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Inhibition of monoamine oxidase–A activity in rat brain by synthetic hydrazines: Structure-activity relationship (SAR)

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Abstract
A series of hydrazine derivatives was synthesized in order to evaluate their monoamine oxidase A (MAO-A) inhibitory effects. MAO-A inhibitory activity of 4-tosyl benzoic acid carbohydrazide was quite potent, similarly to that of the corresponding 4-benzyloxy-benzoic acid carbohydrazide and its N-cyanoethylated derivative. Structural variations of these compounds, such as the replacement of the 4-substitutent, of the aromatic ring on which the carbohydrazide moiety is grafted, as well as cyclization of the hydrazide moiety in five- or six-membered rings caused either significant decline or complete loss of MAO inhibitory properties. The most active compound (4-tosyl benzoic acid carbohydrazide) was also subjected to the forced swim test, an animal model of depression, eliciting a marked reduction in immobility time in rats, without affecting the locomotor activity, implying that it possesses anti-depressant properties due to inhibition of MAO type-A.

Keywords: *Monoamine oxidase-A, rat brain, hydrazides, structure activity, MAO-A, inhibition*

Introduction

It is well established that monoamine oxidase [EC 1.4.3.4; monoamine: O_2 oxidoreductase (deaminating) (flavin containing); MAO] plays a significant role in the control of intracellular concentration of monoaminergic neurotransmitters or neuromodulators such as 5-hydroxytryptamine (5-HT), dopamine, noradrenaline (NA) and dietary amines. It is localized predominantly in the outer mitochondrial membrane of neurons, glial and other cells [1–3]. The enzyme exists in two isoforms, MAO-A and -B, which are identified by their inhibitor sensitivity and substrate selectivity. Monoamine oxidase-A and -B preferentially deaminate 5-HT and phenylethylamine, respectively. In the case of inhibitor sensitivity, MAO-A and -B forms are inhibited irreversibly by low concentrations of clorgyline and selegeline, respectively [4–7]. Currently, the therapeutic interest in MAO inhibitors falls into two major categories. MAO-A inhibitors have been used mostly for the treatment of mental disorders, particularly depression [8], while MAO-B inhibitors are useful for the treatment of Parkinson's disease [9].

In our preliminary studies a carbohydrazide derivative (**1**) showed potent inhibition of MAO-A [10]. In order to further enhance anti-depressant properties of such derivatives, a series of hydrazines of type (**2**–**6**), structural variants of the lead molecule (**1**)

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and some cyclic derivatives (**7**–**16**) were synthesized in a similar fashion as described earlier [10] and tested for their enzyme inhibitory properties. Monoamine oxidase inhibition was analyzed using rat brain mitochondrial preparation and 5-HT as an enzyme source and substrate, respectively. Additionally, *in vivo* experiments such as the forced swim test (FST) and locomotor test (LMT) were also conducted for compound **1** to confirm its anti-depressant properties as observed in *in vitro* experiments [10]. Phenelzine, a well-known anti-depressant agent, was used as a reference drug.

Material and methods

Animals and doses

Protocols used in the paper for the treatment of animals were approved by the ethical commission of our universities. Animals were kept under standard condition with normal light cycle (12 h), with free access to food and water. The NMRI mice and Wistar rats were bred at the animal house of H E J Research Institute of Chemistry, International Center of Chemical Sciences, University of Karachi, and housed in groups of 10 per cage.

Treatment was given in a volume of 5 ml/kg (rats) and 10 ml/kg (mice). For the MAO assay test compounds were solublized either in distilled water (d/w) or 10% DMSO in d/w, whereas, for *in vivo* experiments doses were prepared either in physiological saline (0.9% NaCl) or 10% DMSO in saline. Preliminary experiments (FST, and LMT) were conducted using saline as a vehicle for control animals. While test animals were treated with 10% DMSO in saline. Results obtained from the aforementioned studies showed a non-significant difference in the control and test values. Therefore, control animals were treated only with saline throughout the study.

Acute toxicity test

Either sex of mice (20–30 g) received intraperitoneal injection of saline (control) and compound **1** (5, 10, 15 and 19 mg/kg). Animal behavior was observed for 6 h and mortality, if any, was noted for up to a week [11].

Rat brain mitochondrial preparation

Adult Wistar rats of both sexes were sacrificed by rapid decapitation using a guillotine followed by quick removal of brains. The homogenate was prepared in 20 volumes of 0.32 M ice-cold sucrose using polytron ($8000\,\mathrm{min}^{-1} \times 8$ for 3 s). The homogenate was centrifuged at $1{,}000 \times g$ for 10 min at 4°C, and the supernatant obtained was further centrifuged at $17{,}000 \times g$ for 30 min. The pellet obtained was re-suspended in 10 volumes of sucrose and homogenized using polytron as described above. The brain mitochondrial preparation was used either for the determination of monoamine oxidase-A activity immediately or aliquoted and stored at −50°C till further use [12]. Protein concentrations were determined by the method of Lowry et al. [13] using bovine serum albumin as a standard.

Monoamine oxidase assay

Monoamine oxidase-A activity was determined by the fluorimetric method [14,15] using 5-hydroxytryptamine (500 μM) as a substrate. The mitochondrial preparation (20 μl) was pre-incubated either with buffer or test compound for 30 min at 37°C, followed by the addition of substrate (50 μl). Assay tubes were incubated for 20 min in a final reaction volume of 200 μl. An adrenaline/peroxidase system was used to form the fluorescent adrenolutine, the intensity of the fluorescence being determined using an emission wavelength 405 nm with an excitation wavelength 550 nm. Throughout the experiments hydrogen peroxide (2 n moles) was used as a standard that gave a fluorescence of 251 ± 3.5. The control enzyme activity was expressed as n moles of H_2O_2 formed h^{-1} mg^{-1} protein. The percentage inhibition of enzyme activity in the presence of the test compound was determined by comparing it with the control value. Clorgyline (an irreversible MAO-A inhibitor) at 0.1 μM caused complete enzyme inhibition, confirming the presence of MAO-A in the reaction mixture.

Forced swim test

Male rats (160–180 g) were intraperitoneally treated with saline (control) or phenelzine (4, 7, 10, 13, 16 and 19 mg/kg) or compound **1** (4, 7, 10 and 13 mg/kg) 1 h prior to the test session and the duration of immobility was recorded for a period of 5 min as described before [14,16,19]. The percentage reduction in the immobility time of test animals was calculated as compared to the control. For pre-test session, rats were placed individually in a swimming tank containing 15 cm of water at 25°C. Animals were allowed to swim for a period of 15 min. After that they were removed and blow-dried in a warm environment (30–32°C) for 15–20 min, before returning them to their respective cages.

Locomotor test

A group of five mice of either sex (20–25 g) were intraperitoneally treated with saline 1 h before the observations. Animals were placed in Optovarimax mionor 15 min prior to the observations for acclimatization. Ten-minute locomotor counts were recorded for a period of 1 h. The mean of 6 (10 min) readings

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was noted (control counts). After 24 h, these animals were treated either with phenelzine or compound **1** (10, 13, 16 and 19 mg/kg) and locomotor counts (test counts) were recorded as described above. Test counts at each dose were compared with its respective control [14].

Statistical analysis

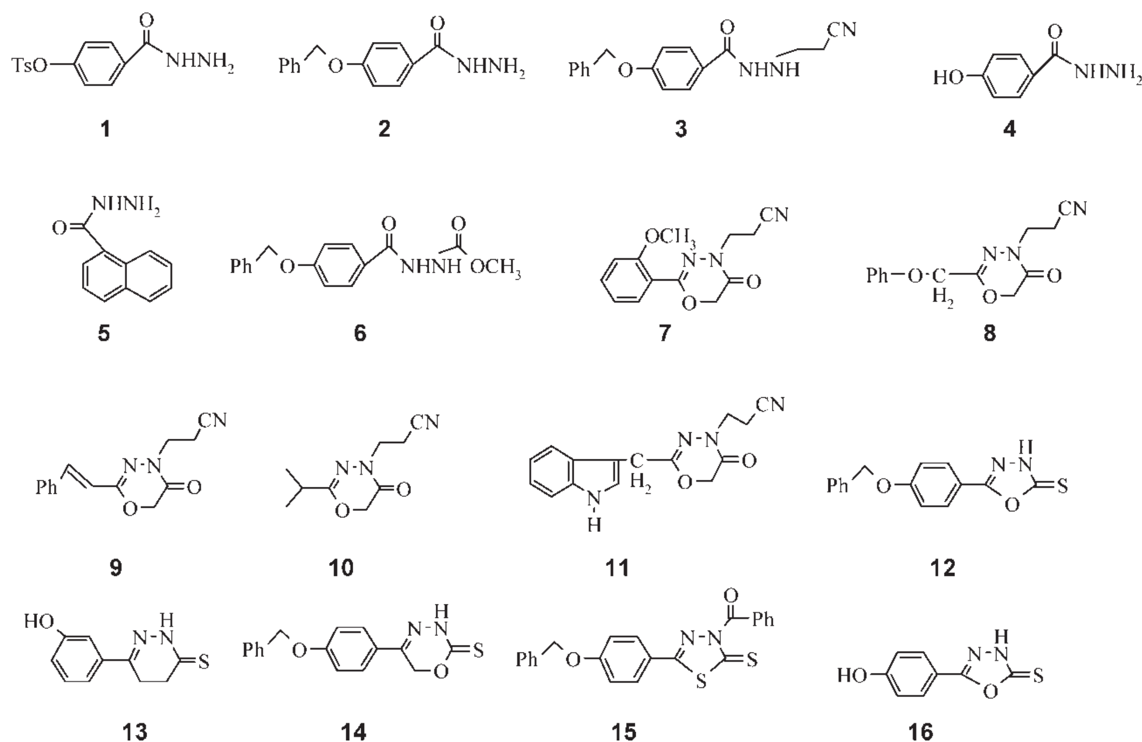
Untransformed data obtained from the forced swim test was analyzed by the Student's *t* test. However, the statistical significance of difference between the control and test locomotor counts was estimated by Student's paired *t* test [20].

Results and discussion

It is considered that depression occurs as a result of functional deficiency of monoamines (*viz* 5-HT and NA) at neuronal levels [21,22]. It is also established that MAO inhibitors (both non-selective and selective) are used for the treatment of depression. Particularly, the selective and reversible MAO-A inhibitors (such as moclobemide) are more effective anti-depressant [23–25] compared to non-selective agents.

monoamine oxidase-A activity was found to produce 19 ± 2.1 n moles of H_2O_2 h^{-1} mg^{-1} protein. This enzyme activity at 100 μM remained unchanged in the presence of hydrazides **4–6**, oxadiazines **7–11**, oxidiazoles **12–14** and thiadiazoles **15–16** indicating that these molecules are devoid of MAO-A inhibiting properties. However, at the same concentration hydrazides **2** and **3** showed a similar magnitude of enzyme inhibition at around 40% (see Tables I and II), thereby reflecting MAO-A inhibiting properties. These compounds appeared to be less potent (about 2.3-fold) as compared to phenelzine (a clinically effective and non-selective MAO inhibitor). Moclobemide (a clinically effective and selective MAO-A inhibitor) caused 26% enzyme inhibition at 100 μM [10] and was less potent than compounds **2** and **3** (Figure 1).

It has been mentioned previously that MAO inhibition is probably due to the presence of the tosyl group at position-4 of the benzene ring that is electron withdrawing in nature. As hypothesized earlier [10] that the presence of the tosyl group is responsible for enzyme inhibition, in the current investigation it was replaced by a benzyl group having electron-donating properties (compound **2**). Interestingly, a dramatic decline of 60% in MAO-A inhibition was evident



As compound **1** demonstrated complete inhibition of MAO-A at 100 μM [10] in an extension of our studies we screened compound **1** with respect to its SAR. In control samples (without test compound)

implying that indeed the tosyl group is crucial for inhibitory activity. On the other hand replacement of one of the hydrogens of N(2) with ethyl cyanide caused no further decline in activity of the molecule clearly

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Table I. Effect of compounds **1**–**16** on the inhibition of MAO-A activities. Samples (26 ± 2.8 mg/ml protein) were pre-incubated with the compound **1**–**16** for 20 min at 37°C before the addition of 5-hydroxytryptamine (500 μM). The inhibition of the enzyme activity is expressed as a percentage of activity relative to the control samples pre-incubated without inhibitor. Each compound was tested in duplicate or triplicate.

COMPOUND	% INHIBITION at 100 μM
1	100
2	43
3	39
4	0
5	0
6	0
7	0
8	0
9	0
10	0
11	0
12	0
13	0
14	0
15	0
16	0

reflecting the importance of the tosyl group for monoamine oxidase inhibition in this class of molecules. In the case of compounds **4**, **5** and **6**, which are structure variants of **1**, they were also devoid of MAO inhibition which advocates that the tosyl group is probably the most preferred moiety for binding at the active site of the enzyme thereby, preventing the deamination of 5-HT. Other derivatives of hydrazides *viz* oxadiazines (**7**–**11**), oxadiazoles (**12**–**14**) and thiadiazoles (**15**–**16**) showed no obvious interaction with the active site of the enzyme indicating the absence of MAO inhibitory activity was absent with these compounds. However, further studies are required to confirm this hypothesis as well as the nature of the inhibition (reversible or irreversible).

Considering that compound **1** at 100 μM was equipotent with phenelzine and 3.8-fold more potent than moclobemide encouraged us to analyze its effect in the *in vivo* system. Animal models of depression *e.g.* forced swim test are extensively used for evaluation of anti-depressant agents [16]. Results obtained from the forced swim test showed that the immobility time of control animals was 170s ± 2.1. Pretreatment of animals with either phenelzine (10–19 mg/kg) or compound **1** (4–13 mg/kg) produced a significant dose-dependent increase in the percentage reduction in immobility time but there was a significant difference in the magnitude of the reduction elicited by them. At 13 mg/kg, phenelzine caused only 38 ± 5.4% reduction in immobility time reflecting anti-depressant property whereas, at the same dose, compound **1** caused marked reduction of 87 ± 2.1%. The IC_{50} values for compound **1** (7 ± 1.8 mg/kg) was found to be 2.2-fold less than that of phenelzine confirming that it is a more potent MAO inhibitor than phenelzine. It has been reported earlier that clorgyline and moclobemide caused significant reduction in the immobility time with an IC_{50} values of 6 ± 0.53 [26] and 5 ± 0.65 mg/kg [14], respectively, that is similar to compound **1**. Thus the anti-depressant property residing in compound **1** can also be demonstrated in the *in vivo* model of depression (forced swim test) with potency equal to or slightly better than some of the commercially available anti-depressants.

Table II. Effect of phenelzine and compound **1** on percentage reduction in the immobility time of rats using the forced swim test.

Dose (mg/kg)	Phenelzine	Compound 1
4	0	19 ± 5.5*
7	1 ± 0.7$^{n.s.}$	49 ± 3.1***
10	20 ± 1.5**	70 ± 1.9***
13	38 ± 5.4***	87 ± 2.1***
16	50 ± 5.1***	N.D.
19	76 ± 4.2***	N.D.

Immobility time of control animals = 170 s ± 2.1; Number of control animals = 55, Animal per dose = 3–6; Values are mean percentage reduction in the immobility time ± SEM. Statistics were done on untransformed data. Asterisks indicate significant ($P < 0.01$*, $P < 0.05$** and $P < 0.001$***) percentage reduction in the immobility time, and n.s. represents non-significant differences compared to the control. N. D. = not done.

It is widely accepted that the locomotor test is a useful tool to distinguish the anti-depressant/psychostimulant or both properties of a test compound. The psychostimulants or locomotor activating compounds display positive effects in the anti-depressant test (*e.g.* FST) by virtue of a direct effect on activity [27,28]. In the present study phenelzine did not produce significant change in locomotor activity of mice at 10 and 13 mg/kg. On the contrary, a corresponding significant decline of 63% and 71% was observed in locomotor activity at 16 and 19 mg/kg. Similar to phenelzine, various anti-depressants *e.g.* MAOIs: clorgyline [21] and TCAs (tricyclic anti-depressants): imipramine have been reported to reduce locomotor activity due to somnolence or lowered muscle tone or both [16,17]. In the case of compound **1**, the locomotor activity of animals remained unchanged up to the dose of 16 mg/kg but at higher dose (19 mg/kg), it produced a significant decline (30%) in motor performance of animals. These results are similar to those reported previously with moclobemide, which exhibited a significant reduction of 53% at 19 mg/kg [14]. Compound **1** caused a marked reduction in the immobility time (13 mg/kg), which does not correlate with the locomotor activity further confirming its anti-depressant property.

In the light of the above results it was essential to determine the acute toxicity of compound **1** and it emerged to be non-toxic up to 19 mg/kg, moreover

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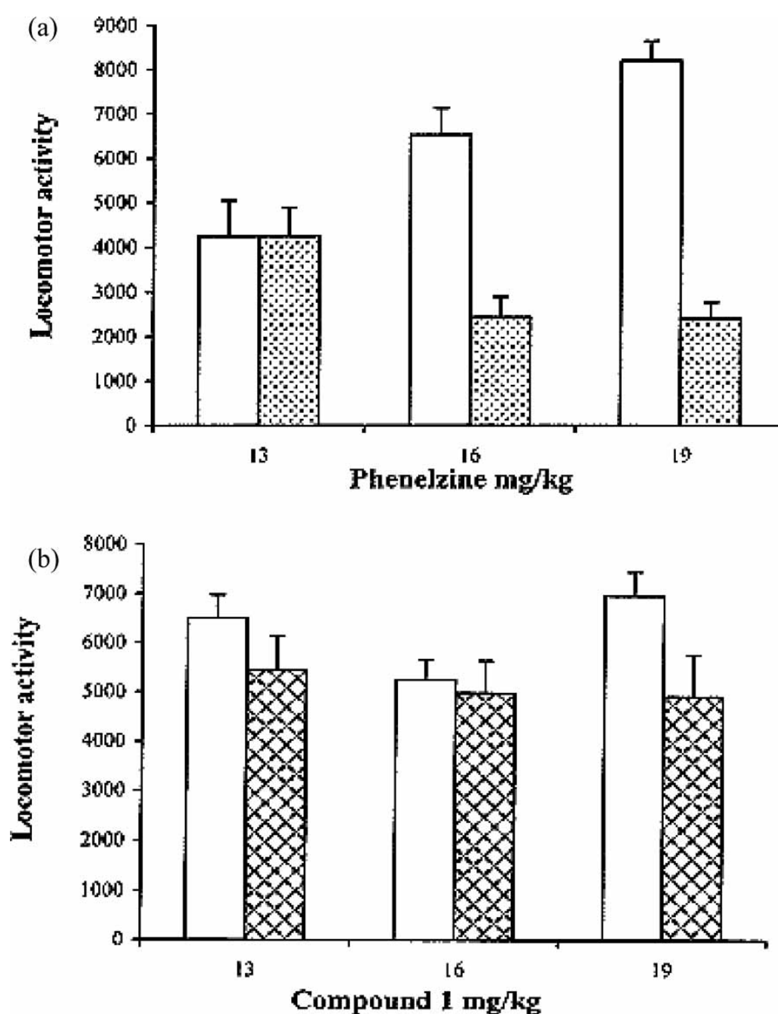

Figure 1. The effect of phenelzine (a) and compound **1** (b) on locomotor activity of mice (n = 5). Open bars depict control counts. Each bar shows the mean locomotor counts/10 min ± SEM.

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it produced no detectable changes in general behaviour of animals as compared to control animals.

In conclusion, compound **1** exhibited excellent MAO-A inhibitory activity [10], whereas compound **2** and **3** showed about 60% less activity by comparison. These results led us to postulate that the tosyl group containing hydrazide appears to be responsible for the MAO inhibition in both *in vitro* and *in vivo* assays. This anti-depressant property manifested by hydrazide justifies its recognition as a potential candidate in the drug discovery program. In the light of these findings it is proposed that replacement of the tosyl group with an appropriate electron withdrawing group or any other electron withdrawing group(s) along with the tosyl group might enhance the MAO inhibitory activity of hydrazides.

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