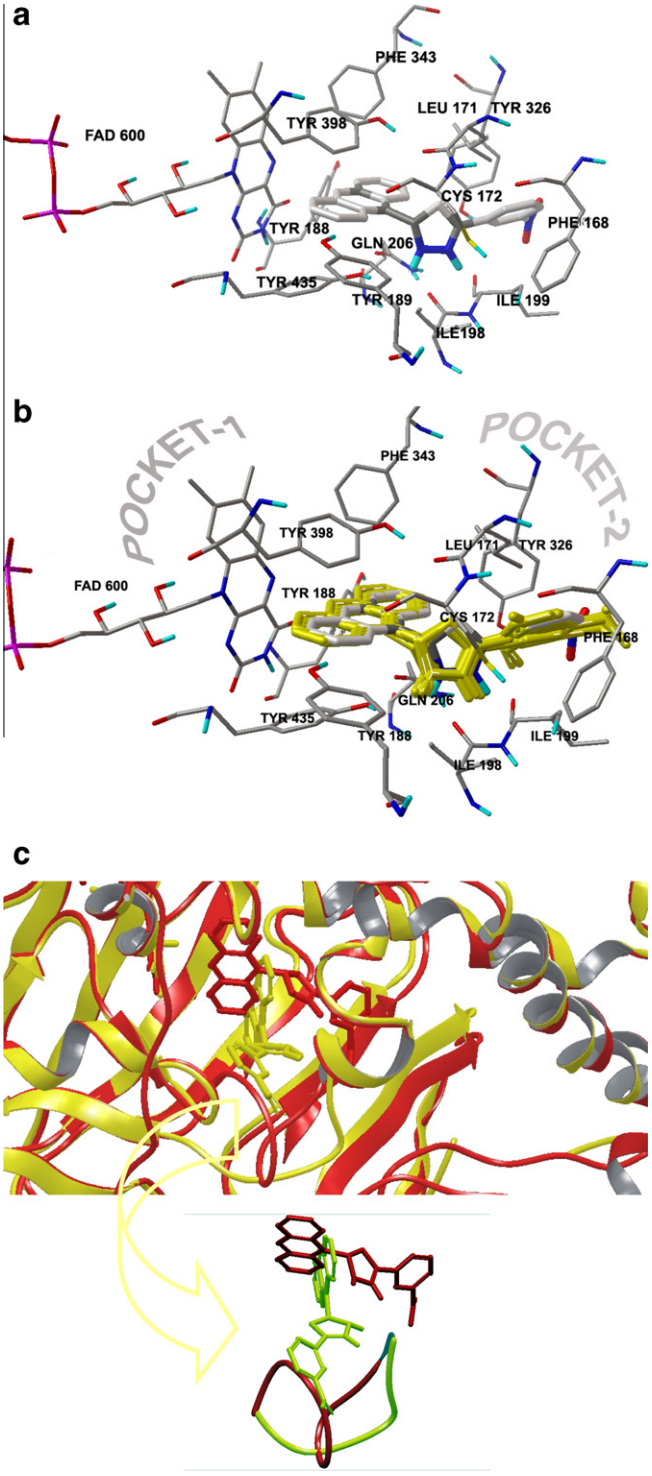
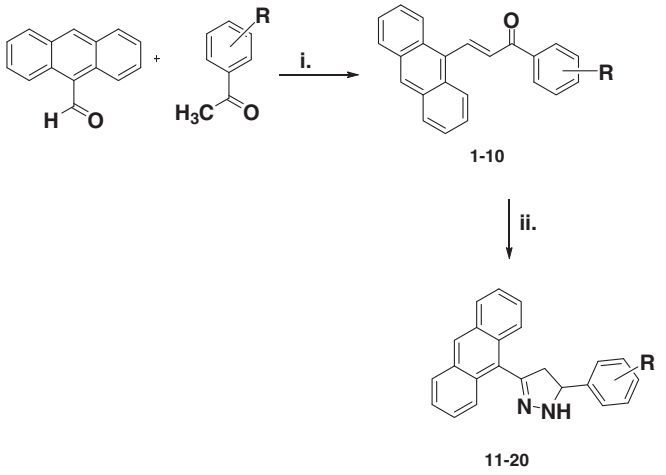


Figure 2. Structural screenshots of the newly synthesized compounds. (a) Most active compound **9** docked in MAO-B active site. (b) Top 10 HITS docked in the MAO-B active site. (c) MAO-A (yellow ribbons) and MAO-B (red ribbons) superimposed, most active MAO-B inhibitor 9 docked to the active site of both the subtypes.

The MAO activity was measured spectrophotometrically according to the method of Holt.[13] Briefly, assay mixture (vanillinic acid, 4-aminoantipyrine and peroxidase type II in potassium phosphate buffer (pH 7.6)) was pre-incubated with substrate *p*-tyramine before addition of enzyme. The reaction was initiated by the addition of enzymes and increase in absorbance was monitored at 498 nm at 37 °C for 60 min. Molar absorption coefficient of 4654 M^{-1} cm



Scheme 1. Synthetic scheme for the newly synthesized 3-(anthracene-9-yl)-5-aryl pyrazolines Reagents and conditions: (i) EtOH, NaOH (60%), 0 °C, stirring 2 h, kept at room temp for 2–4 days; (ii) EtOH, hydrazine hydrate (99%), reflux 1–3 h.

$^{-1}$ was used to calculate the initial velocity of the reaction. Results were expressed as nmol h^{-1} mg^{-1}. For selective measurement of rat MAO-A and MAO-B activities, homogenates were incubated with the substrate *p*-tyramine following the inhibition of one of the MAO isoform with selective inhibitors. Human MAO-A and MAO-B were procured as pure isoforms thus no pre-incubation with selective inhibitor was required. The compounds were dissolved in DMSO and used in the concentration range of 1–1000 μM. The compounds were then incubated with purified MAO at 37 °C for 0–60 min prior to adding to the assay mixture. Reversibility of the inhibition of MAO by these compounds was

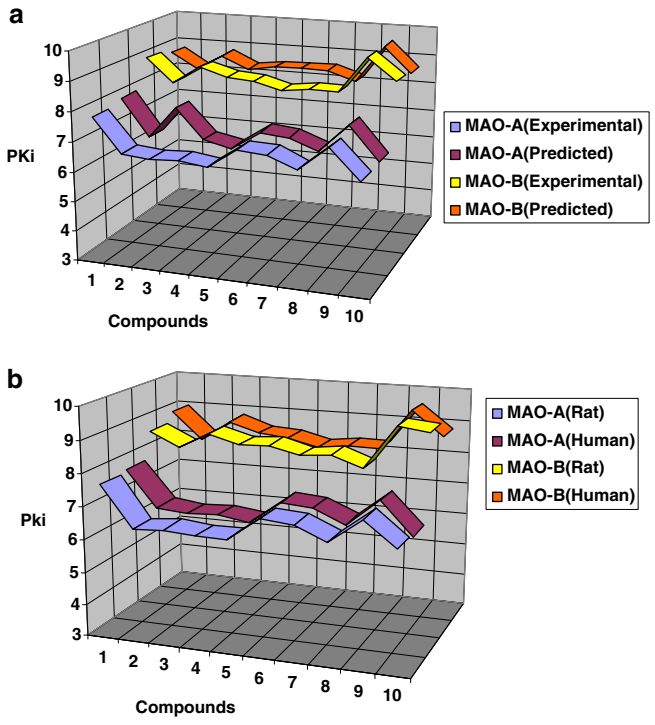
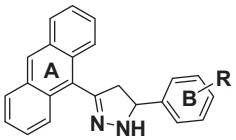


Figure 3. (a) Plot comparing the experimental and predicted pK_i values against both the isoforms of MAO. (b) Plot comparing the pK_i values against both the isoforms of MAO for human and rat enzymes. *pK_i is calculated as negative logarithm of K_i expressed in moles.

Table 1
Structure, physicochemical and spectral properties of the newly synthesized pyrazoline derivatives



Compound	R	MF	MW	Y (%)	MP (°C)	^{1}H NMR (δ ppm)	FAB-MS (m/z)
1	4-NO_2	$C_{23}H_{17}N_3O_2$	367.4	76	178.5	3.38 (H_A, t), 3.87 (H_M, dd, J_{MA} = 17.05 Hz, J_{MX} = 11.4 Hz), 6.32 (H_X, dd, J_{MX} = 9.6 Hz), 7.03–8.6 (13H, m, Ar-H), 10.10 (1H, s, –NH–)	368 (M+H)
2	4-OH	$C_{23}H_{18}N_2O$	338.4	72	148.3	3.63 (H_A, t), 3.79 (H_M, dd, J_{MA} = 16.95 Hz, J_{MX} = 11.4 Hz), 6.30 (H_X, dd, J_{MX} = 9.5 Hz), 6.83–8.77 (13H, m, Ar-H), 9.99 (1H, s, Ar-OH) 10.12 (1H, s, –NH–)	337 (M+H)
3	4-Cl	$C_{23}H_{17}N_2Cl$	356.85	90	151.2	3.71 (H_A, t), 3.77 (H_M, dd, J_{MA} = 16.85 Hz, J_{MX} = 11.4 Hz), 5.87 (H_X, dd, J_{MX} = 9.6 Hz), 7.03–8.6 (13H, m, Ar-H), 9.95 (1H, s, –NH–)	357 (M+H)
4	4-CH_3	$C_{24}H_{20}N_2$	336.43	80	158.8	2.34 (3H, –CH_3, s) 3.66 (H_A, t), 3.71 (H_M, dd, J_{MA} = 16.65 Hz, J_{MX} = 12.4 Hz), 6.39 (H_X, dd, J_{MX} = 9.7 Hz), 7.00–8.74 (13H, m, Ar-H), 8.95 (1H, s, –NH–)	337 (M+H)
5	–H	$C_{23}H_{18}N_2$	332.4	77	125.6	3.67 (H_A, t), 3.79 (H_M, dd, J_{MA} = 16.66 Hz, J_{MX} = 11.7 Hz), 5.96 (H_X, dd, J_{MX} = 9.5 Hz), 7.06–8.75 (14H, m, Ar-H), 10.23 (1H, s, –NH–)	333 (M+H)
6	2-OH	$C_{23}H_{18}N_2O$	338.4	81	130.8	3.55 (H_A, t), 3.91 (H_M, dd, J_{MA} = 16.92 Hz, J_{MX} = 11.34 Hz), 6.16 (H_X, dd, J_{MX} = 10.03 Hz), 7.26–8.55 (13H, m, Ar-H), 9.05 (1H, s, Ar-OH), 10.01 (1H, s, Ar-OH), 11.13 (1H, s, –NH–)	339 (M+H)
7	2,4 Di-OH	$C_{23}H_{19}N_2O_2$	354.4	73	132.6	3.45 (H_A, t), 3.95 (H_M, dd, J_{MA} = 16.85 Hz, J_{MX} = 11.4 Hz), 6.16 (H_X, dd, J_{MX} = 9.6 Hz), 7.26–8.75 (12H, m, Ar-H), 9.03 (1H, s, Ar-OH), 10.33 (1H, s, –NH–)	355 (M+H)
8	Pyridine-2yl	$C_{22}H_{17}N_3$	324.39	70	100.0	3.50 (H_A, t), 3.96 (H_M, dd, J_{MA} = 16.66 Hz, J_{MX} = 11.7 Hz), 5.97 (H_X, dd, J_{MX} = 9.41 Hz), 7.05–8.85 (13H, m, Ar-H), 10.53 (1H, s, –NH–)	325 (M+H)
9	3-NO_2	$C_{23}H_{17}N_3O_2$	367.4	85	109.7	3.41 (H_A, t), 3.77 (H_M, dd, J_{MA} = 17.03 Hz, J_{MX} = 11.54 Hz), 6.38 (H_X, dd, J_{MX} = 9.9 Hz), 7.03–8.72 (13H, m, Ar-H), 10.14 (1H, s, –NH–)	368 (M+H)
10	4-OCH_3	$C_{24}H_{20}N_2O$	352.43	85	107.4	2.54 (3H, –OCH_3, s) 3.76 (H_A, t), 3.87 (H_M, dd, J_{MA} = 16.84 Hz, J_{MX} = 11.23 Hz), 6.42 (H_X, dd, J_{MX} = 10.07 Hz), 7.04–8.81 (13H, m, Ar-H), 9.98 (1H, s, –NH–)	353 (M+H)

Table 2
Calculated and experimental K_i values corresponding to the inhibition of MAO isoforms by the newly synthesized pyrazoline derivatives

Code	Experimental[a] (K_i)				Calculated[b] (K_i)		Inhibition type	Reversibility	MAO inhibitory selectivity
	MAO-A (nM)		MAO-B (nM)		MAO-A (nM)	MAO-B (nM)			
	Rat	Human	Rat	Human					
1	20.14	17.08	2.30	1.15	11.12	1.71	Competitive	Reversible	Selective for MAO-B
2	385.26	225.13	6.75	7.51	203.19	5.97	Competitive	Reversible	Selective for MAO-B
3	320.13	281.19	2.10	1.80	17.30	1.72	Competitive	Reversible	Selective for MAO-B
4	389.49	250.27	3.80	3.56	167.08	4.45	Competitive	Reversible	Selective for MAO-B
5	396.57	332.41	3.43	4.21	269.97	3.31	Competitive	Reversible	Selective for MAO-B
6	77.61	69.91	5.91	7.35	63.26	3.10	Competitive	Reversible	Selective for MAO-B
7	95.65	81.54	4.77	5.54	77.49	3.18	Competitive	Reversible	Selective for MAO-B
8	241.54	205.45	9.91	5.75	183.90	6.41	Competitive	Reversible	Selective for MAO-B
9	43.90	32.16	0.45	0.31	20.49	0.33	Competitive	Reversible	Selective for MAO-B
10	230.3	301.11	0.60	1.70	268.14	2.51	Competitive	Reversible	Selective for MAO-B
SEL	10566.0	67250.0	1350	1960	ND	ND	Competitive	Reversible	Selective for MAO-B

SEL-selegeline, ND-not done.
[a] Values were determined from the kinetic experiments in which *p*-tyramine (substrate) was used at 500 μM to measure MAO-A and 2.5 mM to measure MAO-B. Pargyline or clorgyline were added at 0.50 μM to determine the isoenzymes A and B. Newly synthesized compounds and the known inhibitors were preincubated with the homoganates for 60 min at 37 °C. Each value represents the mean ± SEM of three independent experiments.
[b] Values obtained through AUTODOCK program.

assessed by dialysis. Kinetic data for interaction of the enzyme with these compounds were determined using Microsoft Excel package program. IC_{50} values were determined from plots of residual activity percentage, calculated in relation to a sample of the enzyme treated under the same conditions without inhibitor, versus inhibitor [I] concentration.

All the compounds were found as extremely potent and selective towards MAO-B, (Table 2) with at least 100 times more potent than the positive control selegiline. Compounds **9** and **10** were most promising amongst all, with K_i equal to 0.31 nM and 1.7 nM, respectively for human MAO-B, and 32.16 nM and 301.11 nM for MAO-A, respectively. Both the compounds **9** and **10** were >100 times more selective for MAO-B.

We have made some scrupulous analysis of the observed results. Primarily to check the congruence of the observed results with the predicted one and how much rat and human enzyme activity correlates. For analyzing the results, K_i values were converted to pK_i (negative logarithm of K_i expressed in per moles). These values were then plotted against each compound. In the first plot (Fig. 3a) experimental (human) and predicted values for both MAO-A and MAO-B were compared. Where as in the second (Fig. 3b), experimental values of human and rat enzymes (both MAO-A and MAO-B) was compared.

A superior almost perfect agreement (Fig. 3a) between the experimental and predicted values was obtained. The way, structure based docking with AUTODOCK corresponded the experimental K_i values is truly exceptional. We have been consistently reporting[4–6] and emphasizing on these outstanding correlations. These employed protocols were not only successful in identifying the resulting HITS through a reasonable VS procedure but also,

consolidated the model, giving us enough support, to be used in future as a query model.

Surprisingly, we have obtained a superior correlation (Fig. 3b) between the K_i values obtained from rat and human enzymes. Earlier reports from Son et al.,[11] suggested a close structural match between the human and rat enzymes. However, lot of support is still required to infer whether rat MAO enzyme can replace human enzymes in assays, which can be explored further in future.

From the current work, we have successfully employed a structure based VS protocol and identified 10 selective and extremely potent MAO-B inhibitors. We have made some significant inferences in the process, which can be summarized as follows:

1. HITS were successfully identified through VS with AUTODOCK 4.0. Superior correlation between experimental and predicted K_i values was observed.
2. All the synthesized molecules were reversible and selective inhibitors of MAO-B. They were active in nM range, almost 100 times more potent than selegiline.
3. A superior correlation was observed between the K_i values obtained from rat and human enzymes.

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12. General procedure for the synthesis of chalcones (**1**–**10**).
    To a solution of different acetophenone (0.01 M) and anthracene-9-carboxaldehyde (0.01 M) in ethanol (10 ml) was added aqueous solution of Sodium hydroxide (60%) drop wise with continuous stirring at 0 °C over a period of 2 h. The reaction mixture was kept at room temperature for about 48 h with occasional shaking. After 48 h it was poured into ice-cold water. In case hydroxy chalcone pH was adjusted to 2 using 6 N hydrochloric acid. The yellow precipitate obtained was filtered, washed, dried and recrystallized from dry methanol. The intermediates **1**–**10** were obtained.
    General procedure for the synthesis of 3-anthracene-5-aryl-4, 5-dihhydro-1*H*-pyrazole (**11**–**20**).
    Appropriate chalcone (**1**–**2**) was treated with 10 times excess of hydrazine hydrate (80%) in dry ethanol and refluxed for 3–6 h. The hot reaction mixture was then poured into ice-cold water. The solid separated out was filtered, washed, dried and recrystallized from ethanol to afford respective pyrazoline (**3**–**4**).
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