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In vivo effects of new inhibitors of catechol-*O*-methyl transferase

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1 The effects of two new synthetic compounds showing *in vitro* catechol-*O*-methyl transferase (COMT) inhibitor properties were studied *in vivo* and compared with the effects of nitecapone and Ro-41-0960.

2 QO IA (3-(3-hydroxy-4-methoxy-5-nitrobenzylidene)-2,4-pentanedione), QO IIR ([2-(3,4-dihydroxy-2-nitrophenyl)vinyl]phenyl ketone), nitecapone and Ro-41-0960 (30 mg kg^{-1}, i.p.) were given to reserpinized rats 1 h before the administration of L-DOPA/carbidopa (LD/CD, 50 : 50 mg kg^{-1}, i.p.). Locomotor activity was assessed 1 h later. All the COMT inhibitors (COMTI), with the exception of QO IA, markedly potentiated LD/CD reversal of reserpine-induced akinesia. Similar results were obtained when the COMTI were coadministered with LD/CD. The effect of compound QO IIR was dose-dependent (7.5–30 mg kg^{-1}, i.p.).

3 The COMTI (30 mg kg^{-1}, i.p.) potentiated LD/CD reversal of both catalepsy and hypothermia of reserpinized mice.

4 QO IIR, nitecapone and Ro-41-0960 (30 mg kg^{-1}, i.p.) reduced striatal 3-methyl-DOPA (3-OMD) levels and increased dopamine (DA) and dihydroxyphenylacetic acid (DOPAC) levels. Compound QO IA was devoid of any effect on striatal amine levels. In contrast to the other inhibitors, Ro-41-0961 reduced HVA levels as well. The effect of QO IIR on striatal amine levels was dose-dependent (7.5–60 mg kg^{-1}, i.p.)

5 These results suggest that the new compound QO IIR is an effective peripherally acting COMT inhibitor *in vivo*.

Keywords: Amines; antiparkinsonian therapy; COMT; L-DOPA; Parkinson's disease

Introduction

Catechol-*O*-methyl transferase (COMT; EC 2.1.1.6) is one of the enzymes involved in the deactivation of cathecolamines and drugs with a catechol structure (Guldberg & Marsden, 1975). Levodopa (LD) is widely used in the treatment of Parkinson's disease (PD) (Papavasilous *et al*., 1972; Bartholini & Pletscher, 1975), which is characterized by a marked reduction of dopamine (DA) levels in the striatum. LD is converted by dopa decarboxylase (DDC) to DA, both in peripheral tissues and in brain. To allow higher concentrations of LD to reach the brain and to reduce peripheral side effects of the drug, it is coadministered in combination with a peripheral DDC inhibitor (eg. carbidopa or benserazide). In this situation LD is largely converted into 3-*O*-methyl-DOPA (3-OMD; Sharpless *et al*., 1972; Sandler *et al*., 1974) by COMT. Accumulation of 3-OMD may be detrimental for PD patients since it competes with LD for the active transport through the intestinal mucosa and the blood-brain-barrier (Muenter *et al*., 1973; Reches *et al*., 1982). Thus, selective COMT inhibition improves the bio-availability of LD (Cedarbaum *et al*., 1991; Nissinen *et al*., 1992; Jorga *et al*., 1997) and allows a decrease in its dose as shown in recent clinical trials (Rajput *et al*., 1997; Yamamoto *et al*., 1997; Adler *et al*., 1998; Baas *et al*., 1998).

During the last years, new catechol derivatives that induce potent and selective COMT inhibition both *in vitro* and *in vivo* have been synthesized (Männistö *et al*., 1988; Bäckstrom *et al*., 1989; Borgulya *et al*., 1989; Nissinen *et al*., 1988; 1992). Most importantly, these compounds have been shown to be effective in experimental animals models of PD, following their administration with LD/CD (Lindén *et al*., 1988; Maj *et al*., 1990). Furthermore, clinical trials have demonstrated that they may be succesfully used as coadjuvants in antiparkinsonian therapy (Rajput *et al*., 1997; Yamamoto *et al*., 1997; Adler *et al*., 1998; Baas *et al*., 1998). We have previously reported the synthesis and *in vitro* COMT inhibitor (COMTI) properties of several new synthetic compounds (Pérez *et al*., 1993; 1994). In this work we have studied the effects of 3-(3-hydroxy-4-methoxy-5-nitrobenzylidene)-2,4-pentanedione (QO IA) and [2-(3,4-dihydroxy-2-nitrophenyl)vinyl]phenyl ketone (QO IIR), which showed appropriate *in vitro* potency. The behavioural and neurochemical effects of these new compounds and two known COMTI (nitecapone and Ro-41-0960) were investigated after their administration in combination with LD/CD to rodents. Taken together our results show that QO IIR is an effective COMTI following its *in vivo* administration, showing great similarities with nitecapone. However, QO IA at the dose used (30 mg kg^{-1}) was ineffective in the different assays.

Methods

Animals

Male Sprague-Dawley rats weighing 350 ± 50 g were housed four per cage and male CD1 albino mice weighing 27 ± 3 g were housed 15 per cage (Animal facilities of the University of Santiago de Compostela) at 21 ± 1°C in 12 : 12 h dark:light cycle. The animals had free access to water and food. All experiments were performed according to the guidelines of the EC.

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Drugs

D-amphetamine sulphate, reserpine and LD (3,4-dihydroxyphenylalanine) were from SIGMA; Ro-41-0960 (2-fluoro-3,4-dihydroxy-5-nitrobenzophenone) from Research Biochemicals International (RBI) and S(−)-carbidopa (CD) from Dupont Pharma; while the other COMTI were synthesized at the Institute of Organic Chemistry (CSIC):QO IA, QO IIR and nitecapone (OR-462: 3-(3,4-dihydroxy-5-nitrobenziliden)-2,4-pentanedione).

Amine standards used in the HPLC measurements were purchased from SIGMA; all biochemical reagents, analytical or HPLC grade, and the carboxy-methyl-cellulose were from MERCK.

Reserpine was solubilized in water containing lactic acid (1% v v^{-1}); D-amphetamine sulphate in saline (0.9% NaCl) and all other drugs were suspended in sodium carboxymethylcellulose (1% w v^{-1}). Drugs were prepared immediately before use and were administered intraperitoneally (i.p.) in a volumen of 0.01 ml g^{-1}.

LD (50 mg kg^{-1}, i.p.), CD (50 mg kg^{-1}, i.p.) and the inhibitors (30 mg kg^{-1}, i.p.) were given to rats 2 h before sacrifice. In another experiment the inhibitors were given 1 h before the administration of LD/CD.

Behavioural studies

Locomotor activity Twenty-four hours prior the experiment rats were placed in individual cages, where the locomotor activity was going to be measured and they had free access to food and water. Rats received reserpine 5 mg kg^{-1} i.p. 18 h before measurements. Locomotor activity was measured in activity cages (Panlab Actisystem DAS 16 V.1), which contained an electromagnetic field sensitive to any motion within it. All the experiments were done at the same time of the day (09.00–13.00 h) to avoid alterations due to circadian rythms. Results are accumulated counts during 1 h (mean ± s.e.mean).

Reserpine-induced hypothermia Reserpine 5 mg kg^{-1} was given to mice 18–20 h before drug administration. Rectal body temperature was measured with a rectal probe connected to a thermometer. Three measurements (30 min apart) were averaged and considered as basal temperature. After drug administration (LD/CD alone or coadministered with the COMTI) measurements were done every 30 min for 2 h. Results are expressed as change in temperature (Δ°C) over basal temperature.

Reserpine-induced catalepsy To assess catalepsy after reserpine treatment (see above), mice were placed with their forepaws on one horizontal wire and their hindpaws on another 6 cm apart and 2 cm lower. Time (s) spent in this position was recorded and the cut-off time was 30 s. Catalepsy

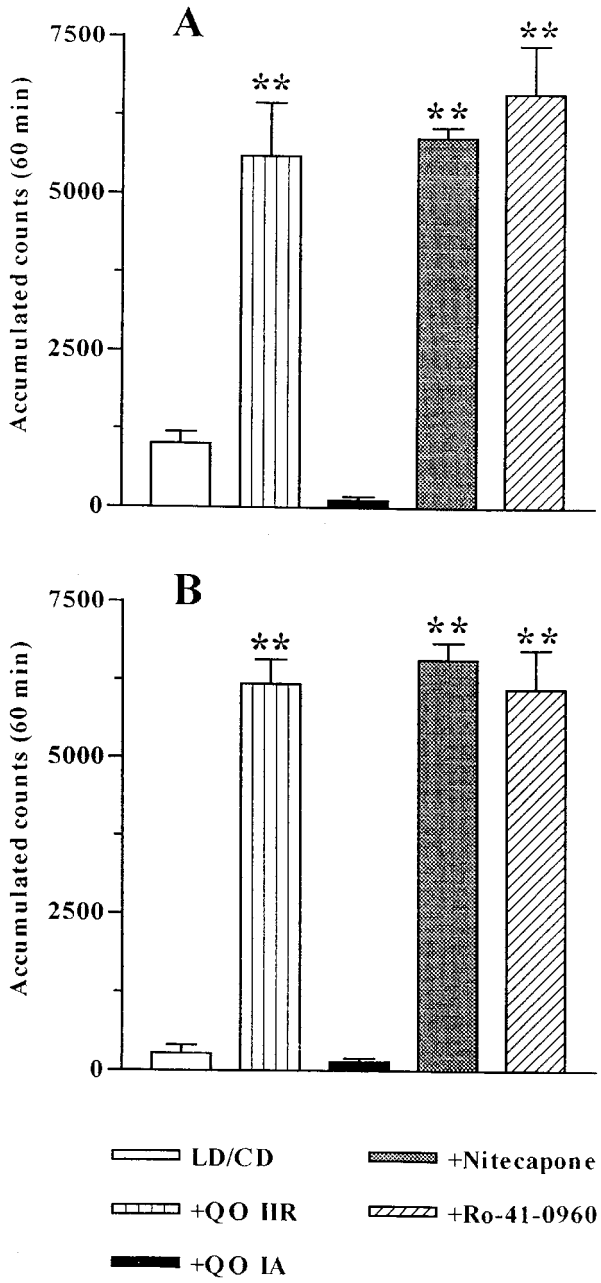

Figure 2 Effect of COMTI on locomotor activity of reserpinized rats treated with LD/CD. (A) COMTI (30 mg kg^{-1}, i.p.) were administered to rats 1 h before LD/CD (50 : 50 mg kg^{-1}, i.p.) and locomotor activity was measured 1 h later for 1 h. (B) COMTI (30 mg kg^{-1}, i.p.) were coadministered to rats with LD/CD (50 : 50 mg kg^{-1}, i.p.) and locomotor activity was measured 1 h later for 1 h. Reserpinized rats showed akinesia (accumulated counts during 1 h : 36.33 ± 9.49). Results are mean ± s.e.mean ($n = 5–8$) of accumulated counts during 1 h. $^{**}P < 0.01$ significant difference versus LD/CD treated rats (one-way ANOVA followed by Dunnett's test).

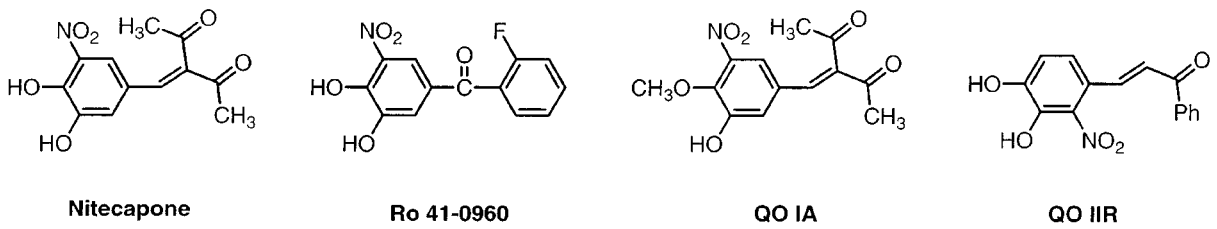

Figure 1 Chemical structures of the COMTI.

was scored every 30 min for 2 h after the administration of LD/CD alone or in combination with the COMTI.

Amines and their metabolites measurements The same animals used in the locomotor activity studies were sacrificed at the end of the experiments for amine measurements. Both striata were removed on ice and homogenized (Ultraturrax, set 5, 10 s) in 20 volumes of 0.1 M perchloric acid containing 40 μM sodium bisulphite. Following centrifugation of samples ($10{,}000 \times g$) aliquots of the supernatants were filtered (Millipore HV 0.45 μm) and analysed or stored at -80°C until analysis. Amines and their metabolites were measured by a standard HPLC with coulometric detection method. In brief, samples were injected onto a reverse phase column (15×0.4 cm, Spherisorb ODS 2, 5 μm) and eluted with 0.1 M citric acid-0.1 M sodium citrate buffer pH 4.0 containing 12% methanol and 0.83 mM octane sulphonic acid, at a flow rate of 1 ml min^{-1}. Results are expressed in μg g^{-1} of tissue. Detection limit was 20 pg for DA, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIIA) and 50 pg for 3-OMD and homovanillic acid (HVA).

Statistical analysis

Statistical analyses and graphs were carried out with GraphPad Prism software (v2.1, Sorrento, U.S.A.). Statistical significant differences were determined by one-way (treatment) analysis of variance (ANOVA), followed by Dunnett's *post hoc* test or by repeated measures ANOVA followed by Dunnett's multiple comparison test (catalepsy and hypothermia).

Results

Effects of COMTI on locomotor activity of reserpinized rats treated with LD/CD

The structures of the reference COMTI and the two new compounds shared some similarities, as shown in Figure 1.

The doses of LD/CD (50 : 50 mg kg^{-1}, i.p.) and the COMTI (30 mg kg^{-1}, i.p.) used in this study were based in those previously shown to be effective by other authors (Männistö *et al*., 1992; Lindén *et al*., 1988). In one experiment the COMTI were administered 1 h before LD/CD administration and locomotor activity was evaluated 1 h later for 1 h. LD/CD (50 : 50 mg kg^{-1}, i.p.) reversed reserpine-induced akinesia ($P<0.01$, Figure 2A). All the COMTI, with the exception of QO IA, markedly potentiated (6- fold increase, $P<0.01$) LD/CD reversal of reserpine-induced akinesia, being equally effective at the dose used (30 mg kg^{-1}, i.p.). When the inhibitors were coadministered with LD/CD similar results were obtained (Figure 2B, $P<0.01$). Again QO IA was without effect, while QO IIR and the reference COMTI markedly potentiated the effect of LD/CD on locomotor activity of reserpinized rats. The effect of increasing doses of the new compound QO IIR on locomotor activity induced by LD/CD is shown in Figure 3. QO IIR dose-dependently potentiated (11.25 – 30 mg kg^{-1}, $P<0.01$) the LD/CD reversal of reserpine-induced akinesia. The lowest dose assessed was without effect, as was the dose of 60 mg kg^{-1}, the highest dose tested.

Effect of COMTI on reserpine-induced catalepsy

The effect of COMTI on catalepsy displayed by reserpinized mice is shown in Figure 4. LD/CD induced a significant ($P<0.01$), although transient, reversal of reserpine-induced catalepsy, observed 30 min post-administration. In contrast, the COMTI markedly potentiated the LD/CD effect up to 2 h after their administration. However, while nitecapone only induced a partial reversal (around a 40% reduction in time spent in the forced posture, $P<0.01$), Ro-41-0960 and compound QO IIR fully reversed catalepsy (90% reduction, $P<0.01$).

Effect of COMTI on reserpine-induced hypothermia

Reserpinized mice showed a marked decrease in body temperature, approximately 4°C. LD/CD non-significantly

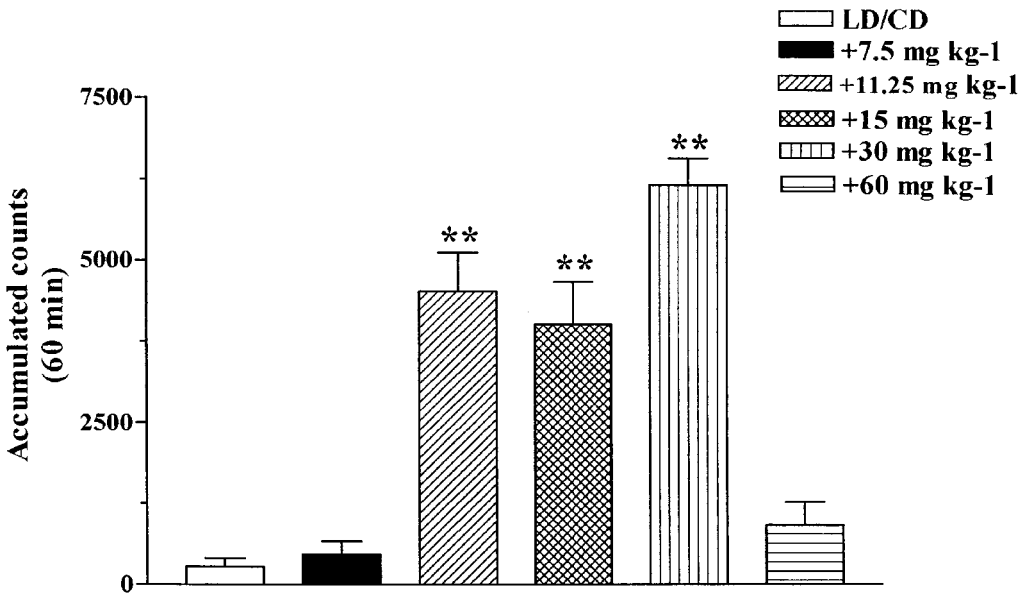

Figure 3 Dose-response curve of the effect of QO IIR coadministered with LD/CD on locomotor activity of reserpinized rats. Increasing doses (7.5 – 60 mg kg^{-1}, i.p.) of QO IIR were coadministered to rats with LD/CD (50 : 50 mg kg^{-1}, i.p.) and locomotor activity was measured 1 h later for 1 h. Reserpinized rats showed akinesia (accumulated counts during 1 h : 36.33 ± 9.49) Results are mean $\pm$ s.e.mean ($n = 5-8$) of accumulated counts during 1 h. $^{**}P<0.01$ significant difference versus LD/CD treated rats (one-way ANOVA followed by Dunnett's test).

increased the body temperature (around 1°C) of reserpinized rats. This effect was significantly potentiated (4–5 fold increase, $P<0.01$) by all the COMTI to the same extent (Figure 5).

Effect of COMTI on striatal amine levels of reserpinized rats treated with LD/CD

Treatment of reserpinized rats with LD/CD resulted in high 3-OMD striatal levels (Table 1). Striatal levels of 3-OMD were significantly reduced by nitecapone, Ro-41-0960 and QO IIR (30 mg kg^{-1}, i.p.). This dose of QO IA was devoid of any effect. The 3-OMD/LD ratio obtained for the different treatments is shown in Table 1. The high ratio observed following LD/CD treatment was not modified by compound QO IA, but was effectively reduced ($P<0.01$) by the other three COMTI under study. The decrease (to 20%) induced by QO IIR was similar to that induced by nitecapone. However, Ro-41-0960 showed a higher effect (to 5%). The decrease in 3-OMD/LD ratio induced by QO IIR treatment tended to be dose-dependent (Table 1), reflecting the dose dependent decrease in 3-OMD striatal levels (data not shown).

As shown in Figure 6, treatment of reserpinized mice with the COMTI 1 h before LD/CD administration induced significant increments in striatal DA (3–6 fold, $P<0.05$–0.01) and DOPAC levels (2–3 fold, $P<0.01$), with the exception of QO IA, which had no effect. HVA levels were only modified by Ro-41-0960, that markedly reduced its levels (to 5%, $P<0.01$).

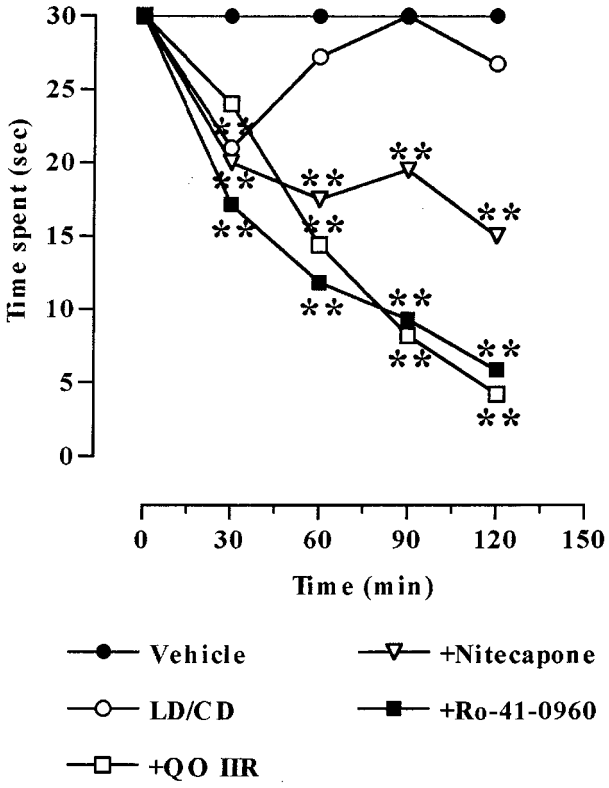

Figure 4 Effects of COMTI (30 mg kg^{-1}, i.p.) coadministered with LD/CD (50:50 mg kg^{-1}, i.p.) on reserpine-induced catalepsy. Results (time spent in the forced posture) are mean ($n=5-8$); s.e.mean which ranged between 0.00–5.55, were omitted for the sake of clarity. $^{**}P<0.01$ versus reserpinized vehicle-treated animals (repeated measures ANOVA followed by Dunnett's multiple comparison test).

At 2 h after LD/CD administered in combination with QO IIR a dose-dependent increase in DA (3–9 fold) and DOPAC (52–98%) levels was observed (Figure 7) compared to LD/CD treatment. However, the dose-response curve in the case of DA

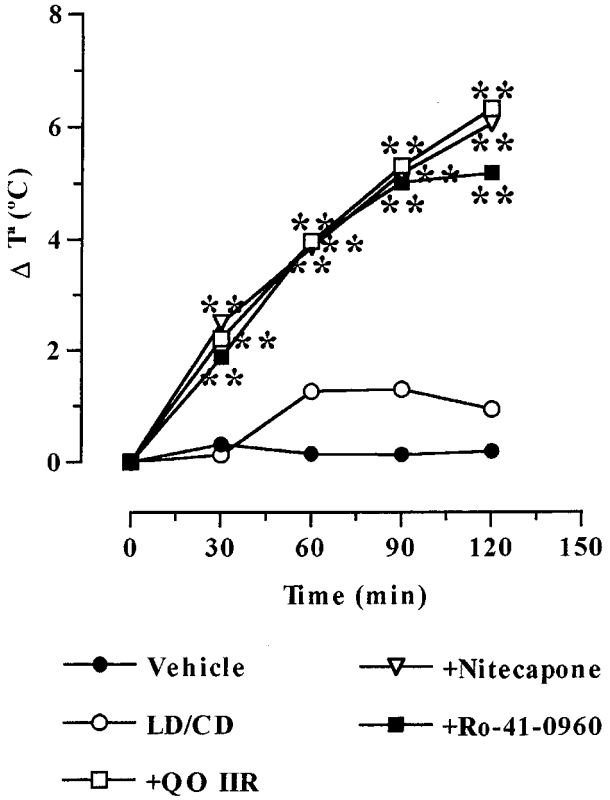

Figure 5 Effects of COMTI (30 mg kg^{-1}, i.p.) coadministered with LD/CD (50:50 mg kg^{-1}, i.p.) on reserpine-induced hypothermia. Results (increments in temperature: Δ°C) are mean ($n=5-8$); s.e.mean, which ranged between 0.12–1.73, were omitted for the sake of clarity. $^{**}P<0.01$ significant difference versus reserpinized vehicle-treated animals (repeated measures ANOVA followed by Dunnett's multiple comparison test).

Table 1 Effect of COMTI on 3-OMD/LD ratio in reserpinized rats treated with LD/CD

Treatments	*3-OMD/LD*
Vehicle + vehicle*	0.00
Vehicle + LD/CD†	10.73 ± 2.87
QO IIR + LD/CD	2.03 ± 0.93
QO IA + LD/CD	9.16 ± 3.32
Nitecapone + LD/CD	2.07 ± 0.98
Ro-41-0960 + LD/CD	0.47 ± 0.38
Vehicle + LD/CD$^{\#}$	10.59 ± 1.58
QO IIR 7.5 + LD/CD	2.94 ± 0.78
QO IIR 11.25 + LD/CD	3.80 ± 0.81
QO IIR 15 + LD/CD	2.89 ± 0.40
QO IIR 30 + LD/CD	1.27 ± 0.30
QO IIR 60 + LD/CD	1.78 ± 0.40

*COMTI (30 mg kg^{-1}, i.p.) were administered to reserpinized rats 1 h before LD/CD (50:50 mg kg^{-1}, i.p.) administration and rats were sacrificed 2 h later. †Striatal basal levels of LD and 3-OMD in reserpinized rats treated with LD/CD were 29/02 ± 2.06 and 2.70 ± 0.53 μg g^{-1}, respectively. $^{\#}$Increasing doses of QO IIR (mg kg^{-1}, i.p.) were coadministered with LD/CD (50:50 mg kg^{-1}, i.p.) and rats were sacrificed 2 h later. All treatments, with the exception of QO IA, significantly reduced 3-OMD/LD ratio versus LD/CD treated rats ($P<0.01$, one way ANOVA followed by Dunnett's test).

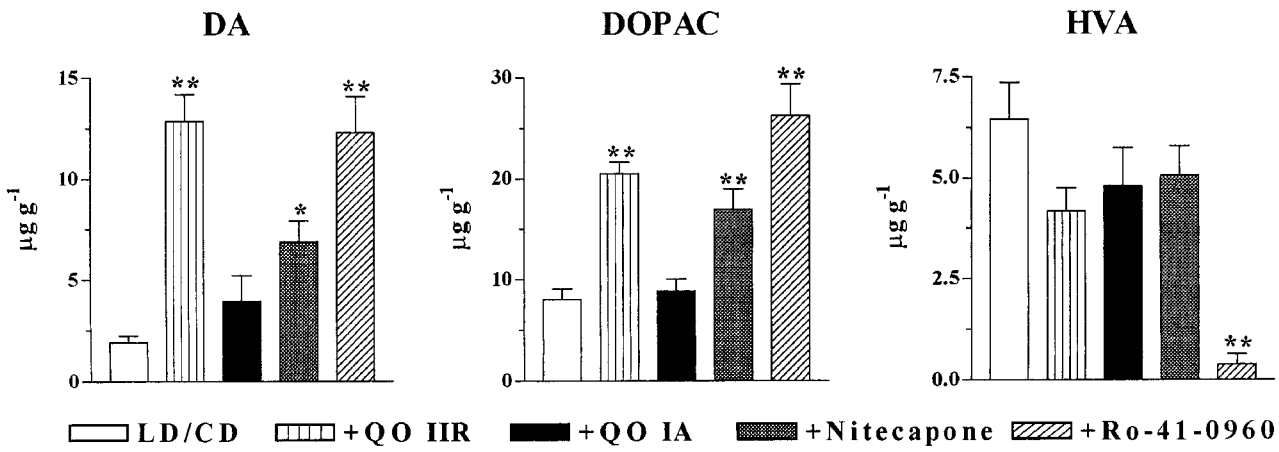

Figure 6 Striatal amine levels following COMTI and LD/CD administration to reserpinized rats. COMTI (30 mg kg^{-1}, i.p.) were administered 1 h before LD/CD (50:50 mg kg^{-1}, i.p.) and rats were sacrificed 2 h later. DA: dopamine; DOPAC: dihydrophenylacetic acid; HVA: homovanillic acid. Results expressed in $\mu g\ g^{-1}$ are mean ± s.e.mean ($n = 5-8$). $^{*}P < 0.05$ $^{**}P < 0.01$ significant difference versus LD/CD treated rats (ANOVA followed by Dunnett's test).

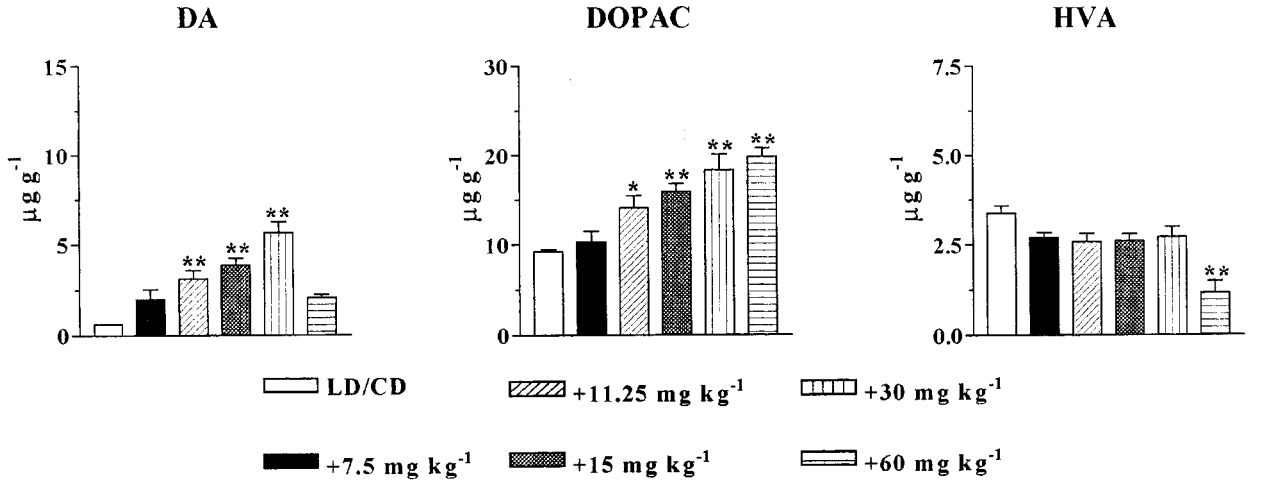

Figure 7 Striatal amine levels following increasing doses of QO IIR (7.5–60 mg kg^{-1}, i.p.) coadministered with LD/CD (50:50 mg kg^{-1}, i.p.) to reserpinized rats. Rats were sacrificed 2 h after drug administration. DA: dopamine; DOPAC: dihydroxyphenylacetic acid; HVA: homovanillic acid. Results expressed in $\mu g\ g^{-1}$ are mean ± s.e.mean ($n = 5-8$). $^{*}P < 0.05$; $^{**}P < 0.01$ significant difference versus LD/CD treated rats (ANOVA followed by Dunnett's test).

levels was bell-shaped. Indeed the highest dose of QO IIR tested (60 mg kg^{-1}, i.p.) induced a much lower effect (only 3 fold increase) compared with the dose of 30 mg kg^{-1}. HVA levels were only decreased (to 34%) after the dose of 60 mg kg^{-1}. Levels of 5-HIAA, the major serotonin metabolite, were unaffected by QO IIR treatment (data not shown).

Discussion

The aim of the present work was to study the possible *in vivo* COMT inhibition of two new synthetic compounds that inhibited COMT *in vitro*. Both compounds are modified nitrocatechols (Figure 1), showing different *in vitro* profiles. While compound QO IIR is a reversible tight-binding inhibitor, moderately potent ($IC_{50} = 3.2\ 10^{-7}$ M; Pérez *et al.*, 1993; 1994), compound QO IA is an irreversible weak COMTI ($IC_{50} = 5.5\ 10^{-5}$ M; Pérez *et al.*, 1993). The behavioural and neurochemical effects of both compounds in reserpinized rats treated with LD/CD have been compared with nitecapone and Ro-41-0960, effective COMTI *in vivo*.

In agreement with previous studies, nitecapone and Ro-41-0960 (30 mg kg^{-1}) markedly potentiated LD/CD reversal of reserpine induced akinesia (Lindén *et al.*, 1988; Maj *et al.*, 1990). Compound QO IIR, at the same dose, potentiated to a similar extent the LD/CD effect. However, compound QO IA, was devoid of any effect on locomotor activity. The effect of QO IIR was dose-dependent, its maximal effect being observed at the dose of 30 mg kg^{-1}. Reversal of reserpine-induced akinesia could be adscribed to effective *in vivo* COMT inhibition. In fact, striatal levels of 3-OMD, the major LD metabolite when administered with a peripheral DDC inhibitor (Sharpless *et al.*, 1972; Sandler *et al.*, 1974), were significantly reduced following the administration of the active COMTI (Männistö *et al.* 1988; 1992, Männistö & Tuomainen, 1991; Lindén *et al.*, 1988; Zürcher *et al.*, 1990), but not modified by QO IA, that neither modified locomotor activity. COMT inhibition may allow higher amounts of LD to enter the brain, that in turn is converted to DA. The COMTI also increased DA and DOPAC striatal levels in reserpinized rats treated with LD/CD, as has been shown in intact rats (Männistö *et al.*, 1988; Männistö & Tuomainen, 1991; Lindén

et al., 1988). As expected, the centrally acting inhibitor Ro-41-0960 was able to decrease HVA levels (Männistö & Tuomainen, 1991). While 3-OMD readily penetrates into the brain (Kopin *et al*., 1985), HVA shows very low, if any, penetration across the blood brain barrier. Thus, a decrease in striatal HVA levels is an index of brain COMT inhibition. However, HVA levels were reduced by the dose of 60 mg kg^{-1} of QO IIR. Similar effects of entacapone, another peripherally acting COMTI, has been occasionally found (Männistö & Tuomainen, 1991; Törnwall *et al*., 1993). Therefore a very high dose of QO IIR would be necessary to attain central COMT inhibition, or, alternatively, now it is able to reach the brain.

It should be mentioned that the highest dose of QO IIR tested (60 mg kg^{-1}) was without effect on locomotor activity. This result is correlated with striatal DA levels, that were only modestly increased in comparison with levels after LD/CD alone. It could be speculated that this high dose of the compound may inhibit DDC activity, resulting in a lower increase in DA striatal levels compared with the other doses tested. Since LD and DOPAC levels were both increased following the administration of 60 mg kg^{-1} of QO IIR, these results suggest that, in this case, LD might be directly converted into DOPAC.

It has been reported that COMTI potentiates LD/benzeraside reversal of catalepsy and hypothermia induced by reserpine administration (Maj *et al*., 1990). Similarly, in the present work a significant potentiation of the LD/CD effect was observed with the active COMTI.

Since the discovery of COMT one of the major goals has been the development of inhibitors that may be clinically useful. However, most of them were ineffective *in vivo*, poorly selective towards COMT or toxic (for review see Männistö & Kaakkola, 1990). Tolcapone (Ro-40-7592: 3,4-dihydroxy-4′-methyl-5-nitrobenzophenone) and entacapone (OR-611: N,N-diethyl-2-cyano-3-(3,4-dihydroxy-4′-methyl-5-nitrobenzophenone) are two commercially available COMTI. However, while entacapone, like nitecapone, does not cross the blood-brain barrier (Nissinen *et al*., 1988; Männistö *et al*., 1992), tolcapone, which is closely related to Ro-41-0960, inhibits brain COMT as well (Borgulya *et al*., 1989; Männistö *et al*., 1992). Although peripheral versus central inhibition differentially alters amine levels after COMTI administration, behavioural effects are quite similar. Indeed, as shown in the present work, the novel compound QO IIR potentiated to a similar extent LD/CD reversal of reserpine-induced akinesia when compared to the two reference COMTI.

In summary, compound QO IA, an irreversible and weak COMT inhibitor *in vitro*, at the dose tested, did not show any behavioural or neurochemical effect suggestive of *in vivo* COMT inhibition. However, the new synthetic compound QO IIR, a more potent (170 fold versus QO IA) and reversible tight-binding COMT inhibitor *in vitro*, has been shown to be an effective COMTI following its *in vivo* administration, with a similar potency to other well known COMTI.

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