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Does 1-(R)-aminoindan Possess Neuroprotective Properties Against Experimental Parkinson's Disease?

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Abstract

The anti-Parkinsonian, monoamine oxidase-B inhibitor drug, rasagiline (Azilect®), is primarily metabolized by hepatic cytochrome P450 isoenzyme 1A2-mediated N-dealkylation to form its major metabolite, 1-(R)-aminoindan. The present study was undertaken to further investigate, for the first time, the possible neuroprotective effect of 1-(R)-aminoindan in two rat models of Parkinson's disease, the 6-hydroxydopamine- and lactacystin (a proteasomal inhibitor)-induced nigrostriatal degeneration. 1-(R)-aminoindan reversed behavioral asymmetry and restored striatal catecholamine levels in these two rat models and significantly protected neurons from hydrogen peroxide-induced oxidative stress. These observations indicate that 1-(R)-aminoindan may contribute to the overall neuroprotective activity of its parental compound, rasagiline. *Antioxid. Redox Signal.* 14, 767–775.

Introduction

THE ANTI-PARKINSONIAN DRUG, rasagiline (Azilect®) (*N*-propargyl-1-(R)-aminoindan) (www.tevapharm.com), is a secondary, cyclic benzylamine and indane derivative, which provides potent, selective, and irreversible monoamine oxidase (MAO)-B inhibition. Rasagiline has demonstrated potent neuroprotective effects in experimental studies against a variety of insults in both *in vivo* and *in vitro* (25). In addition, Zhu *et al.* (30) have reported that rasagiline exerted a significant protective effect against neurodegeneration in nigrostriatal pathways induced by ubiquitin–proteasome system (UPS) impairment. The mechanisms behind the neuroprotection include suppression of permeability transition pore opening, prevention of cytochrome *c* release, caspase-3 activation, and nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase. Recent clinical results of phase III delayed-start clinical study in Parkinsonian subjects (ADAGIO) trial have shown that rasagiline confers significant symptomatic improvement and slows the progression of Parkinson's disease (PD), in the recommended dosage (1 mg daily) (13).

In contrast to the first-generation MAO-B inhibitor, selegiline, which is a propargyl derivative of amphetamine, further metabolized to L-amphetamine and L-methamphetamine (17, 27), rasagiline is metabolized by hepatic cytochrome P-450 isoenzyme 1A2-mediated N-dealkylation to its major metabolite 1-(R)-aminoindan, which is devoid of amphetamine-like effects (3) (Fig. 1). It was demonstrated that selegiline and L-amphetamine could evoke stereotypes behavior in rats, which is indicative of amphetamine-like activity. In contrast, rasagiline and its metabolite 1-(R)-aminoindan did not induce this activity even at high doses (22). A kinetic and crystallographic analysis revealed that 1-(R)-aminoindan is not a substrate for MAO oxidation, but, conversely, a weak reversible inhibitor (3, 22). The neuroprotective properties of 1-(R)-aminoindan have been analyzed and reported (2). 1-(R)-aminoindan not only did not interfere with the neuroprotective activities of rasagiline or selegiline in rat pheochromocytoma PC12 cells exposed to combined serum–nerve growth factor deprivation, but also possessed neuroprotective activity by itself, on the contrary to the neurotoxic effects of L-methamphetamine (2). In accordance, it was reported that, in contrast to L-methamphetamine, which enhanced oxygen–glucose deprivation-induced cell death, 1-(R)-aminoindan did not affect cell death under these conditions (1). Combined exposure of PC12 cells under ischemia to either selegiline and L-methamphetamine or rasagiline and 1-(R)-aminoindan indicated that L-methamphetamine, but not 1-(R)-aminoindan, blocked the neuroprotective effect of the respective parental drug (1). The neuroprotective properties of 1-(R)-aminoindan were further assessed in cytotoxic neuronal models of high-density human SK-N-SH neuroblastoma cell culture and 6-hydroxydopamine (6-OHDA) in PC12 cells (2). Recently,

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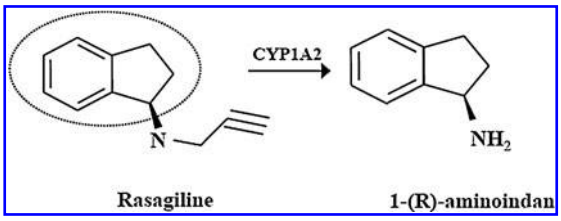

FIG. 1. The chemical structures of 1-(R)-aminoindan and its parental compound, the anti-Parkinsonian drug, rasagiline (Azilect®) (*N*-propargyl-1-(R)-aminoindan). Rasagiline is metabolized by hepatic cytochrome P450 isoenzyme 1A2-mediated N-dealkylation to form 1-(R)-aminoindan. CYP1A2, cytochrome P450 isoenzyme 1A2.

it was demonstrated that rasagiline, selegiline, and 1-(R)-aminoindan decreased ethanol-induced cell death through the inhibition of glyceraldehyde-3-phosphate dehydrogenase–MAO-B-mediated cell death signaling pathway (15). Additionally, it was reported that rasagiline, selegiline, and 1-(R)-aminoindan could significantly prevent dexamethasone-induced neuronal apoptosis in both neuroblastoma and glioblastoma cells (23). Among these drugs, rasagiline possessed the highest neuroprotective effect, compared with either selegiline or 1-(R)-aminoindan. This greater neuroprotective quality of rasagiline may be, in part, due to combined effects of the parent drug and its main metabolite (23).

The present study was undertaken to further investigate, for the first time, the possible neuroprotective effect of 1-(R)-aminoindan *in vivo* in the 6-OHDA-lesioned rat model of PD. As an inhibitor of proteasomal function may contribute to the neurodegeneration process in PD, we also explored the neuroprotective potential of 1-(R)-aminoindan against lactacystin (a proteasome inhibitor)-induced nigrostriatal degeneration in rats.

Neuroprotective Effects of 1-(R)-Aminoindan in 6-OHDA Rat Model of PD

The effect of 1-(R)-aminoindan on motor deficits induced by 6-OHDA was evaluated by two behavioral tests, sensitive to dopaminergic lesions that do not require drug administration: the forced-step test and movement initiation measures. Consistent with previous findings (10), 6-OHDA lesions of the nigrostriatal area produced a deficit in adjusting steps made by the contra forelimb, as measured by the forced-step test during 1–3 weeks post-6-OHDA, compared with respective pre-6-OHDA (Fig. 2B). Administration of 1-(R)-aminoindan (1 and 5 mg/kg) significantly improved the number of adjusting steps made by the contra forelimb during 3 weeks post-6-OHDA-induced lesion, compared with vehicle-treated rats (Fig. 2B). In addition, both 1 and 5 mg/kg 1-(R)-aminoindan significantly improved the ipsi bias, compared with vehicle-treated rats at 1–3 weeks post-6-OHDA-induced lesion (Fig. 2C). The administration of 1-(R)-aminoindan did not change the number of adjusting steps made by the ipsi forelimb during 3 weeks post-6-OHDA-induced lesion, compared with vehicle-treated rats (Fig. 2A). In the post-6-OHDA group, movements initiation test of the ipsi side was not changed, compared with vehicle-treated rats (Fig. 3A), whereas in contra side it was increased from 1 s at pre-6-OHDA lesioned to ~30 s during 1–3 weeks post-6-OHDA-induced lesion (Fig. 3B). Administration of 1-(R)-aminoindan (1 and 5 mg/kg) significantly and dose-dependently reduced the movement initiation time of the contra side, compared with control at 1–3 weeks post-6-OHDA-induced lesion (Fig. 3B). In addition, 1-(R)-aminoindan

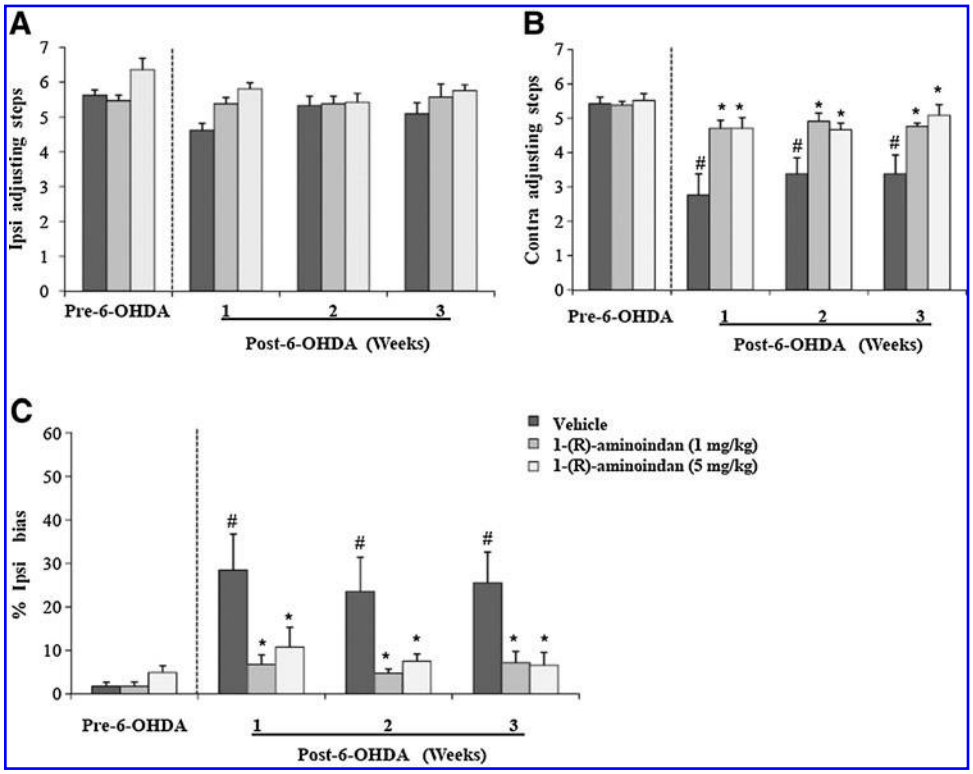

FIG. 2. Forced-step test in unilaterally 6-OHDA-lesioned rats treated with 1-(R)-aminoindan. Behavioral forced-steps test was assessed at 2 weeks pre- and during 3 weeks post-6-OHDA-induced lesion in the following groups: vehicle (saline) and 1-(R)-aminoindan (1 and 5 mg/kg; $n = 7$ in each experimental group). Number of adjusting steps completed, while moving the animal laterally across a surface was recorded for the ipsi **(A)** and contra **(B)** forelimb. The behavioral bias **(C)** is expressed as % ipsi bias (% ipsi steps −% contra steps). A score of 100 represents a complete bias, that is, the animal uses only the ipsi forelimb. Results are the mean ± SEM. $^{\#}p < 0.05$ *versus* respective pre-6-OHDA. $^{*}p < 0.05$ *versus* post-6-OHDA/vehicle-treated rats. 6-OHDA, 6-hydroxydopamine.

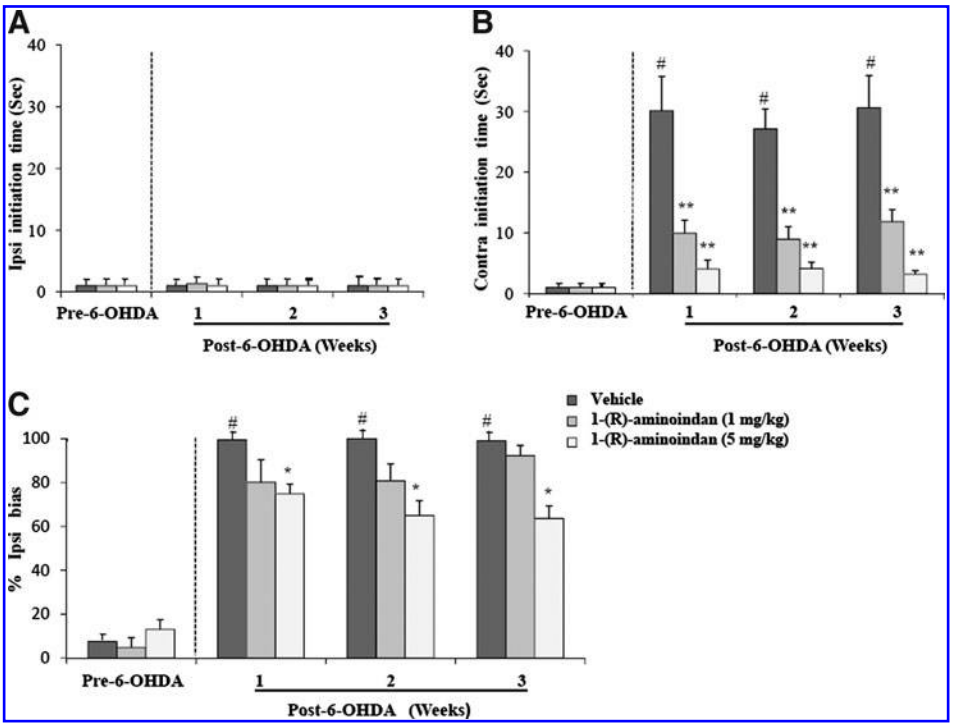

FIG. 3. Movement initiation test in unilaterally 6-OHDA-lesioned rats treated with 1-(R)-aminoindan. Behavioral movement initiation test was assessed as described in Figure 2. The time to initiate a movement (Sec) was recorded for the ipsi **(A)** and contra **(B)** forelimb. The behavioral bias **(C)** was measured and expressed as % ipsi bias within 10 s (% ipsi steps −% contra steps). A score of 100 represents a complete bias, that is, the animal uses only the ipsi forelimb. Results are the mean ± SEM. $^{\#}p < 0.05$ *versus* respective pre-6-OHDA. $^{*}p < 0.05$ *versus* post-6-OHDA/vehicle-treated rats. $^{**}p < 0.01$ *versus* post-6-OHDA/vehicle-treated rats.

(5 mg/kg) was significantly effective in reducing the time of movement initiation bias, compared with control animals during 1–3 weeks post-6-OHDA-induced lesion (Fig. 3C).

As expected, intracerebral unilateral 6-OHDA administration produced, 3 weeks postinjection, a marked reduction in the levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) in the lesioned striatal tissue compared with the nonlesioned (contra) striata (Fig. 4), without affecting serotonin (5-HT) or its metabolite 5-hydroxyindoleacetic acid (data not shown). Treatment with 1-(R)-aminoindan (1 and 5 mg/kg) significantly prevented this decline in the DA, DOPAC, and HVA levels in the lesioned ipsistriatal compared with lesioned striatal of vehicle-treated rats (Fig. 4). In addition, in the 1-(R)-aminoindan (5 mg/kg) group, DA levels were significantly elevated in the nonlesioned side (contra), compared with the respective contra striata in the vehicle-treated rats (Fig. 4). The neuroprotective potential of 1-(R)-aminoindan (1 and 5 mg/kg) was further supported by preventing 6-OHDA-induced increase in striatal DA turnover (ratio of DOPAC+HVA/DA) [6-OHDA-lesioned ipsi side: 7.66 ± 0.01 pmole/mg tissue *versus* nonlesioned contra side: 0.61 ± 0.21 pmole/mg tissue, $p < 0.05$, compared with 1-(R)-aminoindan (1 and 5 mg/kg)-lesioned ipsi side: 0.49 ± 0.06 pmole/mg tissue and 0.17 ± 0.04 pmole/mg tissue, respectively, $p < 0.05$]. In accordance, chronic treatment with 1-(R)-aminoindan at the high, non-clinical dose of 5 mg/kg resulted in inhibition of striatal MAO-B activity (45.9% ± 9.2% of vehicle-treated control rats; $p < 0.01$).

Protective Effects of 1-(R)-Aminoindan Against Hydrogen Peroxide-Induced Cellular Neurotoxicity

There is an increasing amount of evidence that the 6-OHDA neurotoxicity in PD modeling is mainly due to its oxidation, resulting in generation of cytotoxic free radicals, which are believed to play a pivotal role in the degeneration of nigrostriatal system (20). As hydrogen peroxide (H_2O_2) has been extensively used as an *in vitro* model of oxidative stress (OS) for studying neurotoxicity and neuroprotection in the central nervous system (26, 29), we investigated the protective effect of 1-(R)-aminoindan against H_2O_2-induced OS in human SH-SY5Y neuroblastoma cells and rat primary cortical neurons. In SH-SY5Y cells, MTT assay revealed that stimulation with H_2O_2 (200 μM) for 30 min markedly reduced cell viability, whereas pretreatment with 1-(R)-aminoindan (5 and 10 μM) significantly inhibited the neuronal damage (Fig. 5A). Similarly, in rat primary cortical neurons, H_2O_2 (25 μM) exposure for 24 h caused a marked increase in cell death, whereas pretreatment with 1-(R)-aminoindan (5 and 10 μM) significantly reduced cell mortality, as analyzed by an apoptotic cell death detection ELISA (Fig. 5B).

In addition, pretreatment with 1-(R)-aminoindan (5 and 10 μM) led to a significant increase in catalase enzymatic activity, compared with stimulation with H_2O_2 alone (Table 1). The regulatory effect of 1-(R)-aminoindan on mRNA expression levels of phase II enzymes: catalase, peroxiredoxin 1 and NAD(P)H quinone oxidoreductase 1, examined by real-time reverse transcriptase (RT)–polymerase chain reaction (PCR), demonstrated that 1-(R)-aminoindan (5 μM) induced mRNA expression levels of these enzymes compared with H_2O_2 alone (Fig. 5C).

Effect of 1-(R)-Aminoindan Against Lactacystin-Induced Dopaminergic Loss in Rat SN

The neuroprotective efficacy of 1-(R)-aminoindan in ameliorating nigrostriatal dopaminergic degeneration was further examined in the rat model of PD produced by lactacystin-induced nigrostriatal dopaminergic degeneration (30). To determine whether 1-(R)-aminoindan affects forelimb use following lactacystin lesioning, performance was assessed in

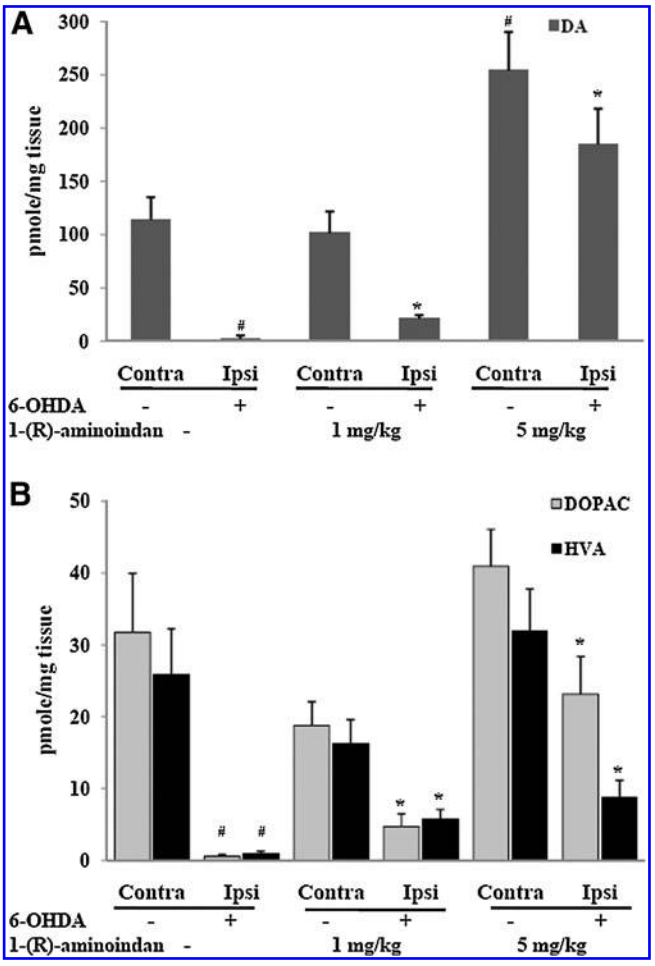

FIG. 4. Catecholamine concentrations in striata of unilaterally 6-OHDA-lesioned rats treated with 1-(R)-aminoindan. Sprague–Dawley rats daily administered by gavage, either vehicle (saline) or 1-(R)-aminoindan (1 and 5 mg/kg), 2 weeks pre- and 3 weeks post-6-OHDA lesion (4 μg/8 μl). The contents of contra and ipsi striatal DA **(A)** and its metabolites, DOPAC and HVA **(B)**, were determined by using HPLC and expressed as the mean ± SEM ($n = 7$ in each group). $^{\#}p < 0.05$ *versus* saline, nonlesioned striatum. $^{*}p < 0.05$ *versus* respective saline, lesioned striatum. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid.

the forelimb grip time test (sensitive to dopaminergic lesions) at 2 weeks after the microinjection of lactacystin. Figure 6A demonstrated that in the lactacystin-injected animals, the forelimb grip time was significantly reduced in the contra forelimb compared with the ipsi forelimb ($p < 0.05$). Administration of 1-(R)-aminoindan (1 and 5 mg/kg) dose-dependently and significantly improved the grip time of the contra forelimb, compared with vehicle-treated rats. In addition, no significant difference was observed between contra and ipsi forelimbs in lactacystin-lesioned rats treated with a dose of 5 mg/kg of 1-(R)-aminoindan (Fig. 6A), similar to that observed in phosphate-buffered saline (PBS)-injected sham controls (data not shown).

Administration of lactacystin resulted in a marked reduction in the levels of DA (Fig. 6B), DOPAC, and HVA in the striatum at the lesioned side (ipsi) (Fig. 6C), compared with the respective contra side. Treatment with 1-(R)-aminoindan (1 and 5 mg/kg) dose-dependently and significantly prevented lactacystin-induced decline in DA (Fig. 6B), DOPAC, and HVA levels (Fig. 6C) in the striatum at the lesioned side (ipsi). Similar to results in the 6-OHDA-treated rats, in the lactacystin experimental model, the striatal DA content was significantly increased in the contra side of the 1-(R)-aminoindan (5 mg/kg) group, compared with the contra striatal tissue in the vehicle-treated rats (Fig. 6B). Lactacystin administration did not affect 5-HT or its metabolite 5-hydroxyindoleacetic acid levels (data not shown).

Concluding Remarks and Future Directions

The present study examined the potential neuroprotective effects of the main metabolite of rasagiline, 1-(R)-aminoindan, in the 6-OHDA and lactacystin rat models of hemi-Parkinsonism.

In the 6-OHDA model, we show that chronic administration of 1-(R)-aminoindan (1 and 5 mg/kg) prevented the reduction in the levels of striatal DA and its metabolites, DOPAC and HVA, and reduced the increase of DA turnover, which is observed following intrastriatal injection of 6-OHDA (19). Accordingly, 1-(R)-aminoindan retarded impaired dopaminergic-related behavioral responses induced by 6-OHDA, including the drug-free behavioral tests: forced step and movement initiation measures. In addition, we have assessed whether 1-(R)-aminoindan is neuroprotective against nigrostriatal degeneration induced by UPS dysfunction. Previous pathological evidence indicated that abnormal structure and dysfunction of the UPS occur in the sabstantia nigra of PD patients (9). Pharmacological inhibition of the proteasome to model such features of PD was found to induce neuronal apoptotic death and the formation of cytoplasmic inclusions that contain, among other proteins, ubiquitin and α-synuclein in cultured neuronal cells (16, 18). DA-induced neuronal degeneration and inclusion formation have also been observed *in vivo* following unilateral stereotaxic injection of the proteasome inhibitor, lactacystin, into the medial forebrain bundle (8). In our study, we have demonstrated that treatment with 1-(R)-aminoindan (1 and 5 mg/kg) dose-dependently and significantly prevented lactacystin-induced decline in DA, DOPAC, and HVA levels in the striatum of the lesioned side. The metabolite also improved the grip time of the contra forelimb *versus* vehicle-treated rats. In this context, Zhu *et al.* (30) have recently provided evidence supporting both neuroprotective and neurorestorative activities for the parental drug, rasagiline, in the lactacystin Parkinsonism model. Compared with selegiline, the higher neuroprotective/neurorescue capabilities against lactacystin-induced neurodegeneration described for rasagiline may in part be associated with the different metabolites of these drugs (30). Thus, the major metabolite of selegiline, L-methamphetamine, has been shown to induce cytosolic inclusions and cause inhibition of the proteasome activity (5–7), whereas the major metabolite of rasagiline, 1-(R)-aminoindan, has been demonstrated to stimulate beneficial neuroprotective effects by itself in animal and cell-cultured models (2).

It may be indicated that these neuroprotective effects observed for 1-(R)-aminoindan are due to its presence in the brain, as inhibition of striatal MAO-B activity was observed following 1-(R)-aminoindan administration. Similarly, it was

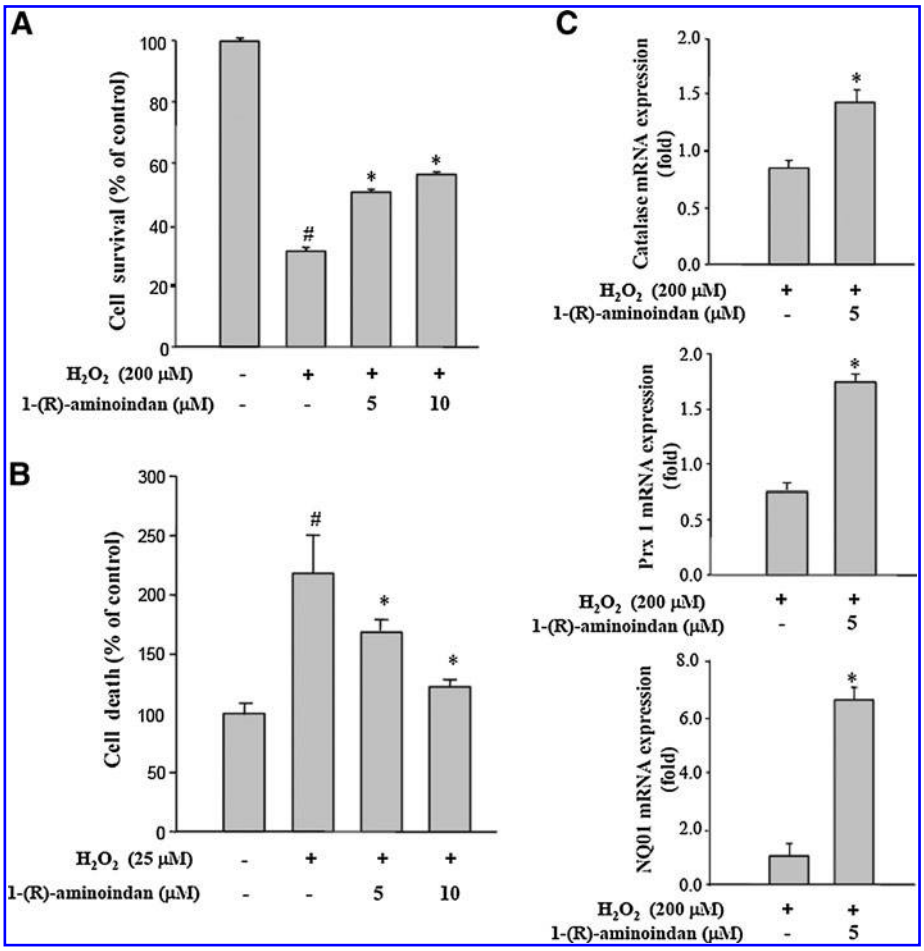

FIG. 5. Neuroprotective effects of 1-(R)-aminoindan against H_2O_2-induced neurotoxicity. (A) SH-SY5Y neuroblastoma cells were treated without (control) or with H_2O_2 (200 μM) in the absence or presence of 1-(R)-aminoindan (5 and 10 μM) for 1 h. Cell viability was evaluated by the MTT assay. **(B)** Rat primary cortical cells were treated without (control) or with H_2O_2 (25 μM) in the absence or presence of 1-(R)-aminoindan (5 and 10 μM) for 24 h. Cell death was assayed using an apoptotic cell death detection ELISA. **(C)** SH-SY5Y cells were treated without (control) or with H_2O_2 (200 μM) for 1 h in the absence or presence of 1-(R)-aminoindan (5 μM). The gene expression levels of catalase, peroxiredoxin 1 (Prx1), and NAD(P)H quinone oxidoreductase 1 (NQO1) were measured by quantitative real-time reverse transcriptase–polymerase chain reaction. The amount of each product was normalized to the housekeeping gene 18S rRNA. The values are expressed as relative gene expression *versus* the respective control. Results are the mean ± SEM ($n = 3$). $^{\#}p < 0.05$ *versus* control. $^{*}p < 0.05$ *versus* H_2O_2 alone. H_2O_2, hydrogen peroxide.

previously demonstrated (21) that treatment with 1-(R)-aminoindan led to 43% inhibition in the brain MAO-B activity in hypoxic rats. Of note, the minimal inhibition of MAO-B required for clinical effects is accepted to be about 80% (24), which is much higher than observed in our study. Moreover, in haloperidol-treated rats, it was shown that 1-(R)-aminoindan provided anti-Parkinsonian benefit, allowing normal locomotion to be initiated and enhancing DA release, thus suggesting that 1-(R)-aminoindan may interact with novel sites controlling MAO release or reuptake (4).

Table 1. Effect of 1-(R)-aminoindan on Catalase Activity in Rat Primary Cortical Cells

Treatment	*Catalase activity (U/mg protein)*
Control	10.1 ± 0.1
H_2O_2 (25 μM)	6.2 ± 0.4[a]
H_2O_2 (25 μM) +1-(R)-aminoindan (5 μM)	8.2 ± 0.7[b]
H_2O_2 (25 μM) +1-(R)-aminoindan (10 μM)	8.3 ± 0.4[b]
1-(R)-aminoindan (5 μM)	11.2 ± 2.0
1-(R)-aminoindan (10 μM)	10.8 ± 0.3

Rat primary cortical cells were treated without or with H_2O_2 (25 μM) in the absence or presence of 1-(R)-aminoindan (5 and 10 μM) for 24 h. Then, cells were homogenized and catalase activity was measured by a specific assay kit (Calbiochem).
[a] $p < 0.05$ *versus* control.
[b] $p < 0.05$ *versus* H_2O_2.
H_2O_2, hydrogen peroxide.

A growing body of evidence suggests OS as a primary cause of neuronal damage implicated in the etiology and progression of PD (11). Thus, based on our previous study, here we considered to investigate whether pretreatment with 1-(R)-aminoindan attenuates H_2O_2-induced neurotoxicity, using SH-SY5Y neuroblastoma and primary culture of cortical neurons. Treatment of neuronal cells with 1-(R)-aminoindan was found to induce a significant neuroprotective effect against H_2O_2-induced damage, associated with increased catalase antioxidant enzyme activity. This is consistent with recent studies in culture of PC12 cells showing that 1-(R)-aminoindan increased cell viability following 6-OHDA-induced neuronal death (2). Further, it was reported that 1-(R)-aminoindan possesses neuroprotective activity against serum and nerve growth factor deprivation and high-density culture conditions-induced apoptosis (2). These studies characterized various mechanisms of action that may be involved in this neuroprotective effect, including reduction of the DNA damage-associated phosphorylated protein, H2A.X (ser139), and cleaved caspase-9 and caspase-3 levels; induction of phosphorylation of protein kinase C; and upregulation of various antiapoptotic Bcl-2 family proteins (2).

Our findings also showed that 1-(R)-aminoindan treatment in H_2O_2-damaged cells stimulated mRNA expression levels of the phase II enzymes, catalase, NAD(P)H

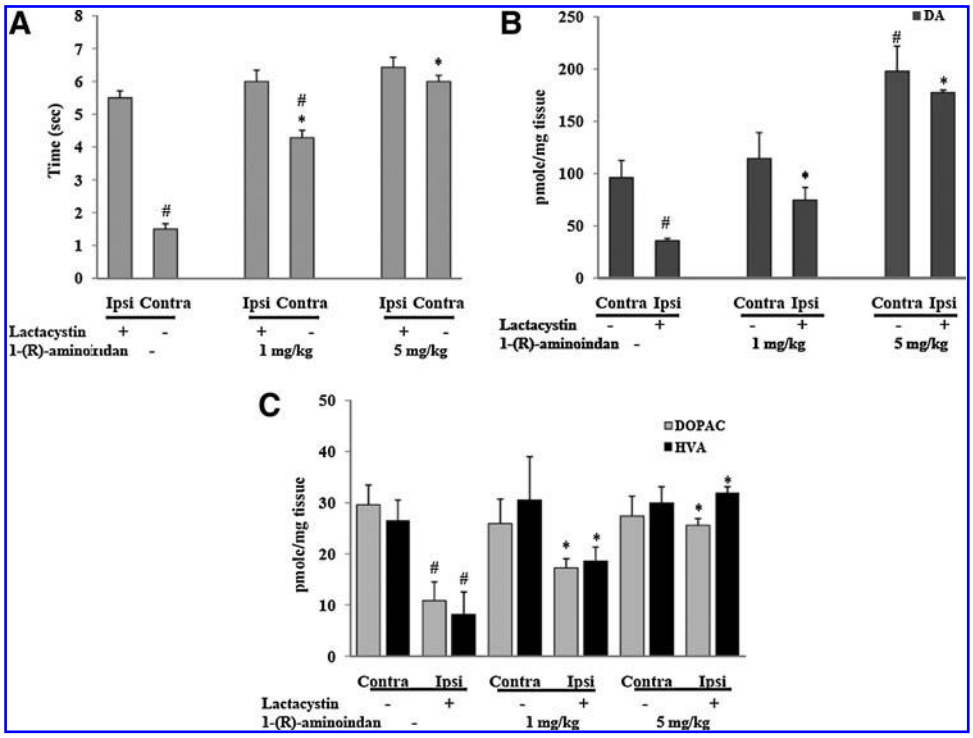

FIG. 6. Neuroprotective effects of 1-(R)-aminoindan in unilaterally lactacystin-lesioned rats treated with 1-(R)-aminoindan. (A) Forelimb grip test. Grip test was assessed 2 weeks post-lactacystin lesion (1.6 μg/4 μl) in the following groups: vehicle (saline) and 1-(R)-aminoindan (1 and 5 mg/kg; $n = 7$ in each group). Animals were tested by placing each paw against the cage grid and allowing them to grasp on to the grid. The time was recorded in seconds from grasp until letting go. Each animal was tested in three independent experiments. Results are the mean ± SEM. $^{\#}p < 0.05$ *versus* respective ipsi side. $^{*}p < 0.05$ *versus* lactacystin/vehicle contra side. **(B, C)** Striata catecholamine concentrations. Sprague–Dawley rats daily administered by gavage, either vehicle (saline) or 1-(R)-aminoindan (1 and 5 mg/kg), 1 week prior and 3 weeks after lactacystin-induced lesions (1.6 μg/4 μl). The contents of striatal DA **(B)** and its metabolites, DOPAC and HVA **(C)**, were determined by using HPLC and expressed as the mean ± SEM ($n = 7$ in each group). $^{\#}p < 0.05$ *versus* saline, nonlesioned striatum. $^{*}p < 0.05$ *versus* respective, lesioned striatum.

quinone oxidoreductase 1, and peroxiredoxin 1, which are important cellular defensive enzymes against oxidative injury. This indicates that an increase of endogenous antioxidants by 1-(R)-aminoindan *via* increased transcription of the respective genes may provide, at least in part, a possible mechanism involved in the protective effects of this metabolite of rasagiline.

Taken together, the results of this study indicate that 1-(R)-aminoindan displayed a neuroprotective activity against 6-OHDA- and lactacystin-induced dopaminergic neuronal degeneration *in vivo* and against OS-induced cytotoxicity in cultured neuronal cells. The complete mechanism by which 1-(R)-aminoindan mediated its neuroprotective activity is considered to be further investigated. It is apparent that besides being valuable by itself as a neuroprotective compound, 1-(R)-aminoindan may also contribute to the overall beneficial neuroprotective/neurorescue effects of its parental anti-Parkinsonian drug, rasagiline.

Materials

1-(R)-aminoindan was kindly supplied by Teva Pharmaceutical Industries Ltd. Tissue culture reagents were obtained from Sigma and Beit-Haemek (Kibbutz Beit). RT reaction mix, random hexanucleotides, dNTP, RNasin inhibitor, and M-MLV RT were purchased from Promega. The LightCycler, DNA Master SYBR Green I ready-to-use PCR mix kit was purchased from Roche Diagnostics. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 6-OHDA, and standards of the monoamines were obtained from Sigma. Lactacystin was purchased from A.G. Scientific. Cell death ELISA kit was obtained from Roche Molecular Biochemicals; catalase assay kit from Calbiochem. PerfectPure RNA cell and tissue kit was purchased from 5Prime.

Animal Procedures: Drug Treatment Regimens and Surgery

Adult male Sprague–Dawley rats, weighing 220–260 g, were purchased from Harlan, Jerusalem, Israel. Rats were allowed to acclimate to the light/dark cycle for 1 week prior to the beginning of the trial and had access to water and food *ad libitum* throughout the whole study period. All animal care and procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

6-OHDA rat model: experimental animals and behavioral testing

Rats were randomly divided into four groups of seven animals in each experimental group, including control. Each group was daily administered by gavage, either 1-(R)-aminoindan (1 and 5 mg/kg) or vehicle (saline), 2 weeks pre- and 3 weeks post-6-OHDA lesion.

Rats were deeply anesthetized using a (2:1) ketamine/xylazine solution. 6-OHDA was dissolved in 0.1% ascorbic acid in 0.9% sterile saline. Intracerebral unilateral stereotaxic injection of 4 μg/8 μl of 6-OHDA was administered into the left rostral substantia nigra pars compacta with coordinates as follows: AP = 3.4 mm from interaural zero; ML = 2.0 mm from midline; DV = 2.3 mm with respect to Lambda with the

tooth bar at −3.3 mm. The injection was conducted with Kopf stereotaxic instrument, using a 24-gauge stainless-steel cannula attached *via* a fine (50 μm in diameter) polyethylene tubing to a 10-μl Hamilton syringe (Hamilton Company), at a speed of 30 μl/h. During the treatment period, rats were weighed once a week. All rats were healthy during the experimental period and no significant change in body weight gain was observed in different experimental groups.

Behavioral forced-step and movement initiation tests were performed at 2 weeks prior to 6-OHDA lesion to assess the behavioral baseline and were further weekly performed for 3 weeks post-6-OHDA lesion to measure the effect of 1-(R)-aminoindan on different behavior parameters.

The forced-step test is a modification of a motor test previously described by Olsson *et al.* (14). Rats were moved laterally for a total distance of 60 cm over approximately 3 s, across a smooth-surfaced table. The experimenter held the rat, allowed its weight to be supported on one of the front forelimbs only and then moved the rat laterally across the table. When testing the right forelimb, the rat was moved from left-to-right and vice versa for the left forelimb. Three trials were performed in each direction and the number of steps made by the forelimb was counted. For each animal, a percentage for each type of forelimb movement was calculated as percentage of ipsilateral (ipsi) bias, calculated as the percentage of contralateral (contra) (% contra) steps subtracted from the percentage of ipsi (% ipsi) steps [% contra = contra/(ipsi + contra)×100; and % ipsi = ipsi/(ipsi + contra)×100].

The test of movement initiation made by each forelimb was assessed as described previously in rats with unilateral 6-OHDA lesion (14). The rat was held by its torso with its hind limbs, while one forelimb lifted above the surface of the table, so that the body weight of the rat was supported by one forelimb only. The experimenter allowed the rat a 10-s period to voluntary initiate a step for one forelimb and then the other forelimb. If no step was initiated, the rat had fixed time of up to 1 min and the time upon first movement initiation was recorded. Three trials were performed and the time to initiate one step was recorded for each forelimb. For each animal, a percentage of step bias within 10 s was calculated as the percentage of contra (% contra) to initiate a step subtracted from the percentage of ipsi (% ipsi) to initiate a step [% contra = contra/(ipsi + contra)×100; and % ipsi = ipsi/(ipsi + contra)×100].

Lactacystin-lesioned rats: experimental animals and behavioral testing

Rats were randomly divided into four groups of seven animals in each experimental group. Each group was daily administered by gavage, either 1-(R)-aminoindan (1 and 5 mg/kg) or vehicle (saline), 1 week prior and 3 weeks after lactacystin-induced lesion. PBS-injected rats were used as sham controls. For the stereotaxic injection of lactacystin, rats were deeply anesthetized and placed in a Kopf stereotaxic frame (Kopf Instruments). An injection cannula was inserted through a hole drilled in the skull, into medial forebrain bundle (MFB), using the following coordinates (in mm): 5.2 posterior; 2.2 left lateral; −7.2 ventral from bregma of each rat. Four microliters of either PBS (0.1 *M*) as control or lactacystin (1.6 μg) in PBS was injected into MFB of each rat. This volume was injected over 5 min, and the cannula was left in for another 5 min, allowing the drug to redistribute. During the treatment period, rats were weighed once a week. No significant changes in body weight gain were observed in the different experimental groups.

To investigate forelimb motor dysfunction following lactacystin-induced lesioning, performance in the grip test was assessed at 2 weeks postsurgery. The animals were tested in three independent experiments by placing each paw against the cage grid and allowing them to grasp onto the grid. The time was recorded in seconds from grasp until letting go.

Analysis of Catecholamine Contents in Rat Striatal

At the end of the experiments, the rats were sacrificed by decapitation. The brains were rapidly removed, and striatal tissues were dissected and homogenized in ice-cold 0.1 *M* perchloric acid with minihomogenizer (Pellet Pestel® Motor; Kontes), followed by centrifugation at 14,000 *g* for 5 min. Supernatants were injected, using an autosampler (Jasco, Inc.), into a high-performance liquid chromatography system (HPLC ESA, Inc.). Separation was performed with 1 ml/min flow rate and the following neurotransmitters and their derivatives were analyzed: DA and its metabolites, DOPAC and HVA. The content was calculated by comparison to catecholamine standards (Sigma) in known concentrations and normalized relative to tissue weight. Determination of MAO-B activity was employed according to Youdim and Tipton (28).

Cell Cultures and Viability Assays

Human SH-SY5Y neuroblastoma cells were obtained from American Type Culture Collection. Cells were routinely cultured in minimum essential medium-Eagle/F-12 (HAM) (1:1), containing 10% fetal calf serum and a mixture of 2% sodium bicarbonate, 1% penicillin/streptomycin/nystatin, and 1% sodium pyruvate. Cell cultures were incubated at 37°C in a humid 5% CO_2–95% air environment.

Primary cerebral cortices were isolated from Sprague–Dawley rat embryonic (E18) pups as previously described (12) and placed in ice-cold Hank's buffered salt solution followed by incubation in 0.25% trypsin at 37°C for 10 min. Trypsin inhibitor and DNase solution were added to terminate trypsin activity, and mechanical dissociation was performed by intensive pipetting until the suspension became homogenous. Then, cells were filtered through 70-μm nylon cell strainer, resuspended in the minimum essential medium supplemented with 10% fetal calf serum and 100 units/ml penicillin/streptomycin solution, and plated in poly-D-lysine–coated culture dishes. Following 4 h of culturing, the medium was replaced with neurobasal medium, supplemented with B27, 2 m*M* L-glutamine, 5 m*M* HEPES, and 100 units/ml penicillin/streptomycin solutions. These conditions support neuronal differentiation and growth, providing primarily neuronal culture. Cultures were maintained at 37°C and 5% CO_2. All experiments were initiated 7–8 days after plating.

A cell death detection ELISA kit was used to detect apoptosis. The assay is based on a quantitative sandwich ELISA, with antibodies directed against DNA and histones to detect mono- and oligonucleosomes in the cytoplasm of cells undergoing apoptosis. The ELISA was performed according to the manufacturer's protocol. The absorption was determined in a Tecan Sunrise Elisa-Reader at $\lambda = 405/490$ nm after

Table 2. Sequences of Primers Used in Quantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction

	Oligonucleotide sequence (5′–3′) forward	*Oligonucleotide sequence (5′–3′) reverse*	*Size of product (bp)*
Catalase	GCGGTCAAGAACTTCACTGA	GCTAAGCTTCGCTGCACAGGT	187
NAD(P)H quinone oxidoreductase 1	CAAATCCTGGAAGGATGGAA	GGTTGTCAGTTGGGATGGAC	199
Peroxiredoxin 1	CACGGAGATCATTGCTTTCA	GACCCCATAATCCTGAGCAA	195
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	171

All templates were initially denatured for 3 min at 95°C. Amplification was done for 45 cycles. To receive melting temperatures of the products, melting curve analysis was done by continuous acquisition from 65°C.

automatic subtraction of background readings. The MTT test was used to determine cellular viability. Absorption was determined in an Elisa-Reader at $\lambda = 570/650$ nm after automatic subtraction of background readings. Cell viability was expressed as a percentage of control, untreated cells.

Catalase Activity

Catalase activity was measured using a catalase assay kit. The measurement of catalase activity was based the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen at 540 nm. One unit was defined as the amount of enzyme that would cause the formation of 1.0 nmol formaldehyde per min at 25°C. The catalase activity was expressed as units/mg protein.

RT and Quantitative Real-Time RT-PCR Detection

Total RNA was purified with PerfectPure RNA cell and tissue kit according to the manufacturer's protocol. Quantitative real-time PCR, using LightCycler and FastStart DNA Master SYBR Green I ready-to use PCR mix, was performed according to the manufacturer's protocol. cDNA (40 ng) was amplified in 20 μl total volume. The sequences of the primers and the size of the products are described in Table 2. The results were analyzed on the provided program of the LightCycler. The relative expression level of a given mRNA was assessed by normalizing to the housekeeping gene 18S rRNA and compared with control values.

Statistical Analyses

For statistical analysis, one-way ANOVA followed by Student's *t*-test was performed and a *p*-value of <0.05 was considered significant.

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Author Disclosure Statement

M.B.H. Youdim together with Teva Pharmaceutical Company (Israel) developed the anti-Parkinson's drug rasagiline and received royalty.

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Abbreviations Used

6-OHDA = 6-hydroxydopamine
CYP1A2 = cytochrome P450 isoenzyme 1A2
DA = dopamine
DOPAC = 3,4-dihydroxyphenylacetic acid
H_2O_2 = hydrogen peroxide
HVA = homovanillic acid
MAO = monoamine oxidase
OS = oxidative stress
PD = Parkinson's disease
RT-PCR = reverse transcriptase–polymerase chain reaction
UPS = ubiquitin–proteasome system

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