

# Catechol-O-methyltransferase Inhibition Attenuates Levodopa Toxicity in Mesencephalic Dopamine Neurons

ALEXANDER STORCH, HEIKE BLESSING, MARKUS BAREISS, SINJE JANKOWSKI, ZAO DUNG LING, PAUL CARVEY, and JOHANNES SCHWARZ<sup>1</sup>

Department of Neurology, University of Ulm Medical School, Ulm, Germany (A.S., H.B., M.B., S.J., J.S.); and Departments of Neurological Sciences and Pharmacology, Neuropharmacological Research Laboratories, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois (Z.D.L., P.C.)

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## ABSTRACT

Inhibition of catechol-O-methyltransferase (COMT; EC 2.1.1.6) is a new therapeutic strategy in the treatment of Parkinson's disease. However, nothing is known about the effects of COMT inhibition on levodopa (L-dopa)-induced toxicity in dopamine (DA) neurons. Therefore we evaluated the effects of the selective COMT inhibitors Ro 41-0960, OR-486, and tolcapone alone and in combination with L-dopa in primary mesencephalic cultures from rat. Neither COMT inhibitor affected the growth of tyrosine hydroxylase immunoreactive (THir) cells with concentrations up to 10  $\mu$ M when studied alone. However, Ro 41-0960 reduced the L-dopa-induced THir cell loss after 24 h in a dose-dependent manner, shifting the TD<sub>50</sub> value from 21  $\mu$ M in the absence to 71  $\mu$ M in the presence of 1  $\mu$ M Ro 41-0960 ( $P$  <

.01) without affecting survival of non-DA neurons. OR-486 and the clinically used COMT inhibitor tolcapone showed similar effects. In contrast, toxicity induced by D-dopa was not altered by COMT inhibitors. Furthermore, the primary metabolite of L-dopa formed by COMT, 3-O-methyldopa, and the methyl group donor S-adenosyl-L-methionine used by COMT did not alter THir neuron survival and L-dopa-induced toxicity, respectively, with concentrations up to 100  $\mu$ M. These data demonstrate that COMT inhibition attenuates L-dopa toxicity toward DA neurons in vitro, but probably not by preventing 3-O-methyldopa production or cellular S-adenosyl-L-methionine depletion.

The introduction of catechol-O-methyltransferase (COMT; EC 2.1.1.6) inhibitors has broadened the spectrum of symptomatic treatment for patients with Parkinson's disease (PD). COMT inhibition is able to increase and stabilize L-dopa plasma levels and consistently enhances the clinical benefits of L-dopa therapy in patients with PD (Kurth and Adler, 1998).

COMT is an enzyme that catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to a hydroxyl group of a catecholic substrate (Männistö et al., 1992). COMT activity is relatively high in several peripheral organs, such as liver and kidney, and is also present in the embryonic and adult central nervous system (CNS), primarily in astroglial

cells (Pelton et al., 1981; Fiszman et al., 1991; Männistö et al., 1992). COMT exists in two forms with different pharmacologic properties: a soluble cytoplasmic form and a membrane-bound form located in the endoplasmic reticulum (Männistö et al., 1992; Vieira-Coelho and Soares-da-Silva, 1999). In the CNS, therefore, glial uptake of substrates into the cytoplasm is necessary for the access to COMT (Männistö et al., 1992). In the dopaminergic system, COMT acts on dopa with stereospecificity for the L-isomer (levodopa) (Gordon-Smith et al., 1982), producing 3-O-methyldopa (3-OMD) and, in addition, in the catabolism of dopamine (DA) resulting in the production of 3-methoxytyramine (Goetz, 1998). 3-methoxytyramine is metabolized further by monoamine oxidase (MAO) to homovanillic acid. DA can also be metabolized by MAO to DOPAC, which is subsequently converted by COMT to homovanillic acid (Goetz, 1998). Thus, inhibition of COMT in the CNS leads to major changes in concentrations and composition of L-dopa, DA, and their metabolites in the striatum (Goetz, 1998), as well as to enhanced levels of the

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<sup>1</sup> Present address: California Institute of Technology, Division of Biology, Pasadena, CA.

**ABBREVIATIONS:** COMT, catechol-O-methyltransferase; CNS, central nervous system; AADC, aromatic amino acid decarboxylase; DA, dopamine; SAH, S-adenosylhomocysteine; DABA, 2,4-diamino-N-butyric acid; GABA,  $\gamma$ -aminobutyric acid; MAO, monoamine oxidase; 3-OMD, 3-O-methyldopa; OR-486, 3,5-dinitrocatechol; PD, Parkinson's disease; Ro 41-0960, 2'-fluoro-3,4-dihydroxy-5-nitrobenzophenone; SAM, S-adenosyl-L-methionine; TH, tyrosine hydroxylase; THir, tyrosine hydroxylase immunoreactivity; tolcapone, 3,4-dihydroxy-4'-methyl-5-nitrobenzophenone.

methyl donor SAM, and to decreased levels of the demethylated product *S*-adenosylhomocysteine (SAH) in all brain regions (Wurtman, 1972; Yassin et al., 1998).

Although some COMT inhibitors alter metabolism of L-dopa in the periphery and are used to increase the availability of L-dopa to the brain, others inhibit metabolism both peripherally and centrally (Männistö et al., 1992). The latter group would not only increase the amount of L-dopa entering the brain, but also increase the amount of DA present in the brain because of reduced metabolism. Numerous investigators have suggested that both L-dopa and DA can be toxic to DA neurons as a result of increased oxidant stress (for review, see Ling et al., 1996). Both substances are known to increase the production of superoxide and hydroxyl radicals as a result of the Fenton reaction, whereas production of  $\cdot\text{OH}$  is diminished by *O*-methylated catecholamines (Nappi and Vass, 1998). It is thus possible that centrally acting COMT inhibitors may potentiate this toxicity by increasing the percentage of released L-dopa or DA metabolized through oxidative pathways. Unfortunately, this possibility has never been evaluated. Therefore, we set out to determine whether the selective COMT inhibitors 2'-fluoro-3,4-dihydroxy-5-nitrobenzophenone (Ro 41-0960), 3,5-dinitrocatechol (OR-486), and 3,4-dihydroxy-4'-methyl-5-nitrobenzophenone (tolcapone) can potentiate the L-dopa-induced loss of DA neurons normally produced by addition of L-dopa to primarily mesencephalic cell cultures. These cultures were prepared from ventral midbrains of embryonic day 14.5 rat embryos, which contain most of the catecholamine-metabolizing enzymes including COMT (Fiszman et al., 1991) and include astroglial cells reported to express COMT activity in primary cultures (Pelton et al., 1981).

## Experimental Procedures

**Materials.** Ro 41-0960 and OR-486 were from Research Biochemical International (Natick, MA). Tolcapone was kindly provided by Hoffmann LaRoche (Basel, Switzerland). L-dopa, D-dopa, and DA were obtained from Sigma (St. Louis, MO). [ $^3\text{H}$ ]DA (48 Ci/mmol) and [ $^3\text{H}$ ]GABA (86 Ci/mmol) were obtained from Amersham International (Braunschweig, Germany). All other chemicals were of analytical grade. Ro 41-0960, OR-486, and tolcapone stock solutions were freshly prepared in ethanol. Final ethanol concentration never exceeded 0.1% (v/v). This concentration did not influence the survival of tyrosine hydroxylase immunoreactive (THir) neurons.

**Cell Culture.** Primary cultures from rat rostral mesencephalic tegmentum were prepared as described previously (Ling et al., 1996). Briefly, the rostral mesencephalic tegmentum from E14.5 rat embryos (Wistar; Charles River, Sulzfeld, Germany) was dissociated using Trypsin/DNase, and the cells were plated out at 125,000 viable cells/cm<sup>2</sup> on poly-L-lysine-coated 48- or 6-well plates in complete media containing DMEM (high glucose)/F-12 mixture (1:1), 1% penicillin/streptomycin (Sigma), and 10% fetal calf serum (PAA, Cölbe, Germany). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. After 4 days in culture, the medium was changed to that containing various concentrations of the COMT inhibitors or 3-OMD and SAM, respectively, combined with L-dopa or D-dopa at various concentrations and incubated for up to 72 h.

**Quantification of Dopaminergic Cell Survival.** After incubation, the cultures were fixed using 3.7% paraformaldehyde and then immunocytochemically processed for tyrosine hydroxylase (TH) using a monoclonal antibody against rat TH (Boehringer Mannheim, Mannheim, Germany) and nickel enhancement. The numbers of

THir cells were assessed by an investigator blinded to treatment history using a Zeiss (Göttingen, Germany) inverted microscope, counting black cell bodies of the whole well surface area. Based on the tissue dissection procedure and the absence of immunoreactivity for the noradrenergic neuron marker DA- $\beta$  hydroxylase in sister cultures, it is likely that THir cells in our cultures were phenotypically DA neurons.

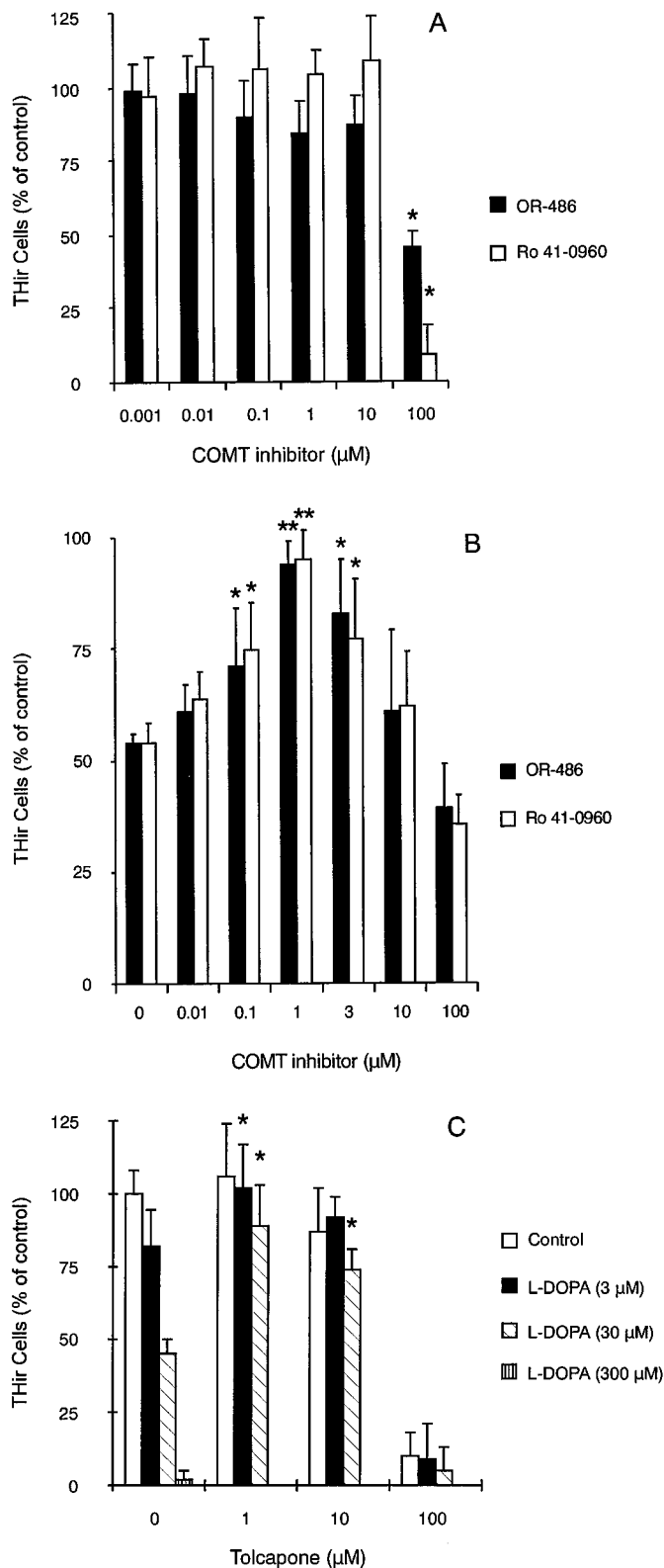
**Neurotransmitter Uptake.** The functional integrity of DA and GABA neurons was evaluated by measuring the uptake of their respective tritiated neurotransmitter, according to Spina et al. (1992). After preincubation for 10 min in incubation buffer containing 100  $\mu\text{M}$  pargyline, 1 mM ascorbate, and 2 mM  $\beta$ -alanine (and for determination of nonspecific uptake, 3  $\mu\text{M}$  GBR12909 and 1 mM 2,4-diamino-*N*-butyric acid; DABA), 50 nM [ $^3\text{H}$ ]DA or [ $^3\text{H}$ ]GABA was added for 15 min at 37°C. Uptake was stopped by washing the dishes with cold PBS, and the remaining radioactivity in the cell lysate was measured using liquid scintillation counting. Specific uptake was defined as the difference between the uptake measured in the absence (total) and the uptake measured in the presence of GBR12909 and DABA (nonspecific).

**Statistical Analysis.** EC<sub>50</sub> and TD<sub>50</sub> values were calculated by nonlinear regression analysis using the iterative curve fitting program Origin (Version 5.0; MicroCal Software, Northampton, MA). Results were expressed as mean  $\pm$  S.E. of at least three independent experiments and compared using unpaired two-tailed *t* tests.

## Results

**Effects of COMT Inhibition on Survival of DA Neurons.** Before investigating the effects of COMT inhibitors on L-dopa-induced toxicity, we determined the effects of the specific COMT inhibitors Ro 41-0960 and OR-486 on THir cell counts in mesencephalic cultures from rat. Ro 41-0960 and OR-486 did not influence THir cell counts in concentrations up to 10  $\mu\text{M}$  after 72 h, whereas 100  $\mu\text{M}$  significantly reduced THir cell survival to  $14 \pm 7$  and  $47 \pm 12\%$  of control for Ro 41-0960 and OR-486, respectively (Fig. 1A). Based on gross microscopic inspection of the cultures, these high concentrations of COMT inhibitors killed all cell types. Furthermore, in cultures treated with 1  $\mu\text{M}$  Ro 41-0960 for 24 h, the uptake of the neurotransmitter DA, an index of both the function and the degree of differentiation of dopaminergic cells, was not significantly affected (Fig. 2). In addition, the uptake capacity of GABA neurons was also not significantly altered (Fig. 2).

**COMT Inhibitors Protect DA Neurons Against L-dopa-Induced Toxicity.** L-dopa caused a dose-related decrease of THir cells in 4-day-old mesencephalic cultures from rat, with a calculated TD<sub>50</sub> value obtained from the toxicity curve by nonlinear regression analysis of  $21.3 \pm 1.4 \mu\text{M}$  after 24 h incubation (Fig. 3). Using the approximate TD<sub>50</sub> value (30  $\mu\text{M}$ ), the effects of various concentrations of both Ro 41-0960 and OR-486 on L-dopa-induced toxicity in THir cells were determined. Both substances showed a concentration-dependent reduction of L-dopa-induced THir cell loss, with a maximum at 1  $\mu\text{M}$  and estimated EC<sub>50</sub> values of  $0.1 \pm 0.02$  and  $0.11 \pm 0.03 \mu\text{M}$  for Ro 41-0960 and OR-486, respectively (Fig. 1B). Similar results were obtained using tolcapone (Fig. 1C), a peripherally and centrally acting COMT inhibitor currently used in the treatment of PD (Kurth and Adler, 1998). Investigating the integrity of DA neurons by measuring the DA uptake revealed a reduction of DA uptake of  $74 \pm 7\%$  after treatment of mesencephalic cultures with 30  $\mu\text{M}$  L-dopa for 24 h (Fig. 2; *P* < .01). This toxic effect was significantly



**Fig. 1.** A, effects of the COMT inhibitors Ro 41-0960 and OR-486 on survival of DA neurons after 72 h. B, effects of various concentrations of the COMT inhibitors Ro 41-0960 and OR-486 on toxicity induced by 30  $\mu$ M L-dopa toward DA neurons after 24 h. C, effects of the COMT inhibitor tolcapone (1–100  $\mu$ M) on toxicity induced by L-dopa (3–300  $\mu$ M) on DA neurons in mesencephalic cultures after 24 h. Please note the toxic effects of tolcapone with concentrations  $>10$   $\mu$ M. In all experiments, the cells were cultured for 4 days before treatment with various concentrations of the respective COMT inhibitor alone for 72 h (A) or with various

reduced by coincubation with 1  $\mu$ M Ro 41-0960 (Fig. 2). In contrast, no significant changes of GABA uptake were observed in cultures treated with L-dopa alone or L-dopa and 1  $\mu$ M Ro 41-0960 (Fig. 2).

Furthermore, a concentration of 1  $\mu$ M Ro 41-0960, which did not affect THir neuron survival when administered alone, shifted the L-dopa survival curve significantly to the right (Fig. 3A). Thus, in the presence of 1  $\mu$ M Ro 41-0960, the 10, 30, and 100  $\mu$ M concentrations of L-dopa exhibited significantly higher THir cell counts relative to cultures treated with L-dopa alone ( $P < .05$ ). The calculated  $TD_{50}$  value obtained from the toxicity curves by nonlinear regression analysis was increased from  $21.3 \pm 1.4$   $\mu$ M for L-dopa alone up to  $71.3 \pm 9.7$   $\mu$ M for L-dopa combined with Ro 41-0960 (significantly different with  $P < .01$ ).

**Ro 41-0960 Does not Affect D-dopa-Induced Toxicity.** To demonstrate the specificity of the described effect of COMT inhibition on L-dopa-induced toxicity, we investigated the effect of COMT inhibition on the toxicity of the metabolically inactive stereoisomer D-dopa. As shown in Fig. 3B, D-dopa showed a dose-dependent toxicity toward THir cells, with a  $TD_{50}$  value of  $77.3 \pm 7.7$   $\mu$ M after 24 h, which is in the same range as that observed with L-dopa in combination with Ro 41-0960. Coincubation with 1  $\mu$ M Ro 41-0960 had no significant effect on D-dopa-induced toxicity ( $TD_{50} = 71.6 \pm 5.2$   $\mu$ M; Fig. 3B).

**Effects of 3-OMD on Survival of DA Neurons and L-dopa-Induced Toxicity.** The primary metabolite of L-dopa formed by COMT is the 3-methylated product 3-OMD. To investigate the involvement of 3-OMD in L-dopa-induced toxicity and the protection of COMT inhibitors, we studied the effects of 3-OMD alone and in combination with L-dopa on survival of DA neurons. As shown in Fig. 4A, 3-OMD displayed no toxic effects toward THir neurons, with concentration up to 1000  $\mu$ M. Furthermore, 3-OMD did not alter L-dopa-induced toxicity significantly (Fig. 4A).

**Effects of SAM on Survival of DA Neurons and L-dopa-Induced Toxicity.** Because COMT uses SAM as the methyl group donor, we tested the hypothesis that the protective effects of COMT inhibitors on L-dopa toxicity are attributable to the prevention of SAM depletion by direct manipulation of SAM levels in our cultures. Before this investigation, we demonstrated that SAM did not affect the growth of THir cells with concentrations up to 100  $\mu$ M when studied alone; only very high doses (1000  $\mu$ M) caused a reduction in THir cell survival to  $24 \pm 7\%$  of control (Fig. 4B). As shown in Fig. 4B, addition of SAM did not show significant protective effects against L-dopa-induced toxicity in mesencephalic cultures after 24 h.

## Discussion

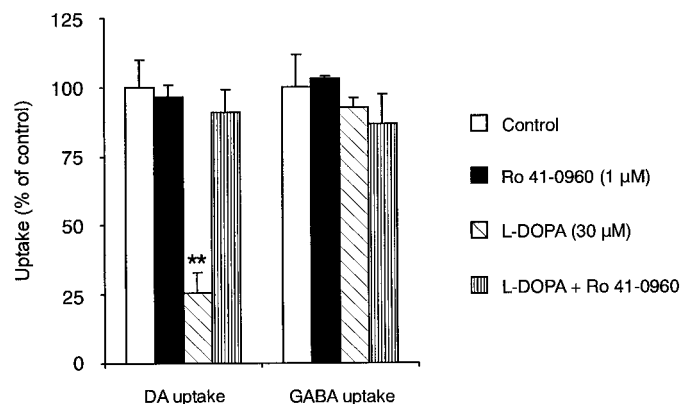
To our knowledge, these results are the first demonstration of protective effects of COMT inhibition on DA neurons in vitro. This study shows that all selective COMT inhibitors tested (Ro 41-0960, OR-486, and the clinically used tolca-

concentrations of L-dopa alone or in combination with the respective COMT inhibitor for 24 h (B and C). Survival of THir neurons is expressed as percentage of untreated control. Control values were  $402 \pm 142$  THir cells/well. Data points represent the mean  $\pm$  S.E. of at least three independent experiments. \* $P < .05$ ; \*\* $P < .01$ , when compared with untreated control (A) or L-dopa alone (B).

pone) produced protection of DA neurons against L-dopa-induced toxicity, with a maximum effect at 1  $\mu\text{M}$ . Ro 41-0960 at a concentration of 1  $\mu\text{M}$  shifted the  $\text{TD}_{50}$  value of L-dopa from 21  $\mu\text{M}$  in the absence to 72  $\mu\text{M}$  in the presence of COMT inhibition. Therefore, Ro 41-0960 reduced the toxic effects of L-dopa more than 3-fold.

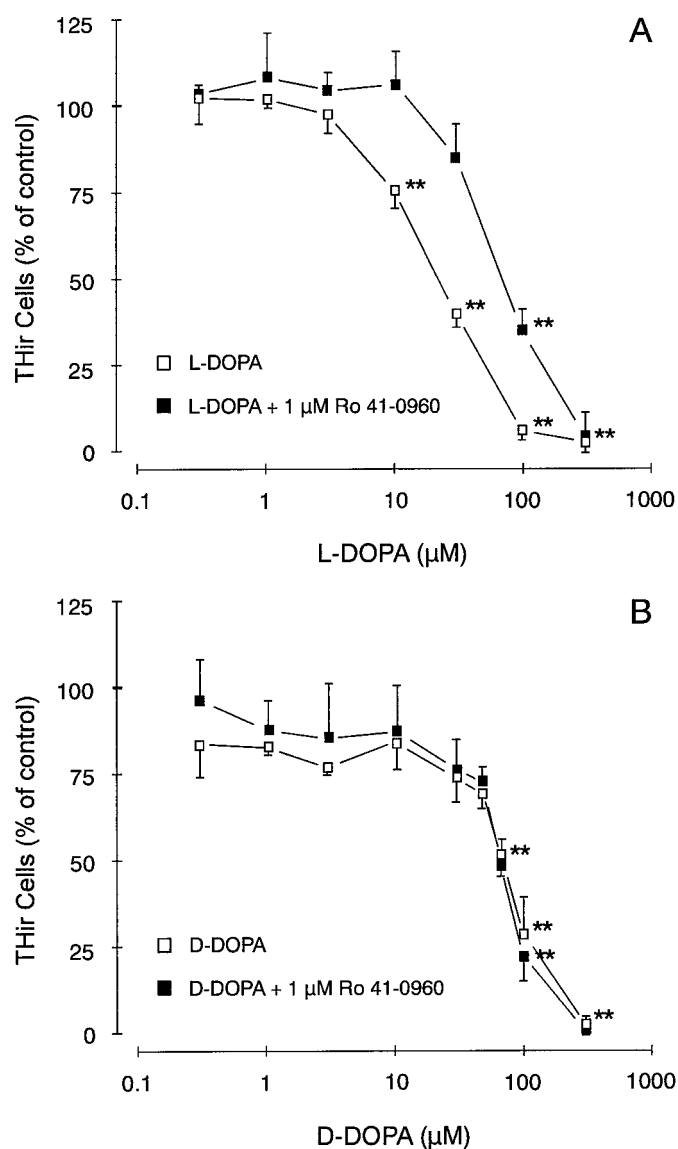
The protective effects of COMT inhibitors against L-dopa-induced toxicity are most likely attributable to their inhibition of COMT activity because the concentrations needed for the reduction of L-dopa-induced toxicity were in the nanomolar range, with estimated  $\text{EC}_{50}$  values of approximately 100 and 110 nM for Ro 41-0960 and OR-486, respectively, in good agreement with the published potencies for inhibition of COMT activity by these compounds (Zürcher et al., 1990; Vieira-Coelho and Soares-da-Silva, 1999); at concentrations needed for attenuation of L-dopa toxicity, both compounds are very specific COMT inhibitors and lack activity on amine-synthesizing and other metabolizing enzymes, including several methyltransferases (Männistö et al., 1992; Nissinen et al., 1988); and, finally, the failure to prevent D-dopa toxicity corresponds to the stereoselectivity of COMT for the L-isomer of dopa (Gordonsmith et al., 1982). In contrast, the toxic properties of the COMT inhibitors at concentrations  $>10 \mu\text{M}$  are probably unrelated to their inhibitory effects on COMT activity, because these concentrations are  $>10$ -fold higher than those needed for complete inhibition of the enzyme (Zürcher et al., 1990; Vieira-Coelho et al., 1999). Furthermore, these toxic effects are rather nonspecific and involve all cell types, including glial cells, in mesencephalic cultures.

The mechanism by which inhibition of COMT can rescue DA neurons from L-dopa-induced toxicity is unclear. The following interactions may be involved. The changes of L-dopa metabolism caused by COMT inhibitors may lead to decreased levels of toxic metabolites or enhanced levels of protective substances. The data presented show that L-dopa is more toxic to DA neurons than D-dopa, which is not a substrate for most of the metabolizing enzymes [such as aromatic amino acid decarboxylase (AADC) and MAO]; however, in the presence of COMT inhibitors, the two stereoisomers are similarly potent, suggesting that in our cell system, L-dopa is toxic owing to both enzymatic (COMT-dependent)



**Fig. 2.** Effects of COMT inhibition on DA and GABA uptake in mesencephalic cultures. Cells were cultured for 4 days before treatment with 30  $\mu\text{M}$  L-dopa with or without 1  $\mu\text{M}$  Ro 41-0960 for 24 h. After treatment, the [ $^3\text{H}$ ]DA and [ $^3\text{H}$ ]GABA uptake was measured in sister cultures. Control values were  $7.8 \pm 0.76$  and  $169.4 \pm 19.7$  pmol/min/dish for specific DA and GABA uptake, respectively. Data points represent the mean  $\pm$  S.E. of three independent experiments. \* $P < .01$  compared with control.

and nonenzymatic pathways, whereas D-dopa is toxic owing to nonenzymatic processes only. These results are in contrast to the data presented by Ling and coworkers showing similar toxic potency of both isomers (Ling et al., 1996), but these discrepancies are probably explained by the different treatment paradigms used in both studies: In contrast to Ling and coworkers (1996), who used mesencephalic cultures 24 h after plating, the present study works with cultures after 4 days in vitro containing higher amounts of proliferating glial cells, which express several enzymes of catecholamine metabolism, including COMT (Pelton et al., 1981; Fiszman et al., 1991; Männistö et al., 1992). However, the primary metabolite of L-dopa formed by COMT, 3-OMD, display no dopaminergic toxicity, showing that conversion of L-dopa to 3-OMD does not contribute to L-dopa toxicity and, consis-



**Fig. 3.** Effects of the COMT inhibitor Ro 41-0960 (1  $\mu\text{M}$ ) on survival of THir neurons in mesencephalic cultures exposed to various concentrations of L-dopa (A) and D-dopa (B), respectively, for 24 h. Cells were cultured for 4 days before drug treatment. Values are expressed as a percentage of untreated controls ( $390 \pm 82$  THir cells/well). Data points represent the mean  $\pm$  S.E. of three to five independent experiments. \* $P < .05$ ; \*\* $P < .01$  compared with untreated control.



tently, attenuation of 3-OMD production is not responsible for the protective effects of COMT inhibition.

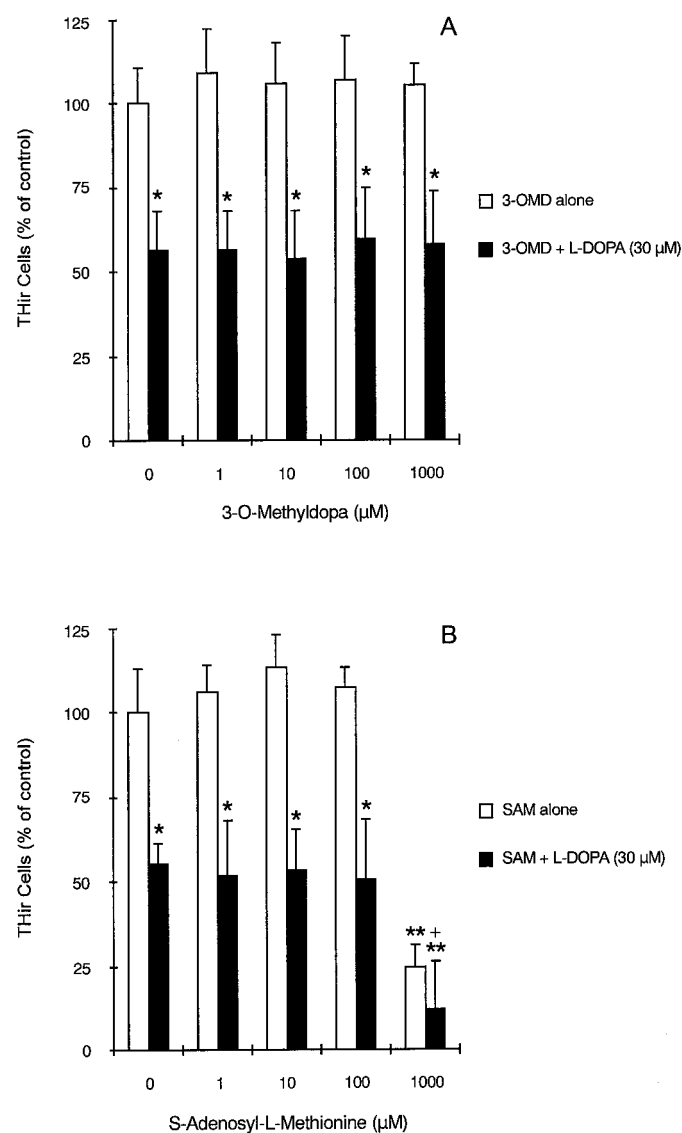
The metabolism of L-dopa by COMT causes decreased levels of the methyl donor SAM and increased levels of SAH in rat brain (Yassin et al., 1998). Thus, it has been hypothesized that by decreasing SAM concentrations, L-dopa renders SAM unavailable for other methylation reactions that are important for structure and function (Wurtman, 1972). Increased SAH may impair these methylations by feedback inhibition (Degushi and Barchas, 1971). Therefore, COMT inhibition may normalize SAM turnover and activity of methylation reactions in L-dopa-treated tissue or cell culture. However,

increasing SAM levels in our cell system do not alter survival of DA neurons or L-dopa-induced toxicity toward DA neurons *in vitro*, suggesting that prevention of SAM depletion plays not a pivotal role for the protection of DA neurons against L-dopa toxicity by COMT inhibition.

L-dopa and 3-OMD compete for various transport systems, including the neutral L-amino acid transport system of glial cells in the CNS (Reches et al., 1982; Männistö et al., 1992). Thus, 3-OMD may be able to block extracellular clearance of L-dopa by glial cells and, therefore, may increase L-dopa concentration acting on DA neurons. Because 3-OMD does not augment L-dopa toxicity, this mechanism seems not to be relevant in our cell system. However, it has been reported that COMT inhibition enhances cellular accumulation of L-dopa in red blood cells and rat striatum (Reches et al., 1982) and may increase intracellular storage and transmembrane uptake kinetic of catecholamines in mammalian cells (Eshleman et al., 1997). These data lead to the hypothesis that inhibition of COMT activity in our cell system increases the uptake of L-dopa or its metabolites from the culture medium into the cytoplasm of glial cells leading to decreased extracellular concentrations of toxic compounds.

In this study, L-dopa induced dose-dependent neurotoxic effects on DA neurons in mesencephalic cultures *in vitro*, as reported previously by Ling et al. (1996) and several other investigators (for review, see Ling et al., 1996). Because several investigators failed to demonstrate similar effects *in vivo* (Hefti et al., 1981; Perry et al., 1984), the relevance of our findings for *in vivo* conditions remains unclear. However, it is important to note that most of these studies used inadequate doses of AADC inhibitor to block peripheral inactivation of L-dopa and, in addition, used young animals with intact nigrostriatal pathway (Hefti et al., 1981; Perry et al., 1984; for detailed discussion, see Ling et al., 1996). A recent study using animals with partially lesioned ventral mesencephalon showed additional DA neuron loss after long-term treatment with L-dopa (Blunt et al., 1993). This study, together with the numerous *in vitro* studies showing L-dopa neurotoxicity, suggests that the cytoprotective actions of COMT inhibition against L-dopa-induced toxicity may be relevant in altered DA neurons *in vivo* and therefore would be potentially important in the management of PD.

We conclude that inhibition of COMT activity leads to a protection of DA against L-dopa-induced toxicity *in vitro*. This protection is stereoselective for the L-isomer of dopa and therefore probably involves changes of the metabolism and/or uptake kinetics of L-dopa by glial cells. The exact underlying mechanism awaits elucidation, but preventing 3-OMD production or cellular SAM depletion is probably not responsible for the described protective effects of COMT inhibition. Future studies have to clarify whether these protective effects are also present *in vivo* and may play a role as a therapeutic strategy in PD.



**Fig. 4.** Effects of various concentrations of 3-OMD (A) and SAM (B), respectively, on survival of DA neurons and dopaminergic toxicity induced by L-dopa (30  $\mu$ M) after 24 h. Cells were cultured for 4 days before drug treatment. Survival of THir neurons is expressed as percentage of untreated control. Unpaired Student's *t* test revealed no statistical difference in THir neuron survival in cultures treated with various concentrations of 3-OMD or SAM alone or in combination with 30  $\mu$ M L-dopa relative to untreated control cultures and L-dopa-treated cultures, respectively (except 1000  $\mu$ M SAM). Data points represent the mean  $\pm$  S.E. of at least three independent experiments. \**P* < .05; \*\**P* < .01 compared with untreated control; + indicates *P* < .05 compared with L-dopa-treated cultures.

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**Send reprint requests to:** Alexander Storch, M.D., University of Ulm Medical School, Department of Neurology, Oberer Eselsberg 45, 89081 Ulm, Germany. E-mail: alexander.storch@medizin.uni-ulm.de

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