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MARKUS FORSBERG

Comparison of Pharmacokinetics and Pharmacodynamics of Catechol-*O*-Methyltransferase (COMT) Inhibitors

Non-Clinical and Safety Aspects

Doctoral dissertation

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ABSTRACT

Parkinson's disease is a progressive neurodegenerative disorder. The treatment is still based on symptomatic dopamine-replacement therapy, and levodopa remains the most effective drug. Second-generation catechol-*O*-methyltransferase (COMT) inhibitors entacapone and tolcapone were launched in clinical use as adjuncts to levodopa-dopa decarboxylase inhibitor therapy of Parkinson's disease in the late 1990s. Though knowledge about the properties of entacapone and tolcapone has expanded, some controversy still remains about the potency, efficacy and safety of these drugs. Recently, also new COMT inhibitors have been introduced.

The objective of this non-clinical study was to compare the pharmacokinetic and pharmacodynamic properties of entacapone and tolcapone as well as to assess the role of physicochemical properties on their behavior in rats. Also the role of COMT in dopamine-linked oxidative stress was studied in COMT-deficient mice.

It was shown that above a certain threshold, the improved pharmacokinetic profile (AUC) of entacapone does not provide any further increase in pharmacodynamic response (AUE). COMT inhibition studies revealed that entacapone and tolcapone are equally potent *in vitro*. However, tolcapone had a longer duration of action in rat peripheral tissues and it showed a higher potential to accumulate than entacapone. Drug concentration measurements and COMT inhibition studies both confirmed that tolcapone penetrates better into the brain than entacapone, evidently due to its higher lipophilicity. In contrast, when these drugs were administered intrastriatally, entacapone appeared to inhibit COMT more effectively. This points to different local distributions of entacapone and tolcapone. Entacapone and tolcapone had similar EC_{50} values *in vivo*. The inhibitory E_{max} model (pharmacokinetic-pharmacodynamic modeling) also showed that concentrations above about 2000 ng/ml produce only a minor further benefit suggesting that peak tolcapone concentrations reached in clinical practice are higher than needed to reach maximal degree of COMT inhibition in the periphery. Finally, despite potentiation of the hyperthermic effect of methamphetamine, COMT-deficiency did not increase methamphetamine-induced hydroxyl radical production. Furthermore, it did not change the activity of glutathione S-transferase or quinone reductase.

In conclusion, the present non-clinical results confirm the earlier suggestions about different behavior of entacapone and tolcapone *in vivo* and provide further evidence that the reasons for the higher potency of tolcapone *in vivo* are pharmacokinetic rather than pharmacodynamic. Different physicochemical properties explain partly the differences between entacapone and tolcapone. This study also provides comparative new information about entacapone and tolcapone, which may help to understand the potential safety risks associated with these drugs and to develop new COMT inhibitors to achieve an optimal outcome as an adjunct to levodopa-dopa decarboxylase inhibitor therapy. The preliminary studies on the role of COMT in dopamine-linked oxidative stress did not reveal any potential concerns. However, further studies are needed to exclude the possibility that COMT-deficiency may increase dopamine-dependent formation of reactive oxygen species and affect the activity of antioxidant defense mechanisms.

National Library of Medicine Classification: WL 359, QU 143, QV 80, QV 38, QZ 180
Medical Subject Headings: catechol O-methyltransferase/antagonists & inhibitors; pharmacokinetics; pharmacology; chemistry, physical; oxidative stress/drug effects; hydroxyl radical; reactive oxygen species; enzyme inhibitors; antiparkinson agents; Parkinson disease/drug therapy; drug evaluation; safety; comparative study; rats; mice, knockout

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Among intensive research, also balancing activities are needed. Playing golf is one of the best ways to get something else to think about. Special thanks to Ewen MacDonald! I owe my sincere thanks to my friends outside the University, especially Timo Salo, Juha Jussila and Päivi Lammi with their families. You all have been great friends during these years. Also, I want to thank those who may not find their names here, but who have contributed in some way during these years.

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Kuopio, September 2005

Markus Forsberg

ABBREVIATIONS

ALAT	Alanine aminotransferase
AUC	Area under the concentration-time curve
AUE	Area under the effect-time curve
BBB	Blood-brain barrier
BZ	Benserazide
CL	Total clearance
C_{max}	Maximal plasma concentration
COMT	Catechol-*O*-methyltransferase
CD	Carbidopa
DA	Dopamine
DAT	Dopamine transporter
DHBA	Dihydroxybenzoic acid
DDC	Dopa decarboxylase (aromatic amino acid decarboxylase)
DOPAC	3,4-dihydroxyphenylacetic acid
EC_{50}	Inhibitor concentration producing 50% of the maximum attainable inhibition
6-FDOPA	6-[^{18}F]-fluorodopa
GSH	Glutathione (reduced)
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HET	Heterozygote (COMT +/−)
HOM	Homozygote (COMT −/−)
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
IC_{50}	Inhibitor concentration producing 50% inhibition of enzyme activity
K_i	Inhibitor constant (dissociation constant of an inhibitor)
K_m	Substrate concentration that produces half-maximal reaction velocity
LD	Levodopa
L-DOPA	L-3,4-dihydroxyphenylalanine
LNAA	Large neutral amino acid
log P_{app}	Apparent partition coefficient
MAO	Monoamine oxidase
MB-COMT	Membrane-bound COMT
METH	(+)-S-Methamphetamine
MPP^+	1-methyl-4-phenyl-pyridium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
$O_2^{\bullet-}$	Superoxide anion

$OH^{\bullet}$	Hydroxyl radical
6-OHDA	6-Hydroxydopamine
3-OMD	3-*O*-methyldopa
PD	Parkinson's Disease
PET	Positron emission tomography
ROS	Reactive oxygen species
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine (AdoMet)
SN	Substantia nigra
S-COMT	Soluble COMT
STN	Subthalamic nucleus
UPDRS	Unified Parkinson's disease rating scale
$t_{1/2}$	Half-life
$t_{1/2\beta}$	Elimination half-life (rapid elimination phase)
t_{max}	Time required to reach maximal plasma concentration (C_{max})
V_{max}	Maximal reaction velocity
WT	Wild-type (COMT +/+)

LIST OF ORIGINAL PUBLICATIONS

This doctoral dissertation is based on the following publications, referred to in the text by Roman numerals **I – IV**.

I Markus Forsberg, Jouko Savolainen, Tomi Järvinen, Jukka Leppänen, Jukka Gynther, Pekka T. Männistö: Pharmacodynamic response of entacapone in rats after administration of entacapone formulations and prodrugs with varying bioavailabilities.
Pharmacology & Toxicology 90: 327-332, 2002

II Markus Forsberg, Marko Lehtonen, Minna Heikkinen, Jouko Savolainen, Tomi Järvinen, Pekka T. Männistö: Pharmacokinetics and pharmacodynamics of entacapone and tolcapone after acute and repeated administration: a comparative study in the rat.
Journal of Pharmacology and Experimental Therapeutics 304: 498-506, 2003

III Markus M. Forsberg, Marko Huotari, Jouko Savolainen, Pekka T. Männistö: The role of physicochemical properties of entacapone and tolcapone on their efficacy during local intrastriatal administration.
European Journal of Pharmaceutical Sciences, 24: 503-511, 2005

IV Markus M. Forsberg, Risto O. Juvonen, Petra Helisalmi, Jukka Leppänen, Joseph A. Gogos, Maria Karayiorgou, Pekka T. Männistö: Lack of increased oxidative stress in catechol-*O*-methyltransferase (COMT)-deficient mice.
Naunyn-Schmiedeberg's Archives of Pharmacology 370: 279-289, 2004

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1 INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder with motor deficits. The prevalence of PD is about 360 per 100 000 with an annual incidence of about 18 per 100 000 (Shannon 2004). In Finland, there are over 10 000 patients with PD. The mean age of onset is approximately 60 years (Olanow et al. 2001).

The major clinical signs of PD are resting tremor, rigidity, akinesia and bradykinesia with subsequent gait and postural disturbances (Lang and Lozano 1998a; Sian et al. 1999; Shannon 2004). Pathologically, PD is characterized mainly by the progressive degeneration of melanised dopaminergic neurons in substantia nigra (SN) pars compacta together with cytoplasmic eosinophilic inclusions i.e. Lewy bodies (Sian et al. 1999; Shannon 2004). The manifestation of clinical symptoms requires more than 50 – 60% loss of the nigral neurons (Sian et al. 1999).

The degeneration of dopaminergic neurons in SN of PD patients results in a dopamine (DA) deficiency in the striatum, first described by Ehringer and Hornykiewicz (1960). This discovery, followed by the pioneering clinical experiments with the DA precursor L-3,4-dihydrohyphenylalanine (L-DOPA), led to introduction of levodopa (LD) therapy in the treatment of PD (see LeWitt 1989). The next breakthrough in the treatment of PD was the introduction of dopa decarboxylase (DDC) inhibitors in the early 1970s (see LeWitt 1989). They improved greatly the efficacy and safety of LD therapy. The primary cause of PD has not been resolved, and the treatment is still based on symptomatic DA-replacement therapy. LD remains the most widely used and most effective drug in the treatment of PD (Lang and Lozano 1998b; Olanow 2004). Unfortunately, several problems occur during long-term LD treatment.

In 1958, Axelrod and coworkers described *O*-methylation of catechols, including DA and 3,4-dihydroxyphenylalanine, by catechol-*O*-methyltransferase (COMT) (Axelrod et al. 1958; Axelrod and Tomchick 1958). They also found that a compound, pyrogallol (1,2,3-trihydroxybenzene), could inhibit *O*-methylation of noradrenaline both *in vitro* and *in vivo* (Axelrod and Laroche 1959). Also catechol and its derivatives, gallic acid esters and tropolones were shown to inhibit COMT (see Guldberg and Marsden 1975). Soon after the introduction of LD for the treatment PD, a COMT inhibitor *N*-butyl gallate was shown to potentiate the effect of LD and to reduce the side-effects of LD in patients with parkinsonism (Ericsson 1971). Unfortunately, its duration of action was extremely short *in vivo* (Dorris and Dill 1977) and it was relatively uneffective compared to the DDC inhibitors (Ericsson 1971). Also several other first-generation COMT inhibitors (e.g. pyrogallol, catechol, U-0521 and tropolone) were short acting and relatively toxic (Guldberg and Marsden 1975).

In the late 1980s, new COMT inhibitors were introduced by two research groups (Bäckström et al. 1989; Borgulya et al. 1989). It was soon established that some of these new inhibitors possessed clearly higher clinical potential as adjuncts to LD therapy than the first-generation inhibitors. Tolcapone was launched in clinical use in 1997 and

entacapone in 1998. Though the knowledge about the properties of entacapone and tolcapone has expanded, some controversy still remains about the potency, efficacy and safety of these drugs. Recently, also new COMT inhibitors and entacapone derivatives have been described.

Our understanding of the COMT enzyme has increased substantially during the past few years. For example, the development of a COMT-deficient mouse strain by Gogos et al. (1998) provided a new tool to study the importance of COMT in the metabolism of DA in the brain. Also the role of genetic polymorphism of COMT activity in the pathogenesis of several neuropsychiatric disorders has been studied. This new knowledge has provided some new theoretical clinical applications of COMT inhibitors.

In the following review of the literature, an updated overview of the COMT enzyme is given with special emphasis on the characteristics crucial to the behavior of COMT inhibitors. This review also outlines the new knowledge about the physiological role of COMT and consequences of COMT-deficiency. Furthermore, current knowledge about the long-term problems in LD treatment and their association with COMT is summarized. Finally, recent advances in COMT inhibitors are discussed. The objective of the present non-clinical study was to compare the pharmacokinetic and pharmacodynamic properties of entacapone and tolcapone as well as to assess the role of physicochemical properties on their behavior in rats. Also the role of COMT in DA-linked oxidative stress was studied in COMT-deficient mice.

2 REVIEW OF THE LITERATURE

2.1 Updated overview on catechol-*O*-methyltransferase

2.1.1 *Comt* gene and its expression

COMT (EC 2.1.1.6) exists in two forms, as a soluble, cytosolic form (S-COMT) and as a membrane-bound, microsomal (MB-COMT) form (Guldberg and Marsden 1975; Kopin 1985; Roth 1992). The *Comt* gene codes both enzyme forms by two promoters; the P1 promoter regulates the expression of a shorter mRNA (1.3 kb in humans and 1.6 kb in rats) that can code only S-COMT protein whereas the P2 promoter expresses a longer mRNA (1.5 kb in humans and 1.9 kb in rats) that is able to code both S- and MB-COMT (Tenhunen et al. 1993, 1994).

The longer COMT mRNA exists ubiquitously but the shorter version is expressed in a tissue-specific manner, indicative of variation in the activity of the P1 promoter between tissues (Tenhunen et al. 1993, 1994). In human and rat brain, no significant expression of the shorter transcript has been found (Tenhunen et al. 1993, 1994; Hong et al. 1998; Matsumoto et al. 2003a). Interestingly, estrogen seems to down-regulate *Comt* transcription at the promoter level (Xie et al. 1999) and this may explain why female subjects have lower COMT activity than males in liver and prefrontal cortex (Boudíková et al. 1990; DeSanti et al. 1998; Chen et al. 2004).

The rat and human S-COMT contains 221 amino acids and its molecular weight is approximately 25 kDa (see Lundström et al. 1995). MB-COMT has a 43 or 50 residue amino-terminal extension in rats and humans, respectively (see Lundström et al. 1995). It contains a hydrophobic region that acts as a transmembrane anchor associating the MB-COMT with rough endoplasmic reticulum, with the carboxy-terminal portion of the polypeptide being oriented to the cytoplasmic side of rough endoplasmic reticulum (Ulmanen and Lundström 1991; Ulmanen et al. 1997). The amino acid sequences of rat and human COMT are very similar (Vidgren et al. 1994).

2.1.2 COMT polymorphism

A common polymorphism of two codominant alleles, low and high COMT activity allele ($COMT^L$ and $COMT^H$, respectively), has been shown to determine the level of erythrocyte COMT activity in humans (see Weinshilboum et al. 1999). The two COMT alleles have been associated to differential thermal stability of the COMT enzyme; it has been shown that those subjects homozygous for the low activity allele have a thermolabile COMT enzyme and 3 – 4-fold lower COMT activity than that in subjects homozygous for high activity allele (Scanlon et al. 1979; Boudíková et al. 1990).

The variation in thermal stability and COMT activity results from a single nucleotide (G → A) transition leading to substitution of Val at codon 108 for Met in S-COMT or Val158 to Met in the case of MB-COMT (Lotta et al. 1995; Lachman et al. 1996). The catalytic sites of Val108 and Met108 variants (S-COMT) are similar, but the Met108 variant is more thermolabile even at 37°C (Lotta et al. 1995). Homozygosity for the Met allele is associated with enzyme thermolability and 4-fold lower COMT activity in the liver than that in subjects homozygous for Val allele, i.e. it leads to $COMT^{LL}$ phenotype (Lachman et al. 1996). As there is only a single *Comt* gene, the Val108/158 Met polymorphism could be assumed to result in alterations of COMT activity and thermal stability also in the brain. Surprisingly, the individuals with the Val/Val genotype seem to have only 40% higher COMT activity in the postmortem brain in comparison with individuals presenting the Met/Met genotype (Chen et al. 2004). It is noteworthy that the frequency of the Met allele varies between populations; it has a clearly higher abundance in Europeans than in other populations (Palmatier et al. 1999).

Recent studies have identified several new single nucleotide polymorphisms (SNP) within the *Comt* gene (Shifman et al. 2002; Shield et al. 2004). However, functional studies have revealed that these novel SNPs do not change significantly COMT activity, suggesting that Val108/158 Met SNP is the predominant genetic factor in determining the variation in COMT activity (Chen et al. 2004; Shield et al. 2004). Since Val108/158 Met polymorphism does not affect COMT mRNA levels, the differences in enzyme activity between alleles are likely to result from differences in COMT protein integrity and thus thermostability (Matsumoto et al. 2003b; Chen et al. 2004).

The association between genetic polymorphism of COMT (variation of COMT activity) and various neuropsychiatric disorders, such as schizophrenia, aggressive behavior, obsessive-compulsive disorder and alcoholism has been studied extensively (reviewed in Bilder et al. 2004), but many of the reported associations are still not fully established. To date, the most convincing evidence exists for the association of COMT Val allele with increased risk of schizophrenia (see section 2.1.5.2). There is also evidence that the Met allele is associated with aggressive behavior (reviewed in Volavka et al. 2004). There seems to be no general association of COMT polymorphism with PD, although some studies have hinted at increased risk in subjects with low COMT activity allele (see Männistö and Kaakkola 1999; Bialecka et al. 2005). Finally, recent findings suggest that COMT genotype can modify pain perception and pain sensitivity (Zubieta et al. 2003; Rakvåg et al. 2005; Diatchenko et al. 2005).

2.1.3 Active site, reaction mechanism and kinetics

The active site of COMT consists of the more buried S-adenosyl-L-methionine (SAM)-binding motif and the catalytic site located in the vicinity of central Mg^{2+} ion (Vidgren et al. 1994, 1999). Both hydrophobic contacts and hydrogen bonding with several amino acids are involved in the binding of SAM, and the conformation of the

enzyme becomes changed during the binding of SAM and release of S-adenosyl-L-homocysteine (SAH) (Vidgren et al. 1994, 1999). The binding of Mg^{2+} ion is directed by Asp141, Asp169, Asn170, the two hydroxyl groups of the catechol ligand and a water molecule (Vidgren et al. 1994). The Mg^{2+} ion steers the binding of the catechol ligand and the hydrophobic gatekeepers Trp38, Trp143 and Pro174 direct the planar catechol ring into the correct position (Vidgren et al. 1994, 1999). One of the hydroxyl groups of the substrate is surrounded by a Mg^{2+} ion, a methyl donor SAM and the catalytic Lys144, and the other is stabilized by a negatively charged carboxyl group of Glu199 (Vidgren et al. 1994).

In the methyl transfer reaction, Mg^{2+} steers one of the hydroxyl groups into a close interaction with the active methyl group of SAM, Lys144 acts as a general base increasing the nucleophilicity of the hydroxyl group and finally, the electron-deficient methyl group of SAM is transferred to the ionized hydroxyl group in a direct nucleophilic reaction (Vidgren et al. 1994, 1999). COMT can methylate either the *meta*-hydroxyl group or *para*-hydroxyl group of the catechol substrate. The *O*-methylation of the *m*-hydroxyl of catecholamines is dominant *in vivo* (see Guldberg and Marsden 1975). However, the regioselectivities of S- and MB-COMT have been shown to differ; MB-COMT is clearly more selective for *m*-hydroxyl than S-COMT *in vitro* (see Männistö et al. 1992c). In human recombinant S-COMT, the *m/p* ratios vary between substrates (Lotta et al. 1995), probably due to the relative abilities of substrates to bind in two different orientations to the active site of COMT (Vidgren et al. 1999).

Current knowlewge of kinetic reaction cycle of both COMT isoforms originates from the kinetic experiments of Lotta et al. (1995) with recombinant human S- and MB-COMT. It was demonstrated that Mg^{2+} does not need to bind before SAM. Furthermore, it was found that SAM binds alone to the Met variant of COMT and stabilizes it. Additionally, the crystal structure of COMT revealed that the binding site of SAM resides deeper in the active site than that of Mg^{2+} (Vidgren et al. 1994). Guided by these results, Lotta et al. (1995) concluded that the methyl donor SAM has to bind first, then Mg^{2+}, and finally the catechol substrate binds within the catalytic site.

Although the active sites and kinetic mechanisms of S- and MB-COMT are similar (see above), their substrate specificities and kinetic parameters vary. Already early studies with purified enzymes suggested that S-COMT has a lower affinity (K_m) for catecholamines than MB-COMT but it has much higher capacity (V_{max}) than MB-COMT (reviewed in Guldberg and Marsden 1975; Männistö et al. 1992c; Roth 1992). It should be emphasized, however, that the reported V_{max} values seem to reflect the amount of the enzyme in different tissues rather than the actual capacity of the enzyme reaction (Männistö et al. 1992c; Lotta et al. 1995). More recent studies with recombinant human S- and MB-COMT have confirmed that MB-COMT has a higher affinity for catecholamines (Malherbe et al. 1992; Lotta et al. 1995). For dopamine, the K_m values of S-COMT and MB-COMT were 207 and 15.1 μM (Lotta et al. 1995). For LD, the corresponding values were 613 and 266 μM. However, no such difference was

observed with the commonly used exogenous substrate 3,4-dihydroxybenzoic acid (3,4-DHBA); the K_m values of S- and MB-COMT were 38.9 and 30 μM, respectively. On the other hand, S-COMT has twice as high a V_{max} value as MB-COMT. This difference was the same for all substrates tested, including LD and 3,4-DHBA.

Generally, the different kinetic characteristics of S- and MB-COMT strongly suggest that these two isoforms have different physiological functions; at low, physiological substrate concentrations (e.g. at physiological catecholamine levels in the brain) MB-COMT is predominant whereas S-COMT dominates at higher substrate concentrations (e.g. at pharmacologically elevated catecholamine levels) (Roth 1992; Lotta et al. 1995). This hypothesis is supported by the cellular distribution of MB-COMT in the brain and high relative proportion of MB-COMT in the human brain (see section 2.1.4).

The different kinetic behavior of various substrates could be explained by structural factors of COMT. The different affinities of DA and 3,4-DHBA to COMT seem to result from interactions between the polar side chain of the substrate and the hydrophobic gatekeepers of COMT (Lotta et al. 1995; Vidgren et al. 1999). The catalytic sites of S- and MB-COMT are identical, thus it is the membrane anchor region of MB-COMT or the adjacent membrane which causes differences in binding of substrates between the two enzyme forms (Lotta et al. 1995). It has been shown that solubilization of rat brain and liver MB-COMT increases the K_m and V_{max} value and further purification of solubilized MB-COMT lead to kinetic characteristics similar to S-COMT (Bonifacio et al. 2000). Also these findings support the hypothesis that the more favorable substrate binding interactions of MB-COMT may be due to the adjacent membrane.

2.1.4 Distribution

2.1.4.1 Tissue distribution and cellular localization

Peripheral tissues

In mammals, COMT is present in most peripheral tissues (Guldberg and Marsden 1975; Tenhunen and Ulmanen 1993; Karhunen et al. 1994; Tenhunen et al. 1994). The highest COMT activity is found in liver but relatively high activity is present also in kidney, gut, stomach, lungs, adrenal gland and spleen as well as in uterus and ovaries (Guldberg and Marsden 1975; Nissinen et al. 1988; DeSanti et al. 1998; Ellingson et al. 1999). Erythrocytes contain COMT, but in humans the activity is much lower than that detected in the rat erythrocytes (Ellingson et al. 1999). Also mouse, rat and human breast tissue contain COMT (Amin et al. 1983; Weisz et al. 2000).

The COMT proteins are not equally distributed within rat peripheral tissues. In the liver, practically all hepatocytes show intense COMT immunoreactivity (Karhunen et

al. 1994). In the kidney, COMT is located in proximal and distal tubules and collecting ducts (Kaplan et al. 1979; Karhunen et al. 1994). The highest COMT activity in the kidney is found in the outer cortex (Odlind et al. 1999). In the gastrointestinal tissues, COMT immunoreactivity has been detected in the epithelial cells (Karhunen et al. 1994).

Brain

The presence of COMT in various brain areas such as cerebral cortex, striatum, hippocampus, hypothalamus, brain stem, pons-medulla and cerebellum has been revealed in immunocytochemical and immunoblotting studies (Kaplan et al. 1979; Tenhunen and Ulmanen 1993; Karhunen et al. 1994; Tenhunen et al. 1994; Karhunen et al. 1995a) as well as COMT activity assays (Guldberg and Marsden 1975; Hansson 1984). These studies also point to regional differences in COMT activity. Additionally, Matsumoto et al. (2003a) have reported that COMT mRNA levels are significantly higher in prefrontal cortex than in striatum, both in rats and humans. Their results also suggest that the COMT mRNA is not equally distributed within the cerebral cortex; the cortical motor and sensory areas had lower densities than the prefrontal and piriform areas.

In the rat brain, COMT proteins have been identified both in the neuronal and non-neuronal structures. In the light microscopic immunocytochemical studies, the highest COMT immunoreactivity was found in the ventricular ependymal cells (Kaplan et al. 1979; Karhunen et al. 1994). Also the cells of choroid plexus, the astrocyte end feet around the capillaries and to a lesser extent, capillary endothelial cells contain high levels of COMT (Kaplan et al. 1979, 1981; Karhunen et al. 1994, 1995a). Since these sites are associated with the blood-brain barrier (BBB), it appears that COMT plays an important role in segregating peripheral and central catechols (Kaplan et al. 1979, 1981). Oligodendrocytes and astrocytes in the cerebral and cerebellar white matter as well as the Bergman cells in the cerebellum contain COMT (Kaplan et al. 1979; Karhunen et al. 1994). Immunoelectron microscopic studies have revealed that COMT is present in the cytoplasm of astrocytes and in the cell bodies of neurons and their processes, such as large dendritic processes and postsynaptic dendritic spines in parietal cortex, striatum and molecular layer of cerebellum but not in dopaminergic nerve terminals (Kastner et al. 1994; Karhunen et al. 1995a). Human nigrostriatal dopaminergic neurons do not contain COMT, but the cell bodies of some dopaminergic neurons in the ventral tegmental area and SN pars lateralis seem to contain COMT (Kastner et al. 1994; Matsumoto et al. 2003a).

Taken together, the cellular location of COMT in the brain suggests that catecholamines are inactivated by COMT mainly in the glial cells (astrocytes) and to a lesser extent in postsynaptic neurons (Kastner et al. 1994; Karhunen et al. 1995a). This hypothesis is not fully supported by the recent results of Matsumoto et al. (2003a)

which indicated that COMT mRNA levels are higher in neurons than in glial cells, both in the striatum and prefrontal cortex. However, it should be emphasized that the higher COMT mRNA levels do not invariably reflect higher COMT protein levels; the COMT gene expression seems to be regulated also at the translational level (see Lundström et al. 1995).

Relative amounts of S- and MB-COMT

The S-COMT protein is the dominant form in most rat and human tissues (Lundström et al. 1995). However, in the human brain, 70% of the COMT protein is the MB-COMT whereas in the rat brain, MB-COMT represents only about one third of the total. This difference in relative COMT protein levels between humans and rats may result from different translation efficiency of COMT proteins from the longer mRNA (Lundström et al. 1995). Furthermore, the dominance of S-COMT in most other tissues may be due to the higher levels of shorter COMT mRNA, which is able to express the S-COMT protein only. It has been suggested that MB-COMT is located in the postsynaptic neurons whereas S-COMT resides mainly in the glia (Rivett et al. 1983; Kaakkola et al. 1987). However, Western blot analyses have revealed the presence of both S- and MB-COMT proteins in cultured neurons and astrocytes (Karhunen et al. 1995b).

2.1.4.2 Subcellular localization

S- and MB-COMT have different subcellular distributions. In the study of Tilgmann et al. (1992), immunoblotting analysis of COMT proteins from subcellular fractions of baculovirus-infected cell homogenate and rat brain homogenate revealed that S-COMT resides exclusively in the cytoplasmic fraction. The low-density membrane fractions representing plasma membranes and endoplasmic reticulum contained only MB-COMT protein. Furthermore, some MB-COMT was detected in the cytoplasmic fraction. The immunocytochemical studies of Ulmanen et al. (1997) in mammalian cell lines overexpressing recombinant MB-COMT have shown that MB-COMT is located in the intracellular structures, mainly in the rough endoplasmic reticulum. In contrast to some earlier proposals, no COMT immunoreactivity was detected in the plasma membrane. Intracellular rather than plasma membrane-associated COMT immunoreactivity was detected in rat C6 glioma cells with naive COMT by immunoelectron microscopy. In cells expressing recombinant S-COMT, diffuse cytoplasmic but also nuclear COMT immunoreactivity was detected. Western blot analysis confirmed the presence of S-COMT in the nuclei in the rat liver and brain. Recently, nuclear localization of S-COMT has also been demonstrated in hamster kidney cortex (Weisz et al. 1998) as well as in human mammary epithelial cells and breast cancer cells (Weisz et al. 2000).

Since both S- and MB-COMT are located intracellularly, the substrates of COMT need to be transported into the cell (Männistö et al. 1992c; Ulmanen et al. 1997). For example, synaptic DA that has escaped from the neuronal high affinity uptake system is transported to surrounding glial cells via extraneuronal uptake (non-neuronal $uptake_1$; $uptake_2$) systems (see Männistö et al. 1992c) but the characteristics of these mechanisms are not fully understood. There is no COMT activity in extracellular fluids such as plasma and cerebrospinal fluid (Guldberg and Marsden 1975; Männistö et al. 1992c).

2.1.5 Physiological role

2.1.5.1 The role of COMT in peripheral tissues

COMT has an important role in the inactivation of biologically active endogenous catechols but it can also detoxify catechol-containing xenobiotics and endogenous toxic intermediates (Guldberg and Marsden 1975; Kopin 1985; Männistö and Kaakkola 1999; Zhu 2002). In addition to catecholamines (adrenaline, noradrenaline, DA), 3,4-dihydroxyphenylalanine and 3,4-dihydroxyphenylacetic acid (DOPAC) (Axelrod and Tomchick 1958), also other hydroxylated catecholamine metabolites and catecholestrogens are substrates of COMT (Guldberg and Marsden 1975; Männistö et al. 1992c). It is known that several dietary catechols and catechol-structured drugs, such as LD, benserazide (BZ) and carbidopa (CD), can be methylated by COMT (Guldberg and Marsden 1975; Männistö et al. 1992c; Lautala et al. 2001).

COMT forms an enzymatic barrier between different compartments of the body: the gut wall metabolizes exogenous catechols, preventing their entry into the circulation (Männistö and Kaakkola 1999), the liver has a crucial role in the inactivation of circulating catecholamines and dietary catechols (Kopin 1985; Zhu 2002), and several other peripheral organs with COMT activity metabolize endogenous catecholamines locally (Kopin 1985; Karhunen et al. 1994). In the kidney, COMT is known to regulate DA-mediated natriuresis (Aperia 2000).

Estrogens (estradiol and estrone) are hydroxylated to 2-hydroxy and 4-hydroxyestrogens by cytochrome P450 enzymes in the liver, uterus and mammary gland (see Zhu 2002). Oxidization products of catecholestrogens have been linked to the estrogen-dependent tumorigenesis (Cavalieri and Rogan 1998). Since COMT is responsible for the detoxification of catecholestrogens and the formation of tumor-suppressing 2-methoxyestradiol, COMT may play a role in the estrogen-dependent carcinogenesis (reviewed in Männistö and Kaakkola 1999; Zhu 2002). It has been found that both S- and MB-COMT levels are significantly increased in malignant human mammary gland (Tenhunen et al. 1999). It has been also suggested that translocation of S-COMT to the nucleus is a protective response to increased catecholestrogen levels (Weisz et al. 1998, 2000).

2.1.5.2 The role of COMT in the elimination of dopamine in the brain

General role

Neuronal reuptake ($uptake_1$) is generally considered as the main mechanism for inactivation of released catecholamines both in the peripheral nervous system and in the brain (Cooper et al. 2003). Subsequently, catecholamines that have been transported to the nerve terminals are either returned back into synaptic vesicles by vesicular monoamine transporters or metabolized enzymatically. Oxidative deamination by monoamine oxidase (MAO), which is located mainly intraneuronally, is the primary metabolic fate of catecholamines in mammals (Kopin 1985; Männistö et al. 1992c; Cooper et al. 2003). COMT, which is located primarily in glial cells and postsynaptic neurons (see section 2.1.4.1), is generally considered as a secondary enzyme in the metabolism of catecholamines (Kopin 1985; Männistö et al. 1992c; Männistö and Kaakkola 1999; Cooper et al. 2003).

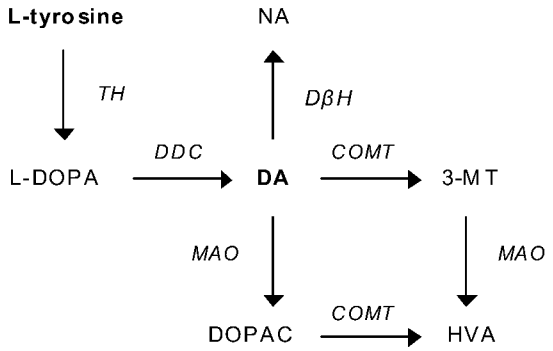


Figure 1. The synthesis and enzymatic metabolism of dopamine in the brain.
L-DOPA, L-3,4-dihydroxyphenylalanine; DA, dopamine; NA, noradrenaline; DOPAC, 3,4-dihydroxyphenylacetic acid; 3-MT, 3-methoxytyramine; HVA, homovanillic acid; TH, tyrosine hydroxylase (the rate-limiting step of catecholamine synthesis); DDC, dopa decarboxylase; DβH, dopamine-β-hydroxylase; MAO, monoamine oxidase; COMT, catechol-*O*-methyltransferase.

Recent studies in COMT-deficient mice have confirmed that COMT does not have a crucial role in the regulation of extracellular and tissue DA levels in striatum under normal conditions. The initial characterization of tissue DA levels in COMT-deficient mice did not reveal any differences between the genotypes in striatum (Gogos et al. 1998). In parallel, Huotari et al. (2002a, 2004) did not detect any significant differences either in striatal extracellular DA levels or in brain tissue DA levels between COMT-deficient (COMT −/−) and wild-type (COMT +/+) mice despite the fact that the

metabolism of DA, as shown by increased DOPAC levels and lack of HVA formation, was significantly altered. These results could be explained by the fact that the dopamine transporter (DAT) is mainly responsible for the regulation of the synaptic DA levels in the striatum and oxidative deamination by MAO is the primary metabolic pathway of DA. In contrast, DAT deficiency has dramatic effects on DA dynamics in striatum (Giros et al. 1996). Moreover, DAT-deficient mice pups gain weight more slowly and show increased mortality than their wild-type counterparts (Giros et al. 1996) whereas COMT-deficient mice pups survive and develop normally (Gogos et al. 1998). It is noteworthy that no compensatory activation in DA synthesizing and metabolizing enzymes or DAT have been detected in COMT-deficient mice (Huotari et al. 2002a, b).

The role of COMT may become more important when DA levels are pharmacologically elevated. However, there seems to be regional differences, probably due to varying capacities of other DA inactivating systems. After LD challenge, brain DOPAC levels but also DA levels in the cortex and hypothalamus were increased more in COMT-deficient mice than in wild-type mice (Huotari et al. 2002a). After inhibition of DAT by GBR 12909, COMT-deficient mice showed higher DA levels in striatum and hypothalamus than their wild-type counterparts but not in cortex (Huotari et al. 2002b). On the other hand, COMT-deficiency did not potentiate the amphetamine-induced increase in brain tissue DA levels or striatal extracellular DA levels (Huotari et al. 2004).

COMT in the prefrontal cortex: a special case

In contrast to striatum, DAT is expressed at low levels and seems to have only a minor effect on the elimination of DA in prefrontal cortex (PFC) (Cass and Gerhardt 1995; Sesack et al. 1998; Wayment et al. 2001; Mazei et al. 2002; Morón et al. 2002). Due to the sluggish DA uptake, released DA can undergo greater extracellular diffusion in PFC than in those brain areas with higher DAT activities (Sesack et al. 1998; Mundorf et al. 2001). These findings strongly suggest that metabolism has an important role in the elimination of DA in PFC. Karoum et al. (1994) have shown that formation of 3-methoxytyramine (3-MT) represents about 60% of total DA metabolism in rat PFC whereas the corresponding proportion is about 15% in striatum suggesting that *O*-methylation by COMT is the primary metabolic pathway of DA in PFC. In parallel, Gogos et al. (1998) reported increased DA levels in cortex of COMT-deficient male mice. After LD challenge, COMT-deficiency led to accumulation of DA in cortex but not in striatum (Huotari et al. 2002a). Finally, inhibition of DAT by GBR 12909 did not influence DA levels in cortex (Huotari et al. 2002b). It should be noted that also the noradrenaline transporter is known to participate in the inactivation of extracellular DA in PFC (Cass and Gerhardt 1995; Mundorf et al. 2001; Wayment et al. 2001; Mazei et al. 2002; Morón et al. 2002).

DA is known to modulate the activity of prefrontal circuits associated with working memory processes via D_1 receptors (see Goldman-Rakic et al. 2004). In primates, impaired DA neurotransmission in PFC leads to cognitive deficits (reviewed in Goldman-Rakic et al. 2004). There is also evidence that working memory deficits characteristic to schizophrenia are associated with dysregulation of DA signaling in PFC (reviewed in Goldman-Rakic et al. 2004). Finally, cognitive deficits are common in patients with PD (Dubois and Pillon 1997) and they are, at least in part, associated with impaired prefrontal cortical DA modulation and decreased blood flow in PFC (Cools et al. 2002; Mattay et al. 2002).

Since COMT has an important role in the regulation of prefrontal cortical DA levels and is thus associated with the regulation of cognitive functions subserved by PFC (see above), variation in COMT activity due to genetic Val108/158 Met polymorphism could influence cognitive performance. In fact, numerous studies have examined the association of COMT polymorphism (COMT genotype) with performance in various cognitive tasks assessing executive functions and working memory as well as with PFC physiology, both in normal subjects and patients with schizophrenia (Egan et al. 2001; Bilder et al. 2002; Malhotra et al. 2002; Shifman et al. 2002; Goldberg et al. 2003; Tsai et al. 2003; Rosa et al. 2004). Most of these studies have suggested that the high COMT activity genotype (Val/Val; Val allele) is linked to decreased performance as well as to increased risk of schizophrenia but the results are not fully consistent. In the study of Shifman et al. (2002), a gender-dependent association between COMT Val108/158 Met polymorphism and schizophrenia was found, but it was also suggested that more than one SNP in the *Comt* gene is contributing to schizophrenia susceptibility. Furthermore, Tsai et al. (2003) did not find any association between COMT polymorphism and executive cognition in young females. It should also be noted that the part of the variance in cognitive performance and the increase in the risk of schizophrenia explained by the effect of COMT genotype has varied between studies (see also Bilder et al. 2004; Goldman-Rakic et al. 2004). On the other hand, though the COMT genotype clearly influences DA transmission in PFC, all of the observed effects on cognitive functions could not be explained at the level of the prefrontal cortex (see Bilder et al. 2004).

COMT inhibitors and cognition

By increasing DA levels in PFC, central COMT inhibitors could have positive effects on cognitive performance. To date, there are no results in healthy human subjects but tolcapone improved the performance of normal aged rats performing tasks (Liljequist et al. 1997). However, it did not reverse the improved performance of scopolamine-treated or nucleus basalis-lesioned rats. Similarly, central COMT inhibitors may also provide new means of modulation in schizophrenia, especially in patients with the high activity COMT phenotype ($COMT^{HH}$; Val/Val genotype). Direct

evidence is lacking but the COMT genotype seems to be associated with the therapeutic effect of antipsychotics. Illi et al. (2003) have suggested that low COMT activity genotype (Met/Met) is associated with a poor response to standard antipsychotics (D_2 antagonists), and the low MAO-A activity genotype further worsens the response. In the study of Bertolino et al. (2004), patients with Met/Met genotype showed the greatest improvements in working memory performance after 8-week olanzapine (atypical antipsychotic) treatment, probably as a result of increased DA levels in the PFC. In contrast, however, inhibition of central COMT could increase violent and aggressive behavior, obsessive-compulsive disorder, and alcohol abuse since low activity Met/Met genotype has been associated with these disorders (Bilder et al. 2004; Volavka et al. 2004).

In PD patients, DA replacement therapy seems to have cognition-improving effects (Cools et al. 2001, 2002; Mattay et al. 2002). In principle, central COMT inhibitors could provide further benefits by extending LD/DA levels in the brain. There is only one published study in which a 6-month tolcapone treatment (as an adjunct to LD-DDC inhibitor and selegiline treatment) improved the performance of advanced PD patients in cognitive tasks (Gasparini et al. 1997). On the other hand, Nutt (1998) has reported increased confusion in PD patients treated with tolcapone. Additionally, inhibition of central COMT could promote DA-linked oxidative stress in PD patients (see section 2.3.2.3). Thus, further studies are needed to establish the possible benefits and risks of central COMT inhibiton.

2.2 The rationale for the use of COMT inhibitors in the treatment of Parkinson's disease

2.2.1 Levodopa in the treatment of Parkinson's disease

Since COMT inhibitors are being used as adjuncts to LD, the role of LD in the treatment of PD is discussed briefly to illustrate the scientific basis for the clinical use of COMT inhibitors. Currently, LD is combined routinely with a DDC inhibitor, BZ or CD. In addition to LD, selective DA receptor agonists, such as bromocriptine, pergolide, ropinirole and pramipexole, are available for PD treatment (Rascol et al. 2001). Both DA agonists and LD-DDC inhibitor are commonly used as the initial therapy of PD (Olanow et al. 2001; Shannon 2004). Also anticholinergics, amantadine and MAO-B inhibitors may be used as monotherapy in patients with mild symptoms, but a DA agonist or LD is needed for progressively developing disability (Lang and Lozano 1998b). At later disease stages, all patients require LD for symptomatic control.

Despite their lower clinical efficacy (Rascol et al. 2000; Parkinson Study Group 2000, 2004), DA agonists may have some benefits compared to LD in the treatment of early PD. The most convincing evidence is for their ability to delay the development of motor complications (Rascol et al. 2000; Parkinson Study Group 2000, 2004). They

may also slow the progression of PD, but these results are rather controversial (Ahlskog 2003; Weiner 2004). Based on these advantages, it has been suggested that DA agonists would be more suitable for initial treatment than LD (Olanow et al. 2001; Rascol et al. 2001). However, there is no consensus about the best strategy for initial treatment of PD (Ahlskog 2003; Shannon 2004; Weiner 2004).

2.2.2 The role of COMT in metabolism and pharmacokinetics of levodopa

LD is almost completely absorbed from the small intestine, mainly from its proximal part via saturable active transport mechanism for large neutral amino acids (LNAA) (Nutt and Fellman 1984; LeWitt 1989). However, the bioavailability of LD remains low; only about 30% of orally administered LD reaches the systemic circulation (Nutt and Fellman 1984). This is due to extensive first-pass metabolism in the intestinal mucosa and the liver (LeWitt 1989). Food and factors inhibiting gastric emptying delay LD absorption (Nutt and Fellman 1984; Cedarbaum 1987). LD is not bound to plasma proteins (see Nutt and Fellman 1984).

Decarboxylation by DDC is the main metabolic pathway of LD (Figure 2); about 70% of LD is decarboxylated to DA in peripheral tissues (Nutt and Fellman 1984). The DDC activity is high in the gut, liver and kidney. Approximately 10% of LD is *O*-methylated to 3-*O*-methyldopa (3-OMD) by COMT and a certain proportion of the dose seems to be oxidized to DOPA quinone by tyrosinase (Nutt and Fellman 1984). Transamination by tyrosine aminotransferase represents a minor pathway. DDC and COMT are present also in the structures associated with BBB (Hardebo and Owman 1980; Kaplan et al. 1979, 1981). Therefore, only about 1% of orally administered LD reaches the brain (Männistö and Kaakkola 1990).

Administration of a DDC inhibitor, BZ or CD, concomitantly with LD increases the bioavailability of LD by 2- to 3-fold (see LeWitt 1989). Thus, the effective dose of LD can be reduced by 70 – 80%, peripheral adverse effects are decreased and the fluctuation of LD levels is also depressed (see Cedarbaum 1987; LeWitt 1989). The proportion of LD penetrating into the brain becomes increased, but it is still only about 5 – 10% (Männistö and Kaakkola 1990) (Figure 2). When DDC is inhibited, *O*-methylation by COMT with the formation of high amounts of 3-OMD becomes the major pathway of LD metabolism (reviewed in LeWitt 1989; Männistö and Kaakkola 1990). Since the half-life ($t_{1/2}$) of 3-OMD is much longer than that of LD (15 – 17 h vs. 1 – 1.5 h), it will accumulate during chronic LD treatment (Cedarbaum 1987; Dingemanse et al. 1996).

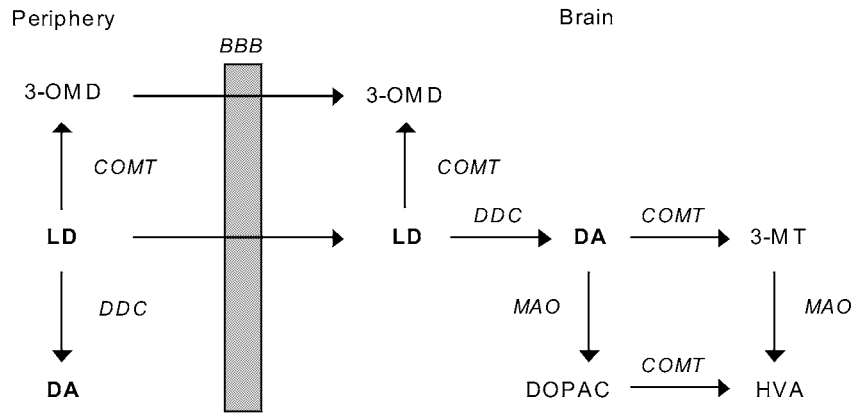


Figure 2. The role of catechol-*O*-methyltransferase (COMT) in the metabolism of levodopa. Only major enzymatic pathways are shown.
LD, levodopa; 3-OMD, 3-*O*-methyldopa; DA, dopamine; 3-MT, 3-methoxytyramine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; DDC, dopa decarboxylase; MAO, monoamine oxidase.

Since the LNAA transport mechanism is rate limiting in the absorption and the BBB penetration of LD, concomitant administration of other neutral L-amino acids (e.g. tryptophan, phenylalanine, tyrosine, leucine and methionine) may reduce plasma and brain LD concentrations by competing with the same transport mechanism (Nutt and Fellman 1984; LeWitt 1989). Also 3-OMD, a BBB-penetrating LD metabolite, may inhibit the LD transport across the gut wall and BBB (reviewed in LeWitt 1989; Männistö et al. 1992c). Thus, 3-OMD can theoretically decrease the clinical benefit of LD in patients with PD. However, this hypothesis remains controversial. There are results suggesting that plasma 3-OMD levels with the LD-DDC inhibitor treatment are not high enough to interfere with the LD (see Männistö et al. 1992c) and 6-[^{18}F]-fluorodopa (6-FDOPA) (Guttman et al. 1992) transport into the brain. On the other hand, it is also clear that 3-OMD has no beneficial effects in the treatment PD (see Männistö et al. 1992c).

Second-generation COMT inhibitors were introduced as adjuncts to LD-DDC inhibitor therapy to further improve the efficacy of LD (Männistö and Kaakkola 1990). When also the peripheral metabolism of LD to 3-OMD is eliminated by a COMT inhibitor, the bioavailability of LD and the proportion of LD penetrating into the brain is further increased (Männistö and Kaakkola 1990; Männistö et al. 1992c). Furthermore, the fluctuation in the subsequent formation of DA in the brain can be decreased. Indeed, several clinical studies have confirmed that inhibition of COMT improves significantly the pharmacokinetics of LD (see section 2.4.4).

2.2.3 COMT polymorphism and levodopa response

It has been suggested that COMT polymorphism (see section 2.1.2) could play a role in determining the individual clinical response to LD in the treatment of PD (Lachman et al. 1996). Accordingly, those individuals with the low activity genotype (Met/Met) would have better LD efficacy and in turn, individuals with high activity genotype (Val/Val) would benefit most from COMT inhibitor therapy. Recent single-dose LD challenge studies have not detected any association between COMT genotype and LD response in PD patients (Lee et al. 2001; Contin et al. 2005). In contrast, Bialecka et al. (2004) found that the frequency of patients homozygous to low activity allele ($COMT^{LL}$; Met/Met) was higher in the low LD dose (<500 mg/24 h) group than that in the high dose (>500 mg/24 h) group. This result suggests that the clinical response of LD may be achieved at lower doses in patients homozygous to low activity allele. Thus, the COMT genotype seems to have a minor impact on clinical benefit of entacapone and tolcapone in LD-treated patients (Chong et al. 2000; Lee et al. 2002).

2.3 Problems associated with the long-term use of levodopa: the role of COMT inhibitors

2.3.1 Clinical problems

2.3.1.1 Levodopa-induced motor complications

The development of motor complications is one of the main problems associated with the long-term use of LD (Marsden and Parkes 1977; Lang and Lozano 1998b; Obeso et al. 2000). Motor complications consist of two subgroups: motor fluctuations (wearing-off, predictable periods of parkinsonian symptoms; on-off, rapid and unpredictable motor fluctuations) and dyskinesias (peak-dose dyskinesias, choreiform involuntary movements; diphasic dyskinesias, mixed involuntary movements) (Lang and Lozano 1998b; Obeso et al. 2000).

In the early stages of the disease, the clinical benefits of a LD dose last several hours, even days after discontinuation of LD treatment (the long-duration response), although LD has a short $t_{1/2}$ (Obeso et al. 2000; Olanow et al. 2001). As the disease progresses, the motor benefit of an LD dose shortens and begins to wear off before the next scheduled dose i.e. the short-duration response begins to emerge (Obeso et al. 2000; Olanow et al. 2001). Concomitantly with the motor fluctuations, LD may induce dyskinesias, which are often associated with peak plasma LD levels (peak-dose dyskinesias) (Obeso et al. 2000). Finally, the motor response of LD begin to mirror the half-life of LD, which leads to unpredictable fluctuations in the patient's motor status between good antiparkinsonian effect (on) and parkinsonian symptoms (off) (Obeso et

al. 2000; Olanow et al. 2001). After being treated for 5 years with LD, over 50% patients experience motor complications (Marsden and Parkes 1977; Schrag and Quinn 2000). The risk of motor complications increases with increasing disease severity, duration of LD treatment and LD dose (see Obeso et al. 2004). Also an early age of onset is a risk factor.

Guided by new knowledge of basal ganglia functions, it has been hypothesized that LD-induced motor complications are associated with dysfunction of the basal ganglia network through pulsatile stimulation of striatal DA receptors (Calon et al. 2000; Olanow et al. 2000). During the early stages of PD, the normal basal ganglia output activity can be maintained due to compensatory mechanisms such as volume diffusion of released DA by remaining neurons and increased postsynaptic receptor sensitivity (see Obeso et al. 2004). However, as hypothesized by Obeso et al. (2004), increasing DA deficiency in the striatum leads to increased overactivity of basal ganglia output neurons and consequent worsening of the parkinsonian motor symptoms. A further loss of dopaminergic neurons reduces the capacity of the striatal neurons to compensate for changes in DA levels, and the output activity of basal ganglia begins to fluctuate depending on the availability of LD. Thus, oral administration of standard LD does not restore the normal function of basal ganglia but instead causes motor complications via pulsatile stimulation of DA receptors. The exact molecular and biochemical mechanisms of motor complications have not been established, but pulsatile stimulation is known to induce adaptive changes in basal ganglia downstream mechanisms such as upregulation of transcription factors, changes in neuropeptide levels, sensitization of glutamate receptors and firing activity of basal ganglia output neurons (Calon et al. 2000; Chase and Oh 2000; Olanow et al. 2000).

According to Obeso et al. (2004), increased disease severity and pulsatile stimulation of DA receptors by LD are the main determinants of induction of short-duration response and motor fluctuations. In contrast, pulsatile dopaminergic stimulation induces dyskinesias without having any direct association with the underlying disease. In fact, LD and short-acting DA agonists induce dyskinesias in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated primates, but this does not occur with continuous infusion of these agents or long-acting DA agonists (reviewed Calon et al. 2000; Olanow et al. 2000). Furthermore, LD induces dyskinesias also in normal monkeys (Pearce et al. 2001).

2.3.1.2 The role of COMT inhibitors in the prevention and management of levodopa-induced motor complications

DA agonists are being used as adjuncts to LD-DDC inhibitor treatment to manage the motor fluctuations in patients with advanced PD (Rascol et al. 2001; Olanow et al. 2001; Olanow 2004; Shannon 2004). They decrease Unified Parkinson's Disease Rating Scale (UPDRS) scores, decrease off-time and allow reduction of LD dose. Also titration

of the LD dose and dosing interval as well as the use of controlled-release LD-DDC inhibitor preparations have been employed to manage motor fluctuations (Olanow et al. 2001). LD-induced dyskinesias are treated first by reducing the LD dose and dose interval and then by adding a DA agonist (with concomitant reduction of LD dose), amantadine or an atypical antipsychotic as an adjunct to LD (Olanow et al. 2001; Shannon 2004).

COMT inhibitors entacapone and tolcapone provide a powerful means for the management of motor fluctuations. According to most recent placebo-controlled, multiple-dose studies, entacapone (200 mg concomitantly with LD-DDC inhibitor preparation) increases on-time by 0.8 – 1.2 hrs, decreases off-time by 0.7 – 1.2 hrs and permits a reduction in the mean total daily LD dose by 59 – 202 mg vs. placebo group in fluctuating patients (Rinne et al. 1998; Poewe et al. 2002; Brooks and Sagar 2003). In contrast, 100 mg tolcapone three times daily increases on-time by 1.8 hrs, decreases off-time by 0.9 – 1.7 hrs and allow for a reduction of mean total daily LD dose by 80 – 185 mg vs. placebo (Baas et al. 1997; Rajput et al. 1997a; Adler et al. 1998). For 200 mg of tolcapone t.i.d., the corresponding outcomes range from +1.8 to +2.4 hrs, from −0.9 to −2.2 hrs and from −82 to −251 mg, respectively (Baas et al. 1997; Myllylä et al. 1997; Rajput et al. 1997a; Adler et al. 1998). However, the incidence of dyskinesias is increased if there is no reduction of the LD dose.

Entacapone and tolcapone seem to offer benefits also in non-fluctuating patients; they have been shown to decrease UPDRS activities of daily living subscores (in some studies also motor) and to allow for a reduction in the LD dose (Waters et al. 1997; Suchowersky et al. 2001; Poewe et al. 2002; Brooks and Sagar 2003). In the large study of Olanow (2004) with stable early-stage PD patients only, health-related quality of life measures but not UPDRS scores were improved with entacapone.

The results presented above indicate that tolcapone may be slightly more efficacious than entacapone. However, tolcapone studies have been shorter (6 weeks – 3 months) than entacapone studies (6 months), and the influence of decreasing benefit of LD with the progression of the underlying disease may remain smaller (Nutt 2000). As a result of liver toxicity concerns (see section 2.6) and suspension of the marketing authorization for tolcapone in Europe during the years 1999 – 2004 (EMEA 2004), only a few long-term studies with tolcapone or comparisons of entacapone and tolcapone have been published during the past five years. The results of the 36-month study by Factor et al. (2001) suggest that tolcapone is more efficacious than entacapone as assessed by decrease in UPDRS motor scores, off-time and LD dose. However, the data was obtained from open-label extensions of placebo-controlled studies with a small number of patients with different baseline UPDRS motor scores. Additionally, European Public Assessment Report for Tasmar presented the results of an entacapone-tolcapone "switch" study that has not been published (EMEA 2005c). In that study, 150 entacapone-treated (200 mg with each LD dose) patients who had at least 3 hours off-time with the optimal LD dose, were randomized into two equally-sized groups, one

continuing with entacapone and another switching to tolcapone (100 mg t.i.d.) for 3 weeks. Surprisingly, the on-time was improved both in entacapone group and tolcapone group, but the increase was apparently greater in the tolcapone group. This result was one of the main reasons for lifting the suspension of marketing authorization for tolcapone in April 2004 (EMEA 2004, 2005c). However, the therapeutic indication of tolcapone was restricted to PD patients with motor fluctuations who failed to respond to or are intolerant of other COMT inhibitors (EMEA 2005d). In contrast, entacapone can be used as an adjunct to LD-DDC inhibitor preparations in all patients with motor fluctuations who have not been stabilized on these preparations (EMEA 2005a) and therefore it is currently the COMT inhibitor of choice. It should be emphasized that the treatment strategies of entacapone and tolcapone are different (see section 2.4.3.2).

It has been hypothesized that avoiding pulsatile stimulation of DA receptors is crucial to preventing of the LD-induced motor complications (Olanow et al. 2000). Selection of a long-acting DA agonist (ropinirole or pramipexole) as a start-up treatment instead of LD-DDC inhibitor treatment has been shown to result in a lower incidence of motor complications both in MPTP-lesioned primates (Pearce et al. 1998; Maratos et al. 2001) and PD patients (Rascol et al. 2000; Parkinson Study Group 2000, 2004). The use of controlled-release LD-DDC inhibitor preparations could theoretically provide more stable LD plasma levels and prolonged LD delivery into the brain than can be achieved with the standard preparations. However, it seems that dyskinesias are not prevented by this strategy, probably due to fact that sufficiently stable LD levels are not achieved (see Olanow et al. 2001).

Since entacapone and tolcapone extend LD plasma half-life and reduce fluctuations in plasma LD levels (see section 2.4.4), it has been hypothesized that COMT inhibitors reduce the risk of LD-induced motor complications, particularly when added to LD-DDC inhibitor therapy already at onset (Olanow and Obeso 2000). There is some non-clinical evidence to support this hypothesis. Smith et al. (2004) have shown that administration of LD-CD (12.5/12.5 mg/kg p.o.) twice daily for 16 days induced dyskinesia in MPTP-lesioned primates, with or without entacapone (12.5 mg/kg p.o.). However, they found that the same daily LD dose divided to four small doses (LD/CD 6.25/12.5 mg/kg p.o.) administered concomitantly with entacapone (12.5 mg/kg p.o.) provided more stable motor improvement and a lower incidence of dyskinesias than the same treatment regimen without entacapone. To date, clinical evidence is lacking, but a clinical study has been initiated to compare the incidence of motor complications between LD-CD-entacapone and LD-CD treated patients with early PD (Orion Pharma 2004).

2.3.2 Levodopa neurotoxicity: theoretical aspects

2.3.2.1 Oxidative stress, dopamine and Parkinson's disease

Familial forms of PD that may have autosomal dominant or recessive inheritance have been identified (Warner and Schapira 2003). These rare variants of PD involve gene mutations (e.g. in the α-synunclein or parkin gene) that induce typically early-onset PD pathology (reviewed in Warner and Schapira 2003; Schapira 2004). However, most PD cases occur sporadically, and it has been suggested that both genetic and environmental factors are involved in the disease process (Warner and Schapira 2003; Shannon 2004).

It seems that several biochemical processes may contribute to the neurodegeneration in sporadic PD cases (Jenner 2003; McNaught and Olanow 2003; Dawson and Dawson 2003; Schapira 2004). One of the leading hypotheses has been that oxidative stress and subsequent oxidative damage are involved in the cascade leading to degeneration of nigrostriatal dopaminergic neurons. It is based on the establishment that metabolism of DA can lead to formation of reactive oxygen species (ROS) (Olanow 1990). ROS can damage several biomolecules such as lipids, proteins and DNA (see Halliwell 1992; Berg et al. 2004). Thus, LD treatment could contribute to neurodegeneration by increasing DA turnover and subsequently DA-dependent formation of ROS (Olanow 1990). On the other hand, it is known that the 1-methyl-4-phenylpyridium ion (MPP^+), the oxidized metabolite of MPTP, induces oxidative stress (see Jenner and Olanow 1996).

Oxidative deamination of DA by MAO in dopaminergic nerve terminals leads to formation of hydrogen peroxide (H_2O_2), 3,4-dihydroxyphenylacetaldehyde (DOPAL) and ammonia (Kopin 1985; Olanow 1990). DOPAL is further oxidized to DOPAC by aldehyde dehydrogenase (Kopin 1985). Normally, H_2O_2 is cleared by catalase or glutathione peroxidase (see Olanow 1990, 1993; Mayes 1993) (Figure 3). However, initiation of dopaminergic cell degeneration in PD leads to compensatory increase in presynaptic DA turnover (Hornykiewicz and Kish 1986; Sossi et al. 2002), which can lead to increased formation of H_2O_2 (Olanow 1990). If H_2O_2 formation exceeds the capacity of the brain glutathione (GSH) system or the GSH system is deficient, H_2O_2 may be converted to highly oxidizing cell-damaging hydroxyl radical ($OH^\bullet$) by an iron-mediated Fenton reaction (Olanow 1990; Halliwell 1992; Jenner and Olanow 1996) (Figure 3). An increase in the level of reactive iron may further promote the formation of $OH^\bullet$ from H_2O_2 (see Olanow 1993). DA is also known to undergo auto-oxidation with the formation of semiquinones, quinones, H_2O_2, superoxide anion ($O_2^{\bullet-}$) and $OH^\bullet$ (Graham 1978; Graham et al. 1978).

Enzymatic oxidation of DA by MAO

$$DA + O_2 + H_2O \rightarrow DOPAL + NH_3 + H_2O_2$$

Auto-oxidation of DA

$$DA + O_2 \rightarrow SQ^{\bullet} + O_2^{\bullet-} + 2\,H^+$$
$$DA + O_2^{\bullet-} + 2\,H^+ \rightarrow SQ^{\bullet} + H_2O_2$$

Elimination of H_2O_2 by catalase

$$2\,H_2O_2 \rightarrow 2\,H_2O + O_2$$

Elimination of H_2O_2 by glutathione peroxidase

$$H_2O_2 + 2\,GSH \rightarrow GSSG + H_2O$$

Reduction of GSSG by glutathione reductase

$$GSSG + NADPH + H^+ \rightarrow 2\,GSH + NADP^+$$

Formation of $OH^{\bullet}$ from H_2O_2 by Fenton reaction

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^- + Fe^{3+}$$

Figure 3. The pathways for oxidation of dopamine, formation and elimination of hydrogen peroxide and generation of hydroxyl radical (modified from Olanow 1993).
DA, dopamine; MAO, monoamine oxidase; DOPAL, 3,4-dihydroxyphenylacetaldehyde; H_2O_2, hydrogen peroxide; $SQ^{\bullet}$, dopamine-semiquinone; $O_2^{\bullet-}$, superoxide anion; GSH, reduced glutathione; GSSG, oxidized glutathione, $OH^{\bullet}$, hydroxyl radical.

There is postmortem evidence of oxidative stress and subsequent oxidative damage in the SN of patients with PD. It is generally accepted that iron levels are increased (Dexter et al. 1989; Riederer et al. 1989) and GSH levels are decreased (Riederer et al. 1989; Sofic et al. 1992; Sian et al. 1994a). However, with the exception of increased γ-glutamyltranspeptidase activity (GSH degradative enzyme), it seems that the activities of other GSH-related enzymes, such as γ-glutamylcysteine synthetase (GSH synthetizing enzyme), glutathione peroxidase, glutathione S-transferase (GST) and glutathione reductase are not changed in PD (Marttila et al. 1988; Sian et al. 1994b). On the other hand, the activity of superoxide dismutase (SOD) that converts $O_2^{\bullet-}$ to H_2O_2, is increased in the SN of PD patients (Marttila et al. 1988; Saggu et al. 1989). Dexter et al. (1994) detected increased lipid hydroperoxide levels indicative of increased lipid peroxidation. Also increased levels of protein carbonyls, which are markers of protein oxidation, have been detected in SN and other brain regions (Alam et al. 1997a; Floor and Wetzel 1998). Finally, increased formation of 8-hydroxyguanine or 8-hydroxyguanosine reflecting oxidative DNA damage has been shown in the SN of patients with PD (Alam et al. 1997b; Zhang et al. 1999).

The association between oxidative stress and other pathogenetic factors in Parkinson's disease: current concepts

Also other biochemical processes associated to neurodegeneration in PD seem to be linked to the oxidative stress. Postmortem studies have shown a 30 – 40% decrease in

complex I (NADH-ubiquinone oxidoreductase, EC 1.6.99.3) activity of the mitochondrial respiratory chain in SN of PD patients (Mizuno et al. 1989; Schapira et al. 1990). Cytoplasmic hybrid cells containing mitochondrial DNA from platelets of PD patients express complex I deficiency with increased formation of ROS and increased antioxidant enzyme activities (Swerdlow et al. 1996; Cassarino et al. 1997; Gu et al. 1998). It is also known that MPP^+ inhibits complex I activity and increases $O_2^{\bullet-}$ production (Hasegawa et al. 1990; Cleeter et al. 1992). Recently, it has been shown that the flavin mononucleotide group of complex I is the major pathologically relevant ROS-generating site in the mitochondria (Liu et al. 2002).

Neuroinflammatory mechanisms, which are mediated mainly by cytotoxic effects of activated glial cells, may contribute to the neurodegeneration in PD (Hunot and Hirsch 2003). High amounts of reactive microglia have been detected in the SN pars compacta of patients with PD (McGeer et al. 1988). It is also known that the levels of proinflammatory cytokines are increased markedly in the SN and cerebrospinal fluid (CSF) of PD patients (reviewed in Nagatsu et al. 2000). Activation of astrocytes and microglia by cytokines or lipopolysaccharide increases the production of nitric oxide and H_2O_2 which in turn can lead to formation of reactive nitrogen and oxygen species such as peroxynitrite and $OH^{\bullet}$ (Dawson et al. 1994; Chao et al. 1996; McNaught and Jenner 2000).

Accumulation of cytotoxic proteins due to a failure of ubiquitin-proteasome system (UPS) may play a role in the degeneration of nigrostriatal dopaminergic neurons in PD (McNaught et al. 2001). Incomplete, misfolded and oxidant-damaged proteins are rapidly degraded by the UPS to prevent the accumulation of abnormal proteins that can have deleterious effects on cell functions (Sherman and Goldberg 2001). In familial PD, gene mutations seem to be associated with impaired degradation of cytotoxic proteins by UPS. Mutant α-synuclein may be resistant to proteolysis or it may inhibit the UPS (see McNaught et al. 2001). On the other hand, ubiquitination or de-ubiquitination may be impaired due to mutations in certain enzymes (e.g. parkin that is a ubiquitin ligase (E3) or ubiquitin carboxy-terminal hydrolase L1, respectively) (McNaught et al. 2001). The proteasomal function is also impaired in SN of patients with sporadic PD (McNaught and Jenner 2001; McNaught et al. 2003). The reason for UPS deficiency is unclear. However, *in vitro* results suggest that complex I deficiency decreases proteasomal activity by increasing the levels of oxidized proteins or by modifying the proteasome itself oxidatively (Hoglinger et al. 2003; Shamoto-Nagai et al. 2003). LD did not inhibit proteasomal activity *in vitro* (McNaught and Jenner 2001).

Though oxidative stress seems to be a crucial event in the pathogenesis of PD, it is still not understood whether oxidative stress is the main trigger for the cascade leading to degeneration of nigrostriatal dopaminergic neurons or is a consequence of other pathogenetic processes (Jenner 2003; Berg et al. 2004). It has been suggested that complex I defect (or mitochondrial dysfunction), increased ROS production and UPS dysfunction form a self-amplifying cycle; this being initiated by the complex I defect

and enhanced by other two factors leading to cell death (Schapira 2004; Dawson and Dawson 2003).

2.3.2.2 Evidence for levodopa neurotoxicity

In vitro studies

Several *in vitro* studies indicate that LD is toxic to cultured catecholaminergic cells at high concentrations and this effect has been linked to the formation of quinones and ROS as a result of oxidative DA metabolism (Mena et al. 1992; Mytilineou et al. 1993; Pardo et al. 1993). It has been shown that LD toxicity occurs in cultured cells also without its conversion to DA, probably due to direct auto-oxidization of LD leading to the formation of quinones and ROS (Basma et al. 1995; Han et al. 1996). However, the less active stereoisomer of LD, D-3,4-dihydroxyphenylalanine, is less toxic than LD (Alexander et al. 1997). The LD toxicity against cultured cells can be prevented partly by the antioxidant, ascorbic acid (Pardo et al. 1993; Han et al. 1996) or MAO-B inhibitor deprenyl (Mena et al. 1992; Pardo et al. 1993). Pardo et al. (1995) have demonstrated that LD is toxic both dopaminergic and non-dopaminergic cells in rat mescencephalic culture. In contrast to non-dopaminergic neurons, however, DA neurons were only partly protected by antioxidants.

Weingarten et al. (2001) have demonstrated a clear difference between intracellular and extracellular toxicity of DA in a DDC-expressing, non-catecholaminergic Chinese hamster ovary cell line. LD, in contrast to DA, penetrates the cell membrane and is converted to DA in the cytoplasm. It was found that antioxidants prevented LD toxicity in wild-type non-catecholaminergic cells but not in DDC-expressing cells. This led the authors to suggest that LD and extracellular DA toxicity is mediated by ROS but there is a non-oxidative mechanism responsible for intracellular DA toxicity. They hypothesized that the defect in regulation of cytoplasmic DA could cause the slow and progressive cell death in PD.

It is noteworthy that the EC_{50} of LD for cell death has varied from 60 to 350 μM in cell cultures (Basma et al. 1995; Pardo et al. 1995; Weingarten et al. 2001). However, the LD concentration in the brain of LD-treated PD patients is presumably lower. After oral administration of standard LD-CD preparations in elderly healthy volunteers and PD patients, peak plasma LD concentration varies between 5 – 20 μM (1000 – 4000 ng/ml) (Robertson et al. 1989; Olanow et al. 1991; Benetello et al. 1993) but the concentration in CSF is only about 10 per cent of that in plasma, i.e. 0.5 – 2 μM (Olanow et al. 1991). Thus, most *in vitro* toxicity studies with 10 – 100-fold higher LD concentrations have only limited predictive value in assessing LD toxicity *in vivo*.

In addition to its toxic effect, LD seems to have antioxidant effects. In cell culture, LD exposure has been shown to increase GSH levels that may in turn protect cells from oxidative stress (Mytilineou et al. 1993). This effect was prevented with addition of

ascorbate in the medium but not with addition of MAO inhibitor pargyline, superoxide dismutase or catalase suggesting that both the GSH increase and the toxic effects of LD were mediated by formation of quinones via auto-oxidation. LD also protected rat mesencephalic cells from toxicity of L-buthionine sulfoximine, which is an inhibitor of GSH synthesis. In contrast, when mesencephalic cells were first exposed to oxidative stress by means of glutathione depletion, LD expressed greater toxicity than compared to its toxicity in cells with abnormal GSH content (Mytilineou et al. 2003).

Glia-conditioned medium or addition of glial cells in the cell culture seems to protect dopaminergic neurons from LD toxicity by neurotrophic factors and antioxidant mechanisms such as up-regulation of GSH (Han et al. 1996; Mena et al. 1997a, b). Exposure of mixed mesencephalic cells to 100 μM LD protected them from subsequent oxidative damage by a powerful oxidant *tert*-butyl hydroperoxide but the protective effects of LD were lost when cells were pretreated with both LD and ascorbic acid, a combination that prevented the increase in GSH levels (Han et al. 1996).

In vivo studies

Chronic LD treatment with high oral doses does not induce any significant damage of nigrostriatal dopaminergic neurons in normal mice (Hefti et al. 1981; Reches et al. 1982), rats (Perry et al. 1984) and monkeys (Lyras et al. 2002). In the studies of Blunt et al. (1991a, b), the effect of chronic LD-CD treatment (approximately 200/25 mg/kg/day in the drinking water for five weeks) on the survival of ventral mesencephalic grafts implanted in the striatum of rats with unilateral nigrostriatal 6-hydroxydopamine (6-OHDA) lesions was studied. A reduction of apomorphine-induced circling and a complete reversal of (+)-amphetamine-induced circling was achieved by the graft in the animals with the lesion, and this effect was not altered with LD-CD treatment (Blunt et al. 1991a). Additionally, no signs of decreased amount of host DA cells or reduced survival and fibre outgrowth of implanted cells were observed with LD-CD treatment (Blunt et al. 1991b).

The 6-OHDA toxicity is selective to catecholaminergic neurons since it is transported to the neurons by high affinity uptake systems (Zigmond et al. 1992). ROS are believed to mediate its neurotoxic effects (Zigmond et al. 1992). It has been shown that perfusion of 6-OHDA into the rat striatum increases $OH^{\bullet}$ production and causes DNA damage (Ferger et al. 2001). Thus, 6-OHDA exposure might theoretically make the remaining cells more prone to additional oxidative insult by LD or vice versa. In fact, Ogawa et al. (1994) reported that chronic LD treatment (100 mg/kg i.p. twice daily for 4 weeks) increased significantly the markers of lipid peroxidation in the striatum and cortex of mice pretreated with 6-OHDA (i.c.v.). In intact mice, LD treatment decreased lipid peroxidation. Further, (Naudin et al. 1995) have shown that acute pretreatment with LD-BZ potentiated the 6-OHDA-induced (50 μg i.c.v.) decrease in DA and its metabolite levels in the striatum, indicating dopaminergic neurotoxicity. Finally, a 27-

week LD-CD treatment led to an apparent reduction of the DA cells remaining in the ventral tegmental area ipsilateral to unilateral 6-OHDA lesion of medial forebrain bundle but the numbers of DA cells in intact SN and VTA were not reduced (Blunt et al. 1993). There is also evidence that LD treatment increases the DA turnover particularly in the 6-OHDA-lesioned striatum (Ogawa et al. 2000), which in turn may lead to increased formation of ROS.

There are also reports suggesting that LD is not toxic to surviving dopaminergic cells in animals with 6-OHDA lesions. Murer et al. (1998) demonstrated that immunoradiolabeling for tyrosine hydroxylase, DAT and vesicular monoamine transporter$_2$ in the SN did not differ between LD- and vehicle-treated rats with severe 6-OHDA lesions of mesencephalic DA neurons. Instead, after a 6-month LD-CD treatment (170/17 mg/kg p.o. once daily), there was partial recovery of dopaminergic function in the denervated areas of the striatum of moderately lesioned animals. Also in the study of Datla et al. (2001), chronic LD-BZ treatment (approximately 200/50 mg/kg/day in the drinking water for 6 months) did not decrease the amount of tyrosine hydroxylase-immunopositive neurons in the lesion-side substantia nigra of 6-OHDA- or $FeCl_3$-lesioned rats.

Direct injection of DA into the rat striatum has been shown to increase by 7 – 20 fold the levels of both free and protein bound cysteinyl-DA and cysteinyl-DOPAC that are oxidization products of DA and DOPAC, respectively (Hastings et al. 1996). The formation of cysteinyl-catechols and specific damage to DA terminals was greatly reduced when DA was injected concomitantly with GSH or ascorbic acid. Thus, selective degeneration of DA neurons in PD may be due to an imbalance between oxidation and available antioxidant defenses (Hastings et al. 1996). In contrast, subcutaneously administered LD did not cause damage to striatal DA neurons or changes in DA levels in intact rat pups despite induction of oxidative stress by GSH depletion (Mytilineou et al. 2003).

LD may also have some influence on other biochemical processes associated with oxidative stress. In the study of Przedborski et al. (1993) in normal rats, chronic LD methyl ester-BZ treatment (50/12.5 mg/kg i.p. twice daily for 40 days) was shown to decrease significantly mitochondrial complex I activity. However, the activity was progressively recovered within 7 days after the last dose. This result suggests that LD treatment may further intensify the complex I deficiency observed in PD patients (see section 2.3.2.1). Also the results of Ben-Shachar et al. (1995) suggest that DA may express neurotoxicity via a direct interaction with mitochondrial electron transport chain.

Also extracellular fluid (ECF) or tissue levels of $OH^{\bullet}$ have been used to assess the LD toxicity in the rat brain. Smith et al. (1994) found that an enormous LD dose (200 mg/kg i.p. with 25 mg/kg of CD i.p.) increased extracellular $OH^{\bullet}$ levels in the rat SN as assessed by the salicylate trapping method. Concomitant inhibition of mitochondrial complex I further increased the $OH^{\bullet}$ production. However, little effect was observed

with lower LD doses (25 – 100 mg/kg i.p.). It was concluded that after high LD doses, formation of ROS may exceed the capacity of nigral antioxidant defenses. The effect of LD on $OH^{\bullet}$ production has also been studied in rats with unilateral 6-OHDA lesion but neither systemically (100 mg/kg i.p. with 25 mg/kg of CD i.p.) nor intrastriatally (1 mM in perfusion medium) administered LD could induce $OH^{\bullet}$ production (Camp et al. 2000). Furthermore, repeated administration of LD-CD (50/5 mg/kg i.p. twice daily for 5 days) had no effect on $OH^{\bullet}$ production in striatum, ventral midbrain and cerebellum.

Clinical studies

Current evidence suggests that LD is not toxic to normal SN. Quinn et al. (1986) did not find any postmortem evidence of SN damage in a subject with essential tremor who had been treated with LD-CD for over four years. Recently, Rajput (2001a, b) published a clinical and pathological summary of six patients who had been on LD treatment for 11 – 30 years. Three patients with essential tremor (mistakenly diagnosed as PD) did not develop any parkinsonian features indicative of SN damage and an autopsy in one patient revealed a normal SN. The findings were parallel also in two patients with dopa-responsive dystonia.

LD treatment has been shown to prolong the life expectancy of PD patients, but only when started before advanced stages of the disease (Rajput et al. 1997b; Rajput 2001a). Since this strategy leads to larger total LD doses than in the case of later onset of LD treatment, Rajput et al. (1997b, 2001a) concluded that LD may actually have protective effects on SN neurons in PD. On the other hand, recent studies comparing LD and DA agonist therapies suggest that nigrostriatal DA function declined faster in LD treated patients than in DA agonist treated patients. In a study of the Parkinson Study Group (2002), DAT function and degeneration of dopaminergic neurons was assessed by single-photon emission computed tomography with iodine 123 2β-carboxymethoxy-3β-[iodophenyl]tropane ([^{123}I]β-CIT). The decrease in [^{123}I]β-CIT uptake in striatum, putamen and caudate was significantly lower in the pramipexole group than in the LD group during the 46-month follow-up. The percentage loss of uptake from baseline uptake was correlated with the corresponding increase in the total UPDRS score at the end of study. In the REAL-PET study, the loss of DA storage capacity in putamen, as assessed by 6-FDOPA uptake, was slower in the ropinirole group than in the LD group within a 2-year period (Whone et al. 2003). It has been postulated that the results of these studies may also reflect the pharmacological effects of dopaminergic drugs into the regulation of DAT and the proteins involved in the metabolism of 6-FDOPA (Ahlskog 2003; Weiner 2004). Thus, these imaging studies without a placebo-treated control group do not provide unambiguous evidence for any toxic or disease-progressing effects of LD.

The effect of LD on the progression of PD has also been evaluated in the ELLDOPA study (Fahn et al. 2004). Within 42 weeks, the disease severity increased

significantly more in the placebo-treated patients than in LD-treated patients as assessed by change in the UPDRS scores. The effect of LD was dose dependent. This result suggests that LD may actually slow the progression of the disease. On the other hand, the greater decline of striatal [^{123}I]β-CIT uptake in LD-treated patients at the highest dose level is evidence that LD hastened the degeneration of nigrostriatal dopaminergic neurons. The authors concluded that LD does not have a clear disease-progressing effect and suggested that the imaging data may also indicate LD-induced modulation of DAT function (Fahn et al. 2004).

In conclusion, the results of both animal and human studies indicate that LD is not toxic to normal SN, probably due to normal dopaminergic function/DA turnover and antioxidant defense mechanisms. Some animal studies have suggested that LD may have deleterious effects when the SN is damaged and antioxidant defense mechanisms are deficient. Thus, one could speculate that in the degenerated SN of a PD patient, LD-induced formation of ROS could become deleterious only when it exceeds the capacity of cellular antioxidant defenses. This hypothesis also emphasizes the importance of non-oxidative metabolic pathways of LD and DA, such as COMT, as detoxification mechanisms in PD patients. On the other hand, current *in vivo* evidence indicate that LD may actually have a protective effect in the degenerating SN. Also the latest consensus meeting concluded that there is no firm evidence of LD causing or accelerating neuronal death in PD (see Agid et al. 2002). However, the proposed LD toxicity, in addition to motor complications associated to long-term treatment, has triggered interest in developing an LD sparing strategy in the drug treatment of PD.

2.3.2.3 Does inhibition of COMT have an influence on the levodopa neurotoxicity?

Central COMT inhibition and production of ROS

It has been suggested that inhibition of central COMT could induce oxidative stress in neurons and glial cells due to enhanced oxidative metabolism of DA via MAO or auto-oxidation, which may in turn lead to increased formation of H_2O_2 and ROS (Miller et al. 1996; Kuhn et al. 1998b). It is known that the *O*-methylated reaction products of LD, DA and DOPAC (i.e. 3-OMD, 3-MT and HVA, respectively) are less susceptible to auto-oxidation and they may even possess some antioxidative properties (Miller et al. 1996; Nappi and Vass 1998). Przedborski et al. (1993) found that LD and DA, but not 3-OMD, DOPAC and HVA, inhibited dose and time dependently the rat brain mitochondrial complex I activity *in vitro*. The inhibitory effect of LD and DA was prevented by several antioxidant mechanisms indicating that ROS formed as a result of oxidation of LD and DA could mediate this effect. Recently, Offen et al. (2001) found that 3-OMD, in contrast to LD, was not toxic to mice cerebral granule neurons or PC12 and neuroblastoma cells. In the PC12 cell line, auto-oxidation and cytotoxicity of LD

was abolished by addition of purified COMT into the incubation medium. This rescue effect was antagonized by a COMT inhibitor tolcapone though tolcapone itself was not toxic to PC12 cells. It was concluded that COMT may partly prevent the potential toxic effects of chronic LD treatment *in vivo* (Offen et al. 2001). These findings suggest that *O*-methylation of LD, DA and DOPAC by COMT is an important antioxidant defense mechanism.

Weingarten and Zhou (2001) have shown that reduction of free cytoplasmic DA concentrations by expression of vesicular monoamine transporter$_2$ and MAO-A may protect cells against intracellular DA toxicity in DDC-transfected Chinese hamster ovary cell line. Also expression of macrophage migration inhibitory factor and GST M2-2, which seem to be involved in the detoxification of oxidized DA metabolites (Baez et al. 1997; Segura-Aguilar et al. 1997; Matsunaga et al. 1999), provided protective effects. These results suggest that disruption of DA disposition or metabolism may contribute to LD cytotoxicity and thus play a role in the degeneration of dopaminergic neurons in PD (Weingarten and Zhou 2001). It could be hypothesized that also expression of COMT would decrease DA toxicity in this setup by decreasing oxidation of DA.

Activated glial cells may contribute to the neurodegeneration in PD (see section 2.3.2.1). On the other hand, they may also have a neuroprotective role by decreasing DA-dependent formation of ROS in the neuronal environment due to their ability to metabolize DA intracellularly both by MAO and COMT (Hirsch 2000). Additionally, glial cells express high levels of glutathione peroxidase, the enzyme which converts H_2O_2 to water, preventing the formation of $OH^{\bullet}$ (Damier et al. 1993). Finally, glial cells are known produce neurotrophic factors that have neuroprotective effects on dopaminergic neurons (see Hirsch 2000). In contrast, nigrostriatal dopaminergic neurons do not contain COMT suggesting that DA is degraded by oxidative pathways only with formation of ROS (Kastner et al. 1994). These neurons also lack glutathione peroxidase (Damier et al. 1993; Trepanier et al. 1996). Therefore, they may be particularly vulnerable to increased oxidative stress (e.g. as a result of increased DA turnover).

To date, only two groups have studied the effect of COMT inhibitors on the LD-induced oxidative stress in experimental animals. Lyras et al. (2002) concluded that chronic high dose LD therapy (80 mg/kg plus 20 mg/kg CD p.o. once daily for 13 weeks) did not induce any significant oxidative damage in the brain of normal cynomologus monkeys and this effect was not significantly potentiated by the peripheral COMT inhibitor entacapone (80 mg/kg p.o. once daily for 13 weeks). In a microdialysis study of Gerlach et al. (2001) centrally acting COMT inhibitor tolcapone (10 mg/kg i.p.) increased marginally striatal $OH^{\bullet}$ production in LD-CD treated animals (50/50 mg/kg). However, no such effect was observed with entacapone. Furthermore, Oechsner et al. (2002) hypothesized that inhibition of COMT with tolcapone could contribute to production of ROS and induction of oxidative stress. This assumption was

based on the fact that tolcapone increased serum DOPAC levels, decreased HVA levels as well as decreased HVA/DOPAC ratio in LD-DDC inhibitor-treated PD patients indicating that the direction of LD metabolism had been shifted to the oxidative pathway. However, the formation of ROS was not monitored.

Although there is a theoretical basis to hypothesize that inhibition of central COMT may promote formation of ROS and contribute to LD toxicity in dopaminergic neurons in SN, clinical evidence in PD patients is lacking. Especially if the SN is in a state of oxidative stress due to increased LD levels and/or defective antioxidant mechanisms, *O*-methylation by COMT could be considered as an important detoxification mechanism (Kuhn et al. 1998b). On the other hand, it is still unclear whether LD is actually toxic to SN in humans and whether it enhances the progression of the disease (see section 2.3.2.2).

Homocysteine, levodopa and COMT

In the *O*-methylation of catecholamines catalyzed by COMT, the methyl donor SAM is converted to SAH and further to homocysteine (see Zhu 2002). Correspondingly, SAH is reconverted to SAM via homocysteine and methionine intermediates.

Rat studies indicate that LD treatment decreases SAM levels and increases SAH levels in the brain (Zürcher et al. 1993; Miller et al. 1997). LD also increases plasma homocysteine levels in rats (Miller et al. 1997; Nissinen et al. 2005). In parallel, it has been shown that plasma homocysteine levels are increased in LD-treated PD patients compared with healthy controls or PD patients not taking LD (Kuhn et al. 1998a; Müller et al. 1999). These could be explained by increased SAM-consuming *O*-methylation due to high LD concentrations (Männistö and Kaakkola 1999).

Recently, LD-induced hyperhomocysteinemia has been linked with increased risk of vascular disease (Rogers et al. 2003) and dysfunction of peripheral sensory nerves (Müller et al. 2004) and should this occur also in the CNS then the elevated homocysteine levels may promote neurodegeneration. Since COMT inhibitors (entacapone, tolcapone and Ro 41-0960) attenuate the effects of LD on striatal SAM and SAH levels as well as on plasma homocysteine levels in rats (Zürcher et al. 1993; Miller et al. 1997) and in PD patients (Lamberti et al. 2005; Valkovic et al. 2005), they might have protective effect against proposed detrimental effects of LD-induced hyperhomocysteinemia (O'Suilleabhain and Diaz-Arrastia 2004). However, further studies are needed to confirm the consequences of increased homocysteine levels (O'Suilleabhain and Diaz-Arrastia 2004). In general, due to important role of SAM in methylation reactions, the SAM-sparing effect of COMT inhibitors can be considered as being beneficial.

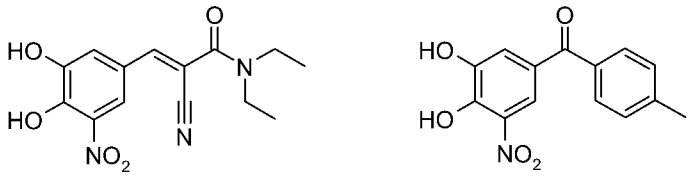
2.4 Characteristics of clinically available second-generation COMT inhibitors: an updated overview

2.4.1 Potency *in vitro* and mechanism of inhibition

Both entacapone (OR-611, Figure 4) and tolcapone (Ro 40-7592, Figure 4) are potent COMT inhibitors. Against rat liver COMT (containing mainly S-COMT), the IC_{50} values of entacapone and tolcapone are 160 nM and 36 nM, respectively (Zürcher et al. 1990b; Nissinen et al. 1992). Entacapone inhibits rat brain S-COMT with an IC_{50} value of 20 nM (Nissinen et al. 1992). However, the IC_{50} values determined in different experimental conditions are not fully comparable.

The potencies of entacapone and tolcapone have been compared only in a few studies. According to Learmonth et al. (2002), tolcapone is more potent than entacapone against both rat brain (2.2 nM vs. 12.8 nM, respectively) and liver COMT (927 nM vs. 2320 nM, respectively). However, the reported values cannot be judged to be significantly different due to the overlapping 95% confidence intervals. On the other hand, De Santi et al. (1998) have reported that entacapone (IC_{50} 110 – 186 nM) is significantly more potent than tolcapone (IC_{50} 773 – 1389 nM) against human duodenal, liver and kidney COMT.

For rat liver COMT, entacapone seems to have slightly lower inhibitor constant (K_i) (14 nM) than tolcapone (30 nM) (Zürcher et al. 1990a; Nissinen et al. 1992). Against human recombinant S-COMT, the K_i values of entacapone and tolcapone were equal (0.3 nM), but tolcapone seemed to possess a lower K_i in MB-COMT than entacapone (0.3 and 2.0 nM, respectively) (Lotta et al. 1995).



Entacapone Tolcapone

Figure 4. The structures of entacapone and tolcapone.

The currently known potent COMT inhibitors share a common feature; they are all catechols with electronegative substituents, such as a nitro group, at the 5-position (Vidgren et al. 1999; Learmonth et al. 2002, 2004). Nitrocatechol-structured inhibitors, as shown with 3,5-dinitrocatechol and 1-(3,4-dihydroxy-5-nitrophenyl)-3-[4-[3-trifluoromethyl)-phenyl]-1-piperazinyl-1-propanone hydrochloride (BIA 3-335), bind to the active site of COMT by chelation of the hydroxyl groups with Mg^{2+} ion (Vidgren et al. 1994; Bonifacio et al. 2002). This confirms also the competitive type of inhibition

with respect to the catechol substrate (Vidgren et al. 1999). Nitrocatechols are not good substrates of COMT but potent inhibitors since the electronegative nitro group decreases the nucleophilicity of ionized catechol hydroxyls in the vicinity of SAM preventing the methylation step (Ovaska and Yliniemelä 1998). A recent structure-activity relationship study of COMT inhibitors supports fully the importance of the interaction between catechol oxygens and Mg^{2+} as well as on the electron-withdrawing property of catecholic substituent (5-position) and suggests that the potency of entacapone could be increased by replacing the 5-nitro group with an even more electronegative group (Tervo et al. 2003).

Since the different affinities of catechol substrates to COMT have been explained by the interactions between the polar side chain of the substrate and hydrophobic gatekeepers Trp38, Trp143 and Pro174 of COMT (see section 2.1.3), these interactions likely affect the binding of catechol-structured inhibitors as well. In fact, it is known that 3,5-dinitrocatechol has almost an ideal interaction with Trp38 and Trp174 (Vidgren et al. 1999). However, also rather large side chains attached to 1-position, as in BIA 3-335, seem to have enough space to be accommodated within the active site (Bonifacio et al. 2002).

2.4.2 Pharmacokinetics

2.4.2.1 Animal studies

The animal pharmacokinetic data on entacapone and tolcapone are scarce but it seems that entacapone is eliminated faster than tolcapone and its oral bioavailability is lower than that of tolcapone in rats. After intravenous administration of 10 mg/kg, the elimination half-life ($t_{1/2\beta}$) of tolcapone was 0.9 h and the total clearance (CL) 470 ml × h^{-1} × kg^{-1} (Funaki et al. 1994, 1995). The oral bioavailability of tolcapone was 48% and 56% after administration of 20 mg/kg and 40 mg/kg, respectively (Funaki et al. 1994). After intravenous administration of 5.7 mg/kg, the $t_{1/2\beta}$ and CL of entacapone were 0.4 h and 829 ml × h^{-1} × kg^{-1}, respectively (Savolainen et al. 2000a). According to Savolainen et al. (2000a), the oral bioavailability of entacapone (5.7 mg/kg p.o.) is 10 – 35% depending on the entacapone formulation.

Studies in experimental animals, as judged by inhibition of brain COMT, indicate that tolcapone penetrates into the brain whereas entacapone has a mainly peripheral action (Zürcher et al. 1991; Nissinen et al. 1992; Männistö et al. 1992b; Learmonth et al. 2002). In parallel, some 6-FDOPA positron emission tomography (PET) studies in primates have revealed the central action of tolcapone (Doudet et al. 1997; Holden et al. 1997).

2.4.2.2 Human studies

After administration of a therapeutic single oral dose (entacapone 200 mg; tolcapone 100 or 200 mg) to healthy volunteers, the maximal plasma concentration (C_{max}) is reached within 0.7 – 1.8 hrs (Table 1). The plasma levels and the area under the plasma drug concentration-time curve ($AUC_{0-\infty}$) reached after administration of tolcapone are clearly higher than those of entacapone (Table 1), which reflects the better bioavailability and lower CL of tolcapone (Keränen et al. 1994; Dingemanse et al. 1995a). Tolcapone is almost completely (>99.9%) bound to plasma proteins whereas the corresponding fraction is about 98% for entacapone (Dingemanse 1997; Männistö and Kaakkola 1999). After a single oral dose of 200 mg, both entacapone and tolcapone are rapidly eliminated with a $t_{1/2\beta}$ of 1.8 – 3.4 h (Keränen et al. 1994; Dingemanse et al. 1995a; Jorga et al. 1998a).

Table 1. Pharmacokinetic parameters of entacapone and tolcapone after oral administration of a single dose. Values are means of 5 – 12 subjects

	Entacapone[a,b]	Tolcapone[c]	
	200 mg	100 mg	200 mg
C_{max} (μg/ml)	1.8	4.6	5.7 – 6.3
t_{max} (h)	0.7	1.7	1.8
$AUC_{0-\infty}$ (μg × h × ml^{-1})	1.6	12.2	17.5 – 18.5
Bioavailability (%)	36	n.d.	60

C_{max}, maximal plasma concentration; t_{max}, time required to reach maximal plasma concentration; $AUC_{0-\infty}$, area under curve; n.d., not determined

[a] Data from Keränen et al. (1994)

[b] (*E*)-isomer; the proportion of (*Z*)-isomer of the AUC is approximately 5%, see Keränen et al. (1994)

[c] Data from Dingemanse et al. (1995); Jorga et al. (1998a)

Both entacapone and tolcapone are extensively metabolized. The main entacapone metabolite is the glucuronide (Wikberg et al. 1993). A large proportion of the orally administered entacapone and its metabolites is excreted in bile and feces (Wikberg et al. 1993; Wikberg and Vuorela 1994; Heikkinen et al. 2001b). In addition to glucuronidation, tolcapone is also *O*-methylated by COMT to 3-*O*-methyltolcapone and oxidized by cytochrome P450 enzymes (Jorga et al. 1999). The half-life of 3-*O*-methyltolcapone is extremely long (30 – 40 h) (Dingemanse et al. 1995a, b). However, during repeated administration, 3-*O*-methyltolcapone accumulates less than one would expect on the basis of its half-life suggesting that inhibition of COMT decreases its formation (Dingemanse et al. 1995a, 1996). About 57% of the oral tolcapone dose is excreted in urine and about 41% in feces (Jorga et al. 1999).

Recently, Heikkinen et al. (2001b) have studied the pharmacokinetics of entacapone in detail by giving simultaneously an intravenous dose of ^{13}C-labeled entacapone (20 mg within 20 minutes) and an oral dose (100 mg) of unlabeled entacapone. The

sensitive assay method allowed the determination of entacapone concentrations for up to 12 h after intravenous administration and revealed three phases of elimination. Both the distribution phase from the central compartment with short $t_{1/2\alpha}$ (0.05 h) and rapid elimination phase ($t_{1/2\beta}$=0.38 h) highlight the rapid elimination of entacapone. The terminal elimination phase, which represented only a small part of the total AUC, was characterized by $t_{1/2\gamma}$ of 2.41 h. In the tolcapone study of Jorga et al. (1998a), the $t_{1/2\beta}$ of tolcapone was 1.2 – 1.6 h after intravenous administration of 50 mg within 10 min. The studies of Jorga et al. (1998a) and Heikkinen et al. (2001b) have also confirmed that the CL of entacapone is much higher (11.7 ml × min^{-1} × kg^{-1} or 52 l × h^{-1}) than that of tolcapone (6.2 – 7.3 l × h^{-1}) in healthy male volunteers.

Direct evidence indicating brain penetration of tolcapone in humans is lacking. However, in a recent 6-FDODA PET study of Ceravolo et al. (Ceravolo et al. 2002), the putamen influx constant of 6-FDOPA 180 – 240 min after administration of 6-FDOPA did not differ from the corresponding early constant (30 – 90 min) in tolcapone-treated (a single oral dose of 200 mg) PD patients. In contrast, the late constant was significantly lower than the early constant in placebo-treated PD patients. The ability of tolcapone to prevent the decline of the late influx constant points to the inhibition of central COMT and stabilization of synaptic DA levels. However, it is still unclear whether inhibition of central COMT provides any additional benefits in the treatment of patients with PD; major clinical benefits of COMT inhibitors in the treatment of PD are currently being achieved by peripheral COMT inhibition only (Dingemanse 1997; Kaakkola 2000; Baas et al. 2001).

2.4.3 Pharmacodynamics

2.4.3.1 Animal studies

Both entacapone and tolcapone are potent COMT inhibitors *in vivo*. The ED_{50} values reported for inhibition of rat liver at one hour after administration are 6.7 mg/kg and 6.3 mg/kg, respectively (Zürcher et al. 1990b; Nissinen et al. 1992). However, in the recent study of Learmonth et al. (2002), clearly lower values were observed (1.9 mg/kg and 0.7 mg/kg for entacapone and tolcapone, respectively). The time course of COMT activity in different tissues (liver, kidney, duodenum, brain) has been studied in rats after oral administration of entacapone or tolcapone but at very different doses (entacapone at 10 mg/kg, tolcapone at 4 – 100 mg/kg) (Zürcher et al. 1990b, 1991; Nissinen et al. 1992). These results suggest that tolcapone has generally a longer duration of inhibitory action than entacapone (Zürcher et al. 1990b; Nissinen et al. 1992). Learmonth et al. (2002) have reported that after a single oral dose of 30 mg/kg, tolcapone has a clearly longer duration of action than entacapone (67% vs. 25% inhibition in the liver at 9 h after administration). Multiple dose studies have not been published.

The effects of COMT inhibitors on the levels of DA and its metabolites in the rat brain have been characterized in detail. Entacapone and tolcapone have no significant effect either on striatal extracellular DA or brain tissue DA levels (Zürcher et al. 1990b, 1991; Acquas et al. 1992; Kaakkola and Wurtman 1992; Napolitano et al. 1995). However, inhibition of COMT leads to a significant increase in DOPAC levels and concomitant decrease in HVA levels. In the case of tolcapone, both striatal extracellular and brain tissue DOPAC levels are increased maximally by about 150% and HVA levels are decreased by about 90% at doses 10 – 30 mg/kg (Zürcher et al. 1990b, 1991; Acquas et al. 1992; Kaakkola and Wurtman 1992; Napolitano et al. 1995). Similar effects are seen also after entacapone treatment, but at doses 30 – 100 mg/kg (Kaakkola and Wurtman 1992).

2.4.3.2 Human studies

As assessed by inhibition of COMT activity in erythrocytes, tolcapone has a longer duration of action; after oral administration of 200 mg, the full recovery of COMT activity is achieved within 6 – 8 h after oral administration of 200 mg entacapone whereas over 15 h is needed for recovery after administration of 200 mg tolcapone (Keränen et al. 1994; Dingemanse et al. 1995a, b). After 100 mg, the required time is only slightly shorter. At equivalent oral doses, tolcapone produces a higher maximal degree of COMT inhibition than entacapone but the time to reach this level is longer (approximately 2 h vs. 1 h) (Keränen et al. 1994; Dingemanse et al. 1995a, b). In the case of tolcapone, there is pharmacokinetic evidence to suggest that this lag time may limit the clinical benefit of the first morning LD dose taken simultaneously with tolcapone (Baas et al. 2001).

Due to different pharmacokinetic and pharmacodynamic properties, the treatment strategies of entacapone and tolcapone are different. The entacapone regimen is to take 200 mg with each LD-DDC inhibitor dose, maximally 10 times per day (EMEA 2005a). Due to the longer duration of its COMT inhibiting action, tolcapone is taken three times per day with a 12 h interval between the evening and morning dose (EMEA 2005d). During tolcapone treatment, COMT is continuously inhibited, whereas the entacapone regimen permits the recovery of COMT activity, at least to some extent, between doses (Männistö and Kaakkola 1999).

2.4.4 Effect on levodopa pharmacokinetics

2.4.4.1 Animal studies

In rats, administration of entacapone and tolcapone concomitantly with LD and a DDC inhibitor, decreases the formation of 3-OMD in the periphery and thus increases

LD levels in serum/plasma (Zürcher et al. 1990a, 1991; Nissinen et al. 1992). In the study of Zürcher et al. (1990a), the AUC of LD was increased 4-fold when tolcapone (30 mg/kg p.o.) was combined with LD-BZ (10/15 mg/kg p.o.). About a 2-fold increase was still seen, although the LD dose was doubled in the group receiving LD-BZ only (Zürcher et al. 1991).

Both entacapone and tolcapone increase LD, DA and DOPAC levels and decrease 3-OMD levels in the rat brain when combined with LD-DDC inhibitor (Zürcher et al. 1990a; Männistö et al. 1992b; Nissinen et al. 1992). At doses 10 – 30 mg/kg (i.p./p.o.), entacapone increases brain HVA levels, which is a reflection of the increased DA levels, whereas tolcapone decreases HVA levels as a result of inhibition of brain COMT (Männistö et al. 1992b; Nissinen et al. 1992). In microdialysis studies, entacapone and tolcapone (10 – 30 mg/kg i.p./p.o.) increase striatal extracellular levels of LD, DA and DOPAC when given as an adjunct to LD-DDC inhibitor combination, with tolcapone being more potent (Acquas et al. 1992; Kaakkola and Wurtman 1993; Napolitano et al. 1995). Recently, Napolitano et al. (Napolitano et al. 2003) have confirmed in freely moving rats that both entacapone and tolcapone at 15 mg/kg p.o. increase equally the striatal extracellular LD levels by inhibiting formation of 3-OMD in the periphery but that tolcapone increases more the DA levels as a result of inhibition of central COMT. Finally, 6-FDOPA PET studies in primates indicate that when given with CD, entacapone and tolcapone increase the 6-FDOPA availability into the brain by inhibiting its peripheral metabolism (see Männistö and Kaakkola 1999).

2.4.4.2 Human studies

By inhibiting the peripheral metabolism of LD (see section 2.2.2), entacapone and tolcapone (200 mg) increase the AUC of LD in healthy volunteers after administration of standard LD-CD (100/25 mg) preparation (Table 2) without increase in C_{max} value or time required to reach it (t_{max}) (Keränen et al. 1993; Sedek et al. 1997). Accordingly, the plasma levels of 3-OMD are significantly decreased (Table 2). Additionally, tolcapone prolongs the $t_{1/2\beta}$ of LD (Table 2). COMT inhibitors also increase plasma DOPAC levels and decrease HVA levels (Table 2). Recently, Heikkinen et al. (2002) have shown that 200 mg entacapone increases LD $AUC_{0-12\ h}$ by 30 – 40% independently of the LD-CD dose. In the case of tolcapone, the increase in LD $AUC_{0-\infty}$ varied between 60 – 90% with different LD-CD doses (100/10, 100/25, 200/20, 200/50, 250/25) (Jorga et al. 1998b). Entacapone and tolcapone increase the LD AUC also when combined with controlled-release LD-DDC inhibitor preparation (Ahtila et al. 1995; Jorga et al. 1997, 1998b). As an adjunct to controlled-release LD-CD (200/50 mg), tolcapone seems to be more efficacious than entacapone (LD AUC +69% vs. +21%) (Ahtila et al. 1995; Jorga et al. 1998b), probably due to its longer duration of action.

Table 2. The effect of entacapone and tolcapone on the pharmacokinetics of levodopa and its main metabolites in healthy volunteers after administration of a single dose concomitantly with standard levodopa-carbidopa (100/25 mg) tablet. Values are means of 6 –16 subjects

	Entacapone[a]	Tolcapone[b]	
	200 mg	100 mg	200 mg
LD AUC	+35 –42%	+50%	+69 –88%
LD $t_{1/2\beta}$	n.s.	+32%	+53 –60%
3-OMD AUC	−45[c] –46%	−64%	−62 –64%
DOPAC AUC	+160[c] –214%	+175%	+253%
HVA AUC	−35%[c]	−52%	−54%

AUC, area under curve; $t_{1/2\beta}$, elimination half-life; n.s., no significant change
[a] Data from Keränen et al. (1993); Heikkinen et al. (2002)
[b] Data from Sedek et al. (1997); Jorga et al. (1998b)
[c] Estimated from Figure 2 of Heikkinen et al. (2002)

In PD patients, single entacapone dose (200 mg) given concomitantly with a standard LD-DDC inhibitor preparation, increases LD AUC by 23 –48% and $t_{1/2\beta}$ by 32 –75% but does not significantly change C_{max} and t_{max} of LD (Myllylä et al. 1993; Nutt et al. 1994; Ruottinen and Rinne 1996). In the case of tolcapone (200 mg, single dose), LD AUC and $t_{1/2\beta}$ are increased by 32 –58% and 77 –79%, respectively (Tohgi et al. 1995; Limousin et al. 1995). In parallel with entacapone, the C_{max} and t_{max} of LD are not changed. After repeated administration for 6 –8 weeks with the recommended dosage regimen (200 mg entacapone with each individual LD dose or up to 200 mg tolcapone t.i.d.), entacapone and tolcapone increased LD AUC similarly (by 43% and by 33 – 62%, respectively), but tolcapone decreased the 3-OMD levels more than entacapone (Nutt et al. 1994; Yamamoto et al. 1997; Napolitano et al. 1999). It is noteworthy that the recommended starting dose of tolcapone is 100 mg t.i.d. (EMEA 2005d). In a recent two-week, placebo-controlled crossover entacapone study with PD patients (entacapone given 4 –6 times daily with each individual LD-CD dose), the LD AUC was increased by 27% with 200 mg entacapone (Heikkinen et al. 2001a).

When LD-CD is administered at 2 – 4 h intervals, entacapone increases mean plasma concentration (+23%) i.e. it elevates the LD trough concentration but does not elevate peak concentrations even though the LD dose has been reduced (Nutt et al. 1994). Thus, also the fluctuations in the LD levels are significantly reduced (Nutt 2000). Entacapone decreases daily variation of LD concentration also when administered with controlled-release LD-CD (100/25 mg q.i.d. at 4 h intervals) in healthy volunteers (Paija et al. 2005). During repeated administration of LD-BZ four times daily with tolcapone treatment (100 mg t.i.d.) the peak concentration seen after each dose is increased by 20 –114% but the trough concentrations are as much as 1.6 –2.7-fold higher as compared to values without tolcapone (Baas et al. 2001).

In humans, the effect of entacapone on LD transport into the brain has been evaluated by 6-FDOPA PET studies (Ruottinen et al. 1995, 1997, 2001). The results indicate that inhibition of peripheral COMT by entacapone increases the 6-FDOPA

levels in plasma and thus 6-FDOPA availability to the brains of PD patients. It is noteworthy that the time course of the entacapone-induced increase in striatal 6-FDOPA accumulation is different in healthy controls and in patients with advanced PD; in controls, the 6-FDOPA accumulation was increased both during early (30 – 90 min after injection) and late imaging (150 – 210 min after injection), whereas patients showed significant, comparable increase during the later scan only (Ruottinen et al. 2001).

2.4.5 Levodopa-carbidopa-entacapone combination tablet

Since entacapone is being administered concomitantly with each individual LD-DDC inhibitor dose (EMEA 2005a) and the pharmacokinetic properties of LD and entacapone are similar (Kaakkola 2000), the combination tablet is a rational development step to separately administered LD-DDC inhibitor preparation and entacapone tablet. LD-CD-entacapone combination tablet (Stalevo®) received market authorization in the EU in October 2003. It is available in three dosage combinations (LD/CD/entacapone): 50/12.5/200, 100/25/200 and 150/37.5/200 mg (EMEA 2005b).

The results of bioequivalence studies in healthy volunteers (male/female, n = 44) have been summarized recently by Hauser (2004). At corresponding doses, the combination tablet provided equivalent mean AUC and C_{max} of LD, CD and entacapone with separately administered LD-CD and entacapone. In the case of entacapone, however, some variation in C_{max} values was observed. Mean $t_{1/2}$ values of LD were equal between the combination tablet and corresponding reference product. Also the adverse effect profiles were comparable to separately administered tablets with the exception of nausea in 150/37.5/200 group, which has been explained by slightly higher entacapone C_{max} in combination tablet group than that in corresponding reference group (Hauser 2004). In the LD-DDC inhibitor-treated PD patients with wearing-off fluctuations, combination tablet produced an equal improvement in motor performance and reduction in fluctuations as could be attained with the separately administered LD-DDC inhibitor preparation and entacapone but 81% of patients preferred the combination tablet (Brooks et al. 2005).

2.4.6 Optimization of triple treatment

In general, there has been some doubt that the currently used doses of COMT and DDC inhibitors are not optimal in the triple treatment. It has been suggested that the dose of the COMT inhibitor could be reduced if the dose of DDC inhibitor was concomitantly increased (Oechsner et al. 1998; Männistö and Kaakkola 1999). This could further improve the efficacy of LD and reduce the risk of adverse effects associated with COMT inhibitors. However, carefully planned pharmacokinetic (and pharmacodynamic) studies are needed to confirm this hypothesis.

Both CD and BZ are highly effective, pseudoirreversible inhibitors of peripheral DDC, but they do not completely block its activity (LeWitt 1989; Dingemanse et al. 1997). Additionally, inhibition of COMT may increase burden back to the DDC pathway. On the other hand, both CD and BZ are substrates of COMT (Lautala et al. 2001), and inhibition of COMT may thus increase the plasma CD and BZ levels. However, this effect might have clinical importance only in the case of BZ, since it has 10-higher K_m for S-COMT than entacapone (Lautala et al. 2001). In addition to this theoretical basis, there is some clinical evidence, even in the standard LD-DDC inhibitor therapy, that the degree of DDC inhibition might be insufficient at current doses (Oechsner et al. 1998; Olanow et al. 2001). Generally, CD and BZ have been considered as being poorly brain penetrating due to their low lipophilicity (Clark et al. 1973) but studies in experimental animal suggest that also brain DDC may be inhibited at higher doses (especially with BZ), and this would have detrimental effects on LD-induced increase in striatal DA levels (Tedroff et al. 1991; Kaakkola et al. 1992; Jonkers et al. 2001).

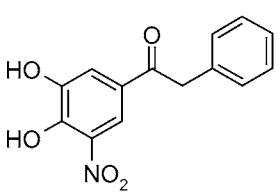
As assessed by the effects on LD AUC and $t_{1/2\beta}$ in PD patients, 200 mg of entacapone (single dose) is only slightly more effective than 100 mg (Ruottinen and Rinne 1996). However, during repeated administration the situation may be different. For example, in a two-week, placebo controlled crossover study of Heikkinen et al. (2001a), 100 mg of entacapone increased LD AUC by 17% and 200 mg by 27%, but 100 mg and 200 mg increased the $t_{1/2\beta}$ similarly.

2.5 New COMT inhibitors and new entacapone derivatives

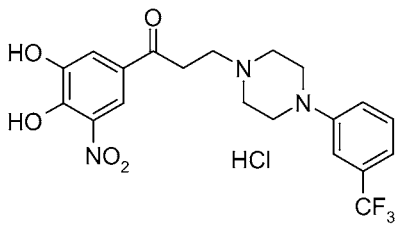
2.5.1 New nitrocatechol inhibitors

Recently, Learmonth et al. (2002, 2004) described two series of new nitrocatechol-structured COMT inhibitors. In the first series, the side chain length of nitrocatechol homologues was varied to find optimal lipophilicity to yield potent, peripheral COMT inhibitor with a longer duration of action than entacapone (Learmonth et al. 2002). 1-(3,4-dihydroxy-5-nitrophenyl)-2-phenyl-ethanone, BIA 3-202 (Figure 5) was found to have a clearly longer duration of inhibitory action in rat liver at an oral dose of 30 mg/kg in contrast to entacapone or other novel compounds. It inhibited also brain COMT, but to a lesser extent than tolcapone (22% vs. 78% inhibition at 9 h after administration, respectively). The ED_{50} values of BIA 3-202 and tolcapone were equal (0.7 mg/kg at 1 h) in the rat liver but BIA 3-202 was significantly less potent in the brain (5.3 mg/kg vs. 1.6 mg/kg). When compared with entacapone, BIA 3-202 was more potent (lower ED_{50} value) both in the liver and brain. Several structural modifications, such as acylation of phenolic hydroxyls, replacement of the C1 carbonyl moiety or substitution of side chain phenyl ring, to BIA 3-202 were made but the parent

compound was proven to be optimal to achieve prolonged peripheral COMT inhibition (Learmonth et al. 2002).



BIA 3-202



BIA 3-335

Figure 5. The structures of new nitrocatechol-structured COMT inhibitors.

Parada et al. (2001) have studied the effects of BIA 3-202 on striatal LD, DA and their metabolite levels in LD-BZ-treated (20/30 mg/kg p.o.) rats. BIA 3-202 (3, 10 and 30 mg/kg p.o.) increased dose and time dependently striatal LD levels (up to 6 h after administration) with a concomitant decrease in the 3-OMD levels. The elevation of HVA levels indicates that striatal COMT was not inhibited. The compound's peripheral effect is supported by the fact that striatal HVA levels were not changed after administration of BIA 3-202 alone. However, BIA 3-202 (10 and 30 mg/kg) increased significantly striatal DA levels 3 – 6 h after administration, which is not in line with a peripheral action. In microdialysis experiments, as expected, BIA 3-202 (30 mg/kg p.o.) increased the striatal extracellular LD levels and decreased 3-OMD levels in LD-BZ-treated (12/3 mg/kg p.o.) rats (Soares-da-Silva et al. 2003), but unexpectedly, extracellular DA levels were not elevated.

BIA 3-202 has also undergone clinical studies in healthy volunteers. After administration of a single dose of 100 or 200 mg, the C_{max} and t_{max} values of BIA 3-202 (Table 3) are similar to those seen after administration of tolcapone but higher than after administration of entacapone (Table 1). At 200 mg, the $AUC_{0-24\,h}$ value of BIA 3-202 is near to that observed after tolcapone ($AUC_{0-\infty}$) but clearly higher than that after entacapone (Tables 1 and 3). Elimination of BIA 3-202 is rapid ($t_{1/2\beta}$ of 2.0 – 2.9 h) (Almeida and Soares-da-Silva 2003b) as is the case with entacapone and tolcapone (see section 2.4.2.2). The bioavailability of BIA 3-202 has not been reported.

When given concomitantly with LD-CD (100/25 mg), BIA 3-202 increased the $AUC_{0-\infty}$ and the $t_{1/2\beta}$ of LD (Table 3). It also decreased dose dependently plasma 3-OMD levels (Table 3). As assessed by the increase in LD AUC, 200 mg BIA 3-202 seemed to be as effective as 200 mg of entacapone but less effective than 200 mg tolcapone (see Tables 1 and 3). The erythrocyte COMT activity was inhibited maximally by 78% at 1.8 h and the activity had fully recovered within 12 – 18 h after administration of 200 mg BIA 3-202 (Almeida et al. 2004). Also these outcomes are close to those of tolcapone (Dingemanse et al. 1995b). BIA 3-202 has a similar effect on the LD AUC, 3-OMD AUC and erythrocyte COMT activity also when given

concomitantly with LD-BZ (Silveira et al. 2003). However, the LD $t_{1/2\beta}$ was not significantly prolonged. Repeated administration of 50, 100, 200 mg b.i.d or 200 mg t.i.d. for 8 days did not result in any significant accumulation of BIA 3-202 in plasma but the levels of its *O*-methylated metabolite were about 10-fold higher, and they declined slowly with a $t_{1/2\beta}$ of 72 –86 h after discontinuation of treatment (Almeida and Soares-da-Silva 2003a).

Table 3. Pharmacokinetic parameters of BIA 3-202 after oral administration of a single dose (values are means of 7 subjects) and the effect of BIA 3-202 on pharmacokinetics of levodopa and 3-*O*-methyldopa in healthy volunteers after administration of a single dose concomitantly with standard levodopa-carbidopa (100/25 mg) tablet (values are means of 17 –18 subjects)

	BIA 3-202	
	100 mg	200 mg
C_{max} (μg/ml)[a]	4.1	5.9
t_{max} (h)[a]	1.5	2.0
$AUC_{0\text{-}24\ h}$ (μg × h × ml^{-1})[a]	7.4	16.0
LD AUC[b]	n.s.	+50%
LD $t_{1/2\beta}$[b]	+75%	+91%
3-OMD AUC[b]	−47%	−61%

C_{max}, maximal plasma concentration; t_{max}, time required to reach maximal plasma concentration; $AUC_{0-\infty}$, area under curve; n.s., no significant change

[a] Data from Almeida and Soares-da-Silva (2003)

[b] Data from Almeida et al. (2004)

In the second series, several homologous heteroatom-containing substituents to the side chain of nitrocatechol moiety, with 1-(3,4-dihydroxy-5-nitrophenyl)-ethanone acting as the parent compound, were made to study the possibilities to decrease further the central effect and prolong the duration of COMT inhibitory action (Learmonth et al. 2004). Oral administration of 1-(3,4-dihydroxy-5-nitrophenyl)-3-[4-[3-trifluoromethyl)-phenyl]-1-piperazinyl-1-propanone hydrochloride (30 mg/kg), BIA 3-335 (Figure 5), to mice resulted in clearly longer duration of COMT inhibitory action in the liver than entacapone (74% inhibition vs. 26% inhibition at 9 h after administration) and superior selectivity for liver COMT inhibition over brain COMT inhibition (82% vs. 14% inhibition at 1 h, respectively) as compared to tolcapone (99% inhibition both in the liver and brain at 1 h).

In conclusion, the BIA compounds have mainly a peripheral action being similar in that respect to entacapone but they have a clearly longer duration of action. Since the pharmacokinetic and pharmacodynamic properties of BIA 3-202 are similar to tolcapone, also the treatment strategy of BIA 3-202 could be assumed to be similar with tolcapone, i.e. the novel drug is administered twice or thrice daily independently of LD doses. However, multiple dose studies with BIA 3-202 as an adjunct to a LD-DDC inhibitor treatment have not been published yet.

2.5.2 Entacapone prodrugs

A prodrug is a pharmacologically inactive compound that is biotransformed to an active compound within the body (Stella et al. 1985). The prodrug technique has been employed to improve the oral absorption of drugs with low aqueous solubility and lipophilicity (reviewed in Stella et al. 1985; Fleisher et al. 1996; Taylor 1996). Recently, several entacapone prodrugs have been introduced by Leppänen et al. (2000, 2001) and Savolainen et al. (2000b). The aim was to increase the aqueous solubility and lipophilicity of entacapone and to evaluate the possibilities to improve the oral bioavailability of entacapone. In the study of Savolainen et al. (2000b), (*E*)-2-cyano-*N,N*-diethyl-3-[3-*N*-t-butylcarbamoyloxy-5-nitrophenyl]propenamide (prodrug A, Figure 6) showed higher apparent partition coefficient (log P_{app}) but also slightly higher aqueous solubility at pH 5.0 and 7.4 whereas (*E*)-2-cyano-*N,N*-diethyl-3-[3-*N*-ethylcarbamoyloxy-5-nitrophenyl]propenamide (prodrug B) had significantly higher aqueous solubility but lower log P_{app} than entacapone. Despite promising *in vitro* data, the pharmacokinetic studies in rats did not show any improvements compared to entacapone; the observed C_{max} and $AUC_{0-2\ h}$ values of these prodrugs were more than 50% lower than those observed after administration of the equivalent dose of entacapone.

Leppänen et al. (2001) have also synthesized a series of lipophilic entacapone prodrugs. One acyl ester prodrug, (*E*)-2-cyano-*N,N*-diethyl-3-[3-pivaloyloxy-4-hydroxy-5-nitrophenyl]propenamide (prodrug C, Figure 6), had clearly higher log P_{app} than entacapone at pH 5.0 (2.24 vs. 1.58) and 7.4 (0.80 vs. 0.18) and similar aqueous solubility as entacapone at pH 7.4. In animal pharmacokinetic studies, it showed a plasma profile resembling entacapone after administration of entacapone suspension but at a lower level. Since the oral bioavailability of entacapone was not improved by lipophilic prodrugs, it was suggested that the relatively low lipophilicity of entacapone at neutral pH is not the main reason for its poor bioavailability (Savolainen et al. 2000b; Leppänen et al. 2001).

A phosphate ester prodrug of entacapone, entacapone monophosphate (prodrug D, Figure 6), has more than 1700-fold and 20-fold higher aqueous solubility than the parent drug at pH 1.2 and 7.4, respectively (>30 000 μg/ml vs. 16.6 and 1750 μg/ml) (Leppänen et al. 2000). Subsequent *in vivo* experiments have shown that the $AUC_{0-2\ h}$ value of entacapone is 2-fold higher after oral administration of entacapone monophosphate solution than after administration of an entacapone suspension (Heimbach et al. 2003). However, plasma levels observed shortly after administration of entacapone solution (pH 7.4) were not reached with the prodrug, suggesting that the pro-moiety may limit the absorption of entacapone or its hydrolysis may be too slow (Heimbach et al. 2003).

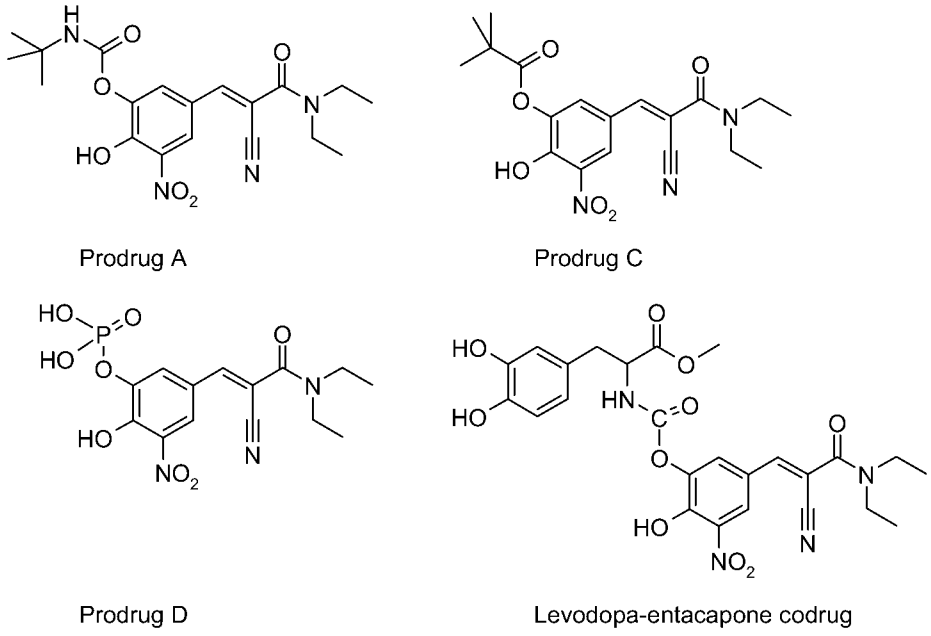


Figure 6. The structures of entacapone prodrugs and levodopa-entacapone codrug.

In some cases an inactive pro-moiety of a prodrug has been replaced with a pharmacologically active compound (see Leppänen 2002). These compounds are called "mutual prodrugs" or as "codrugs". Though this drug development approach has been utilized with antibiotics in order to prevent their degradation or prolong the duration of action, there are rather few codrugs available (see Leppänen 2002). Recently, Leppänen et al. (2002) introduced a novel LD-entacapone codrug in which LD and entacapone are linked with a biodegradable carbamate spacer (Figure 6). The ultimate goal of this drug development strategy was to study the possibility to improve the delivery of LD into the brain by ensuring the COMT inhibition at the site of LD absorption (Leppänen et al. 2002). Theoretically, also the variation in the LD absorption could be diminished. Their *in vitro* studies showed that the LD-entacapone codrug is enzymatically hydrolyzed to yield parent compounds in a rabbit liver homogenate (pH 7.4) with a half-life of 7 min. Unfortunately, *in vivo* pharmacokinetic studies were not performed.

2.5.3 Inhibitors without nitrocatechol-structure

Most known potent COMT inhibitors, including entacapone, tolcapone and BIA 3-202, are nitrocatechol derivatives. As discussed above, they do not have ideal pharmacokinetic properties (e.g. limited bioavailability, rapid metabolism and elimination). Furthermore, it has been suggested that the toxicity of tolcapone may be partly due to conversion of its amine and acetylamine metabolites to toxic species (see section 2.6.3). Thus, there has been some interest to develop new COMT inhibitors that

do not contain the nitrocatechol moiety. Since an electronegative substituent seems to be crucial for the inhibitory activity of catechols (see section 2.4.1), the nitro group is not easily replaced without decreasing the potency. Furthermore, the design of noncatechol-structured inhibitors has proven difficult (Vidgren et al. 1999).

Some COMT inhibitors that lack the nitrocatechol-structure have been published. GCP 28014 (Figure 7) is a hydroxypyridine derivative; it does not contain the catechol-structure at all (Waldmeier et al. 1990). When administered concomitantly with LD-CD at a high dose, it has a similar but weaker effect on brain LD, 3-OMD, DOPAC and HVA levels compared to entacapone and tolcapone (Männistö et al. 1992b). Furthermore, it increases 6-FDOPA availability to the brain in primates as do entacapone and tolcapone (reviewed in Männistö and Kaakkola 1999). However, GCP 28014 does not inhibit COMT *in vitro* at relevant concentrations, which suggests that it is probably a prodrug (Waldmeier et al. 1990) or an inhibitor of the uptake systems involved in the transport of released DA into glial cells (Männistö et al. 1992c; see also section 2.1.4.2). Brevitt et al. (1997) have introduced a series of COMT inhibitors that are of a "bifunctional" nature i.e. they have duplicated catechol substructures for binding to the enzyme. All these inhibitors lack the catecholic nitro group. Unfortunately, they are much less potent COMT inhibitors than entacapone and tolcapone; the K_i value of the best compound, *N,N*-1,3-propanediylbis(3,4-dihydroxybenzamide) (Figure 7) is 300 nM.

2.5.4 Bisubstrate inhibitors

Approaches to develop inhibitors for SAM-dependent methyltransferases have included analogues of the methyl acceptor substrate, the methylated product, SAM or SAH (Borchardt 1980). Most first-generation (see Guldberg and Marsden 1975) and second-generation COMT inhibitors (see Männistö and Kaakkola 1999) are catechol-structured methyl acceptor substrate analogues. In addition, several SAM analogues have been developed and studied as inhibitors of COMT and other SAM-dependent methyltransferases, but they have shown poor substrate and inhibitor properties, highlighting the high specificity of SAM-dependent methyltransferases for the structural features of SAM (see Borchardt 1980). On the other hand, the specificity of SAM analogues among methyltransferases has been a problem. However, recent studies with modern methods suggest that the binding site of SAM and the conformation of bound SAM vary between the different methyltransferases (Männistö and Taskinen, personal communication). Thus, it may be possible to design new, specific SAM inhibitors in the future.

One strategy for COMT inhibitor development has been bisubstrate or multisubstrate inhibition. In the 1980s, these kinds of compounds were designed and synthesized as "transition state analogues" of COMT i.e. by linking SAM or SAH and catechol moieties to a single structure (Anderson et al. 1981; Yau and Coward 1990).

More recently, new bisubstrate inhibitors of COMT have been designed and synthesized on the basis of crystal structure of COMT (Masjost et al. 2000; Lerner et al. 2003). The initial strategy was to connect the C(5′)-hydroxyl group of adenosine or adenosine substitutes to the catechol moiety via a suitable linker to occupy SAM and catechol-binding pockets of COMT (Masjost et al. 2000). The compound with a CH_2-CH_2-NH-CO linker between adenosine (ribose-C(5′)-O) and nitrocatechol (1-position) was considered to be the most promising (bisubstrate inhibitor 1, Figure 7). Masjost et al. (2000) showed that it binds first to the SAM pocket via a competitive mechanism (K_i = 0.3 μM) and then to the catechol pocket of COMT with a noncompetitive mechanism (K_i = 0.55 μM). Unfortunately, this compound is far less potent (IC_{50} = 2 μM) in rat liver COMT than entacapone, tolcapone and BIA 3-202.

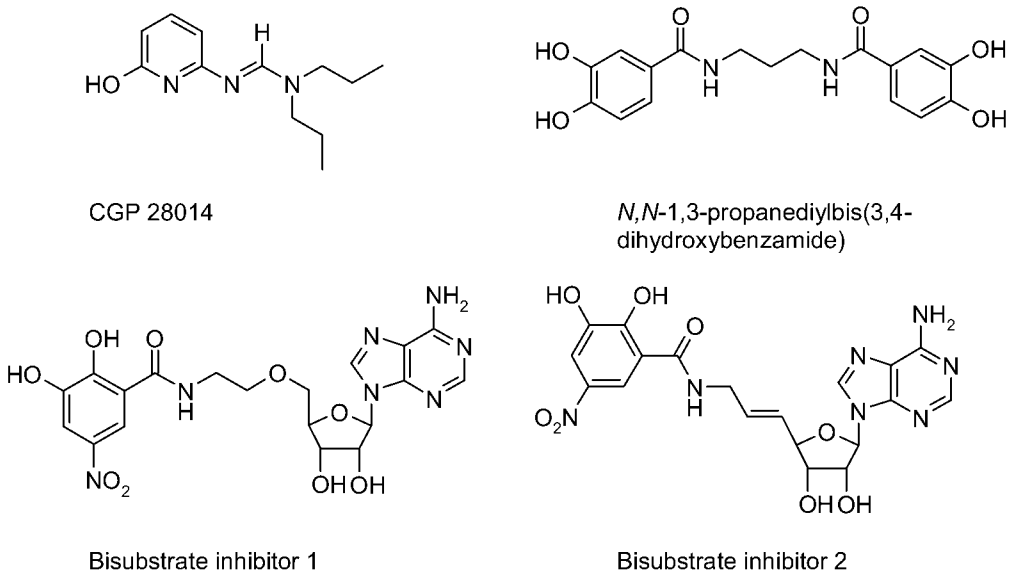


Figure 7. The structures of non-nitrocatechol COMT inhibitors and bisubstrate inhibitors.

Subsequently, Lerner et al. (2003) showed that the affinity of bisubstrate inhibitors for COMT is strongly dependent on the linker between the adenosine and nitrocatechol moiety. Replacement of the CH_2-O-CH_2-CH_2-NH-CO bridge in bisubstrate inhibitor 1 with CH=CH-CH_2-NH-CO (bisubstrate inhibitor 2, Figure 7) led to significantly higher COMT-inhibiting potency. In fact, this compound has an IC_{50} value of 9 nM in rat liver COMT, which is apparently even lower than that of entacapone, tolcapone and BIA 3-202 (see sections 2.4.1 and 2.5.1). Kinetic studies suggest that it has a similar ordered mechanism of inhibition as the parent compound (see above). Recently, Paulini et al. (2004) have shown that the bisubstrate inhibition approach permits the replacement of the catecholic nitro group without decreasing significantly the COMT-inhibiting potency. The IC_{50} value of about 30 – 40 nM in rat liver COMT was achieved by substituting the nitro group of bisubstrate inhibitor 2 with Br, Cl, CN or CF_3

atom/group. However, the most potent derivatives were those with fluorophenyl, methylphenyl and pyridine substituents (IC_{50} = 21 – 23 nM). New bisubstrate inhibitors are promising compounds but *in vivo* pharmacokinetic and pharmacodynamic studies are needed to assess their true potential as novel COMT inhibitors.

2.6 Toxicity of clinically available COMT inhibitors: an update

2.6.1 Non-clinical studies

Unacceptable toxicity was one of the main problems associated with first-generation COMT inhibitors (see Guldberg and Marsden 1975). Toxicity studies with entacapone and tolcapone in rodents indicate low toxicity; the oral LD_{50} values are >2 g/kg and the therapeutic ratio in oral treatment >50 (Männistö et al. 1992c). Preclinical safety data have revealed no special hazards for humans (EMEA 2005a, d). However, in a 24-month carcinogenicity study with tolcapone, 3% and 5% of rats in the mid- and high-dose groups, respectively, suffered renal epithelial tumors (EMEA 2005d). Also an increased incidence of uterine adenocarcinomas was observed with a high dose. In the case of entacapone, decreased fetal weight and slightly delayed bone development has been observed in rabbits (EMEA 2005a). It is noteworthy that no signs of hepatotoxicity were observed with either tolcapone or entacapone in preclinical toxicity studies (Watkins 2000).

Recent studies have noted a difference between the toxicity profiles of entacapone and tolcapone. Haasio et al. (2001) reported that tolcapone (400 or 600 mg/kg/day) increased mortality and clinical signs of toxicity, such as laboured breathing, decreased spontaneous motility and drowsiness, in rats during a 7 – 8-day study. In addition, the rectal temperature was significantly higher in tolcapone-treated animals than in controls throughout the study. Finally, histopathological examination revealed liver cell necrosis in four out of six rats treated with 600 mg/kg/day of tolcapone. In contrast, no signs of toxicity were seen in entacapone-treated animals. It is noteworthy that the calculated exposure factor (i.e. AUC_{rat}/AUC_{man}) of entacapone at 600 mg/kg/day was clearly higher than that of tolcapone at 400 mg/kg/day (21 vs. 14) suggesting a higher safety margin for entacapone. Furthermore, in the study of Korlipara et al. (2004), tolcapone was toxic to cultured human neuroblastoma SH-SY5Y cells at 100 and 200 μM (48 h exposure), whereas entacapone did not decrease cell viability even at 500 μM.

2.6.2 Clinical observations

In phase III clinical studies tolcapone was found to increase serum alanine aminotransferase (ALAT) or aspartate aminotransferase at doses 100 mg t.i.d and 200 mg t.i.d. but the liver function generally normalized within 2 – 3 weeks after

discontinuation of the treatment (Olanow 2000; Watkins 2000). Serum ALAT is a marker for hepatocellular injury; a high level of ALAT in serum is indicative of increased turnover of liver cells as a result of pathological processes (Watkins 2000). Thus, it was originally recommended to monitor liver transaminases for 6 months after the initiation of tolcapone treatment (EMEA 2005c). However, three cases of lethal liver injury linked to tolcapone treatment were reported soon after marketing authorization of tolcapone (reviewed in Olanow 2000). The potential of tolcapone to induce hepatotoxicity and the possible occurrence of rhabdomyolysis and neuroleptic malignant-like syndrome led to the suspension of its marketing authorization in Europe in November 1998 (EMEA 2005d; see also section 2.3.1.2). In the United States, prescribing information was revised and a black box warning was included (see Olanow 2000). After re-evaluation, the suspension of marketing authorization of tolcapone in Europe has been lifted recently (EMEA 2004). However, tolcapone is available only for restricted use (see section 2.3.1.2), and more careful monitoring liver function and checking for possible signs of underlying liver disease are required (EMEA 2005d, 2004). Furthermore, tolcapone is contraindicated in patients who have a history of neuroleptic malignant syndrome symptom complex and/or non-traumatic rhabdomyolysis or hyperthermia.

There are no reports of liver toxicity associated to entacapone treatment either in clinical studies or in postmarketing surveillance (Brooks 2004). No increased ALAT levels have been observed in clinical studies with entacapone (Watkins 2000). Fischer et al. (2002) have reported elevated liver enzyme levels in three entacapone-treated patients but the association with entacapone remains obscure due to other interfering factors (Beck et al. 2002). Thus, it seems that liver toxicity concerns seem to be restricted to tolcapone treatment.

2.6.3 Mechanisms of tolcapone toxicity

There is increasing *in vitro* and *in vivo* evidence that tolcapone impairs mitochondrial energy production by uncoupling oxidative phosphorylation. It has been shown to increase mitochondrial respiration i.e. uncoupling of oxidative phosphorylation *in vitro* at lower concentrations (EC_{50} = 2.6 μM) than a known uncoupler 2,4-dinitrophenol (EC_{50} = 12.5 μM), whereas entacapone is a significantly weaker uncoupler (EC_{50} = 58.0 μM) than either of these compounds (Nissinen et al. 1997). Recently, Korlipara et al. (2004) reported that tolcapone prevented ATP synthesis at concentrations of 50 μM and above in human neuroblastoma cells whereas entacapone had only a partial effect at 200 μM.

Haasio et al. (2002b) have studied the toxicity mechanism of tolcapone *in vivo* by assessing the consequences of uncoupling of oxidative phosphorylation. It was found that the serum ALAT level was increased and protein levels decreased in tolcapone-treated animals (500 mg/kg/day for 15 days) indicative of liver toxicity. Tolcapone (300

and 500 mg/kg/day) and 2,4-dinitrophenol (20 mg/kg) increased rectal body temperature as well as liver weight. The ATP/ADP ratio and energy charge of liver mitochondria was decreased significantly with a high tolcapone dose and 2,4-dinitrophenol. None of these effects were seen with entacapone even at 500 mg/kg/day. It is noteworthy that when the concentrations of entacapone and tolcapone were similar in the rat liver, tolcapone, but not entacapone, exhibited toxicity (Haasio et al. 2002b). There is also direct *in vitro* evidence of uncoupling effects of tolcapone; it disrupted mitochondrial membrane potential at similar concentrations (EC_{50} = 3.6 μM) as 2,4-dinitrophenol (EC_{50} = 1.7 μM) (Haasio et al. 2002a). In accordance with other studies, entacapone showed only a small effect at 100 μM.

Though there is convincing evidence of the uncoupling effects of tolcapone *in vitro* and *in vivo*, the differences between the concentrations inducing uncoupling and the therapeutic plasma concentrations in humans have raised some doubts of the clinical relevance of these findings. Borroni et al. (2001) have shown that the uncoupling effect of tolcapone in isolated rat liver mitochondria is significantly attenuated in the presence of serum albumin. Thus, they claimed that only the free tolcapone is responsible for the uncoupling effect and that toxic free concentrations in plasma are not achieved in clinical practice. However, it has to be stressed that actual tissue concentrations of either entacapone or tolcapone during repeated dosing at therapeutic doses are not known (see also section 6.3.3).

It has also been suggested that tolcapone-induced hepatotoxity may be partly due to the formation of reactive species from tolcapone metabolites (Smith et al. 2003). The 5-nitro group of tolcapone is reduced to yield the amine metabolite, which can be acetylated with the formation of the acetylamine derivative (Jorga et al. 1999). These metabolites can be oxidized to reactive quinone and quinone-imine species *in vitro* (Smith et al. 2003). On the other hand, nitrocatechol-structured COMT inhibitors (entacapone and nitecapone) seem to possess some antioxidant and radical scavenging effects (reviewed in Männistö and Kaakkola 1999). It is noteworthy that the corresponding acetylamine metabolite of entacapone is formed in rats but not in humans (Wikberg et al. 1993). Thus, the formation of reactive species may partly explain the different toxicity profiles of entacapone and tolcapone in humans but not in rats.

In conclusion, the toxicity of tolcapone seems to be associated with the compound itself, not with inhibition of COMT. This is further supported by the fact that COMT-deficiency as such does not induce any marked alterations in tissue histopathology, clinical chemistry or in haematological parameters values in mice (Haasio et al. 2003).

3 AIMS OF THE STUDY

The general objective of this non-clinical study was to compare the pharmacokinetic and pharmacodynamic properties of entacapone and tolcapone under identical conditions, to assess the effect of physicochemical properties on their behavior and to evaluate the role of COMT in DA-linked oxidative stress.

The specific aims of the study were as follows:

1. To evaluate the effect of bioavailability of entacapone on the pharmacodynamic response i.e. inhibition of COMT in erythrocytes
2. To compare the potencies of entacapone and tolcapone *in vitro* with total COMT preparation under identical experimental conditions taking tight binding inhibition into consideration.
3. To compare the pharmacokinetic and pharmacodynamic properties of entacapone and tolcapone under identical experimental conditions to reveal actual differences in their behavior and to evaluate the accumulation of entacapone and tolcapone in different tissues after repeated administration.
4. To assess the correlation between plasma COMT inhibitor concentration and the degree of COMT inhibition in various tissues.
5. To compare the pharmacodynamics of entacapone and tolcapone in the striatum when the influence of systemic pharmacokinetic processes on drug delivery into the brain has been excluded and to evaluate the role of physicochemical properties of entacapone and tolcapone on their behavior.
6. To assess the effect of COMT-deficiency on methamphetamine-induced hydroxyl radical production and the activity of certain enzymatic defences.

4 MATERIALS AND METHODS

4.1 Drugs

Entacapone (**I – III**) was obtained from Orion Pharma (Espoo, Finland). Tolcapone (**II, III**) was synthesised by Ms. Aino Pippuri (Orion Pharma, Espoo, Finland). Entacapone prodrugs (**I**), (E)-2-cyano-*N,N*-diethyl-3-[3-*N*-t-butylcarbamoyloxy-5-nitrophenyl]propenamide (prodrug A, Figure 6) and (E)-2-cyano-*N,N*-diethyl-3-[3-*N*-ethylcarbamoyloxy-5-nitrophenyl]propenamide (prodrug B) were synthesized as described previously (Savolainen et al. 2000b). Hydroxypropyl-β-cyclodextrin (HP-β-CD; Encapsin®) (**I**) was purchased from Janssen Biotech N.V (Belgium). (+)-S-Methamphetamine L-tartrate (METH) (**IV**) was synthesized according to Fogassy et al. (1986) by Dr. Jukka Leppänen with permission of National Agency of Medicine. L-3,4-dihydroxyphenylalanine methyl ester hydrochloride (LD methyl ester) was from Sigma-Aldrich (St. Louis, MO, USA) and sodium salicylate from Merck (Darmstadt, Germany) (**IV**).

4.2 Animals

Male Han/Wistar rats, supplied by the National Laboratory Animal Centre, Kuopio, Finland were used as experimental animals and tissue donors in pharmacokinetic and pharmacodynamic studies (**I – III**). COMT-deficient mice were originally generated in Rockefeller University (New York, NY, USA) by Gogos et al. (1998). Heterozygous (COMT +/−) male and female mice were bred in the National Laboratory Animal Center, Kuopio, Finland to produce mice of all three genotypes, and the strain was enriched by breeding C57BL/6J males or females with heterozygotes. The genotype of mice pups was determined by Southern blot analysis as described earlier (Huotari et al. 2002a). Mice of all COMT genotypes, i.e. wild-type (WT; COMT +/+), heterozygote (HET; COMT +/−) and homozygote (HOM; COMT −/−) were used in the experiments (**IV**).

Both rats and mice were housed in stainless steel cages and kept on a 12-h light – 12-h dark cycle (lights on at 7 am) at an ambient temperature of 22 ± 1°C. Pelleted food (Lactamin R36, Lactamin AB, Södertälje, Sweden) and tap water were available *ad libitum*. The rats used were 7 – 12 weeks old and weighed 130 – 370 g. Mice were 2 – 9 months old with the exception that skin tyrosinase dopa oxidase activity was determined also in 7 – 8-day old hairless pups (**IV**).

All procedures with animals were performed according to European Community Guidelines for the use of experimental animals. The protocols were reviewed by the Animal Ethics Committee at the University of Kuopio in conformance with current legislation and approved by the local Provincial Government when appropriate.

4.3 Inhibition of rat COMT *in vitro* (II, III)

Twenty-five untreated rats were sacrificed to obtain striata and livers. Tissue samples were homogenized in a MOPS buffer (pH 7.4) containing 0.32 M saccharose and 0.5 mM dithiothreitol. Striatal homogenates and the first halves of liver homogenates were centrifuged for 10 min at 900 × g at 4ºC. The supernatants containing both S- and MB-COMT were pooled and used as a total COMT preparation (total protein concentration ~4.4 and ~0.7 mg/ml in striatal and liver preparation, respectively). The other halves of the liver homogenates were centrifuged for 20 min at 15 000 × g, and the supernatants further for 60 min at 100 000 × g at 4°C. High-speed supernatants, enriched with S-COMT, were pooled, diluted and used as a S-COMT preparation (total protein concentration ~0.3 mg/ml). The pellet enriched with MB-COMT was washed, resuspended in homogenisation buffer and used as a MB-COMT preparation (total protein concentration ~5.5 mg/ml).

100 µl of supernatant was incubated for 30 min at 37ºC in 0.1 M phosphate buffer, pH 7.4, containing 200 µM SAM, 5 mM $MgCl_2$ and 240 µM 3,4-DHBA (the substrate) and varying concentrations (3×10^{-11} to 3×10^{-5} M, final concentrations) of entacapone or tolcapone in a total volume of 250 µl. The reaction was stopped by transferring the samples on ice and adding 25 µl of ice-cold 4 M perchloric acid. The samples were centrifuged for 10 min at 5400 × g at 4°C. The supernatants were filtered, diluted and stored at −70°C until analyzed.

For calculation of the inhibitor constant (K_i), substrate concentration that resulted in half-maximal reaction velocity (K_m) and maximum reaction velocity (V_{max}) of rat striatal and liver total COMT were determined without any inhibitor by varying the substrate concentration from 60 to 420 µM.

4.4 Pharmacokinetic and pharmacodynamic studies in rats (I, II)

4.4.1 Drug administration and sampling

Entacapone, tolcapone and prodrug A suspensions for oral administration were prepared by mixing an appropriate amount of drug in potassium biphthalate buffer (pH 3.0, USP 23). Entacapone, tolcapone and prodrug B solutions for oral and intravenous administrations were prepared by dissolving the drug in phosphate buffer (pH 7.4). Entacapone–HP-β-CD inclusion complex solution (entacapone/HP-β-CD) was prepared by dissolving entacapone in 30% HP-β-CD in potassium biphthalate buffer (pH 3.0). Drugs were given in a volume of 0.5 ml/100 g animal weight. In a 7-day study (**II**), entacapone and tolcapone was administered twice daily. Rats were fasted before drug administration (in a 7-day study (**II**) only before the last dose). When blood samples were taken from the retrobulbar plexus (**II**), the rats were anaesthetized with chloral hydrate (350 mg/kg i.p.) before administration of drug.

Animals were decapitated 10, 20, 30, 60, and 120 min (**I**) or 0.5, 1, 2, 4, 6 and 8 h (**II**) after oral administration of single dose. In a 7-day treatment study (**II**), rats were sacrificed 0.5, 1, 2, 4, 6 and 8 h after the last dose of the 7-day treatment. After intravenous administration of entacapone solution (**I**), rats were sacrificed at 2.5, 5, 10, 20, 30, 60 and 120 min. Blood samples were collected into tubes containing Na_2-EDTA. Plasma was separated by centrifugation and stored at −70°C until analyzed. Erythrocytes (**I**) were washed with ice cold 0.9% saline and stored at −80°C until analyzed. After dissection, the tissue samples (striatum, duodenum, cortical part of the kidney and liver) (**II**) were frozen and stored at −70°C. The lumen of duodenum samples was rinsed with 0.9% saline.

For determination of entacapone and tolcapone concentrations in serum (**II**), a blood sample (250 µl) was taken from the retrobulbar plexus before and 5, 10, 15, 30, 60 and 120 min after intravenous administration. Maximally 3 retrobulbar samples were taken from each rat and the last sample was collected after decapitation. Blood was allowed to clot and serum was separated by centrifugation. To determine entacapone and tolcapone concentrations in the perfused rat striatum (**II**), a blood sample was taken from the retrobulbar plexus at 14 min or 59 min after the intravenous injection of entacapone or tolcapone. Immediately after blood sampling, 0.9% saline perfusion was started via the left ventricle (blood was drained out via right auricle). The rats were decapitated after 3-min perfusion and the striata were dissected.

4.4.2 COMT activity assay

Tissue homogenates (see section 4.3) were centrifuged for 10 min at 900 × g at 4°C, and the supernatant containing both S- and MB-COMT was used as total COMT enzyme preparation (**II**). The supernatants of kidney and liver homogenates were first diluted with homogenisation buffer. Erythrocytes were haemolysed with ice-cold ultrapure water. The lysates were centrifuged for 20 min at 20 000 × g at 4°C, and the supernatant, enriched with S-COMT (Ellingson et al. 1999), was used as the enzyme preparation (**I**).

100 µl of the enzyme preparation was incubated for 30 min (erythrocytes 60 min) at 37°C in 0.1 M phosphate buffer, pH 7.4 (erythrocytes pH 7.8), containing 200 µM S-adenosyl-L-methionine, 5 mM $MgCl_2$ and 240 µM 3,4-DHBA in a total volume of 250 µl. The reaction was stopped and the samples were treated as described in section 4.3.

4.5 Determination of aqueous solubility and apparent partition coefficient of tolcapone (III)

To allow comparison of the physicochemical properties of entacapone and tolcapone, the aqueous solubility and the apparent partition coefficient (log P_{app}) of tolcapone were determined equally as those of entacapone in our previous experiments

(Savolainen et al. 2000a, b). Briefly, the aqueous solubility of tolcapone was determined in 0.16 M phosphate buffer solutions (pH 3.0, 5.0, 6.0 and 7.4) and in HCl buffer solution (pH 1.2, USP 23) at room temperature. The log P_{app} of tolcapone was determined from the distribution of the drug between 1-octanol and 0.16 M phosphate buffer at pH 5.0, 6.0 and 7.4.

4.6 Brain microdialysis (III)

Rats were anaesthetized with chloral hydrate (350 mg/kg, i.p.), and further doses were provided as needed. Body temperature was maintained at 36.5°C with a thermal blanket unit (Harvard apparatus Ltd., Edenbridge, Kent, England). Each rat was placed in a Kopf stereotaxic apparatus and a concentric probe (MAB 6, 4 mm exposed membrane, 570 μm outer diameter, 15 000 daltons cut off, AgnTho's, Lidingö, Sweden) was implanted through a burr hole into the striatum to the final coordinates (the tip of the probe): AP 0.5, L 3.2, DV −7.8 (Paxinos and Watson 1982). The probe was perfused with artificial cerebrospinal fluid (138 mM NaCl, 5 mM KCl, 1.1 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM $NaHCO_3$, 1 mM KH_2PO_4, pH 7.40) with a flow rate of 2 μl/min. After an 80 min wash out period, dialysis samples were collected as 20-min fractions and stored at −70°C until analyzed.

Entacapone and tolcapone were administered intracerebrally via the microdialysis probe as described earlier (Huotari et al. 2001). Briefly, the drugs were dissolved in perfusion medium (pH adjusted to 7.40). After 60 min baseline collection, a perfusion solution containing entacapone or tolcapone (10 or 100 μM) was introduced into the microdialysis probe for 60 min (−20 to 40 min). The microdialysis samples for determination of extracellular DOPAC and HVA levels were collected via the same probe.

4.7 Methamphetamine-induced hyperthermia and hydroxyl radical production in COMT-deficient mice (IV)

Male mice of all COMT genotypes (WT, HET, HOM) received four METH injections (2.5, 5 and 10 mg/kg as a free base i.p.) at 2 h intervals in a volume of 0.1 ml/10 g animal weight. The drug solutions were warmed before administration. Salicylate (100 mg/kg) was administered intraperitoneally 1 h after the last METH dose. The animals were sacrificed 1 h after administration of salicylate (i.e. 2 h after the last METH dose). The samples (striata, cortex and hypothalamus) were dissected and stored at −70°C until assayed. Rectal temperature was monitored at 1, 3, 7 and 8 h after the first METH injection.

4.8 Activities of enzymatic defense mechanisms and liver mono-oxygenases in COMT-deficient mice (IV)

Untreated mice of all COMT genotypes (WT, HET, HOM) and both sexes were sacrificed by decapitation. Livers were dissected on ice and placed in ice-cold 0.9% saline. Microsomes were prepared according to Pearce et al. (1996) and stored at −70°C.

The activities of GST and quinone reductase were determined spectrophotometrically with standard methods. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used in both assays. 7-Ethoxy- and 7-pentoxyresorufin O-dealkylation activities were measured fluorometrically with Bio-Tek FL 500 Microplate fluorescence reader (Bio-Tek Instruments, Winooski, Vermont, USA) using excitation at 530 nm and emission at 640 nm on the basis of the method of Burke et al. (1985). The final concentration of 7-ethoxy- or 7-pentoxyresorufin in the assay was 0.1 μM and 4 μM, respectively. The protein contents of the samples were measured spectrophotometrically with Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) using a Bio-Rad protein assay kit and enzyme activities were expressed as mol × min^{-1} × mg $protein^{-1}$.

LD methyl ester (30, 50 and 80 mg/kg as a free base i.p.) was given twice daily for two days to female HOM mice. The animals were decapitated 12 h after the last dose. Brain and liver were dissected, frozen and stored at −70°C until analyzed.

Whole brains were homogenized in a 10 mM MOPS buffer (pH 7.4) containing 0.32 M saccharose and 0.5 mM dithiothreitol. Homogenates were centrifuged for 15 min at 15 000 × g at 4°C and the supernatants were used as the enzyme preparation. Liver microsomes were prepared as described above. The enzyme activities were assayed using standard methods as described above with the exception that glutathione S-transferase activity was measured in an end point assay using 0.625 mM 1-chloro-2,4-dinitrobenzene as the substrate.

4.9 Determination of DOPA oxidase activity of skin tyrosinase (IV)

Particulate tyrosinase was isolated by the method modified from Martínez et al. (1987) and Valverde et al. (1992, 1993). Briefly, untreated hairless mice pups (WT and HOM, aged 7 – 8 days) and adult mice (WT and HOM, aged 14 – 15 weeks) of both genders were sacrified by decapitation. The hairless back skin was cut into small pieces, which were frozen and stored at −70°C. The frozen skin samples were ground with a mortar and the skin powder was extracted with 10 mM sodium phosphate containing 0.25 M saccharose and 0.1 mM Na_2-EDTA (pH 6.8). The homogenates were centrifuged for 10 min at 1000 × g at 4°C. The supernatants were recentrifuged for 30 minutes at 11 000 × g and then ultracentrifuged for 1 h at 170 300 × g. The pellets were resuspended in 0.5 × volume of 10 mM Tris-HCl buffer (pH 6.8) containing 1% Brij 35 and frozen at −70°C until analyzed.

The DOPA oxidase activity of tyrosinase was determined by the method of Winder (1994) with minor modifications (**IV**). The reaction mixture contained 250 µl of assay buffer (100 mM potassium phosphate, 0.1% Triton X-100), 25 µl of 0.5 mM deferoxamine mesylate, 120 µl of 5 mM L-DOPA–25 mM 3-methyl-2-benzothiazoninone mixture, 55 µl of ultrapure water and 50 µl of enzyme preparation. After 30 min incubation at 37°C, the reaction was stopped by the addition of 500 µl of 1 M perchloric acid. The mixture was centrifuged for 3 min at 9000 × g at 4 °C and the reaction product was assayed spectrophotometrically at 505 nm using a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan).

4.10 Analytical

4.10.1 Determination of reaction products of COMT assay (I – III)

The reaction products of COMT assay (vanillic and isovanillic acid) were analyzed with the high performance liquid chromatography (HPLC) method of Reenilä et al. (1995) with minor modifications. The HPLC system consisted of an isocratic Waters 510 pump (Waters, Milford, MA, USA), a Waters 717 plus autosampler with cooler (Waters, Milford, MA, USA), ESA Coulochem 5100A electrochemical detector (ESA, Chelmsford, MA, USA) with a 5021 conditioning cell and a 5011 analytical cell, and Shimadzu C-R6B Chromatopac integrator (Shimadzu, Kyoto, Japan). The potentials applied were +0.47, +0.20 and −0.25 V for conditioning cell, Det 1 and Det 2, respectively. A Purospher RP-18e (125 × 4 mm, 5 µm) column with a Purospher RP-18e (4 × 4 mm) precolumn (Merck, Darmstadt, Germany) was used for chromatographic separations. The chromatographic conditions were as follows: injection volume 10 µl, flow rate 1.1 ml/min. The mobile phase consisted of 0.1 M disodium hydrogen phosphate, 0.15 mM Na_2-EDTA and 15% methanol (v/v). The pH was adjusted to 3.2 with phosphoric acid.

The protein contents of the samples were measured spectrophotometrically with Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) using a Bio-Rad protein assay kit. Specific COMT activities were expressed as pmol vanillic acid × min^{-1} × mg $protein^{-1}$ and transformed as percentages of control when appropriate.

4.10.2 Drug analysis (I – III)

Entacapone and prodrug B concentrations in rat plasma after oral administration (**I**) were determined by the HPLC method of Savolainen et al. (2000a) for *in vivo* samples. For determination of entacapone and tolcapone in rat plasma (**II**), the method was transferred on the Agilent HPLC 1100 Series chromatographic system (Agilent Technologies, Palo Alto, CA, USA), which consisted of a binary gradient pump, a

vacuum degasser, an autosampler, a thermostatted column compartment, a diode-array detector and ChemStation software. A Zorbax SB-C18 (150 × 4.6 mm, 5 μm) column with a guard cartridge (12.5 × 4.6 mm) (Agilent Technologies, Palo Alto, CA, USA) was used for the chromatographic separations. Tolcapone was detected at 280 nm.

Serum and striatal homogenate concentrations of entacapone and tolcapone after intravenous administration (**II**) were determined with a HPLC method described by Karlsson and Wikberg (1992) for entacapone. Only sample volumes were modified. The striata were homogenised in 500 μl of 50 mM phosphate buffer (pH 7.2) and then treated similar to the serum samples.

Tolcapone concentrations in buffer phase (aqueous solubility and log P_{app} studies) (**III**) were determined with a gradient HPLC method of Savolainen et al. (2000b) for *in vitro* samples except that the mobile phase consisted of various percentages of citrate-phosphate buffer (pH 2.2) and acetonitrile-water mixture (75:25). To determine the *in vitro* recoveries of the microdialysis probes for entacapone and tolcapone (**III**), the probes were perfused with perfusion medium containing entacapone or tolcapone (10/100 μM) and the concentrations in the perfusate were determined by a HPLC method described earlier for *in vitro* samples (Savolainen et al. 2000a).

4.10.3 Determination of extracellular DOPAC and HVA levels (III)

The DOPAC and HVA contents of microdialysis samples were measured by a HPLC method modified from Huotari et al. (2002b). The method optimized for fast DOPAC and HVA analysis did not enable quantitation of dialysate DA levels. The HPLC system consisted of an ESA 582 pump (ESA, Chelmsford, MA, USA), an ESA 542 autosampler with cooler, and an ESA 5600 A CoulArray® electrochemical array detector (eight-channel) equipped with a 5014B microdialysis cell. The potentials applied were −175 (Ch. 1), +200 (Ch. 2) and +450 mV (Ch. 4), respectively. The analytes were separated on an Inertsil ODS-3 column (150 × 4.0 mm, 5 μm, GL Sciences Inc., Japan). The mobile phase consisted of 75 mM sodium phosphate, 1 mM sodium dodecylsulphate, 18% (v/v) acetonitrile and 12% (v/v) methanol. The pH was adjusted to 2.5 with phosphoric acid. The flow rate was set at 1.0 ml/min. Samples were injected into the HPLC system without any pretreatment.

4.10.4 Assay of 2,3-DHBA, 2,5-DHBA and salicylate concentrations (IV)

The tissue concentrations of 2,3-DHBA, 2,5-DHBA and salicylate were determined according to the method described by McCabe et al. (1997) with minor modifications. Tissue samples were homogenized in 0.2 M perchloric acid containing 100 μM Na_2-EDTA and sodium metabisulfite. Homogenates were centrifuged for 10 min at 15 000 × g at 4°C and 10 μl of supernatants were injected immediately into a HPLC system, which consisted of an isocratic Shimadzu LC-10 ADvp pump and a CTO-10 ASvp

column oven (Shimadzu, Kyoto, Japan), a Waters 717 plus autosampler with cooler (Waters, Milford, MA, USA), an ESA Coulochem II electrochemical detector equipped with a 5020 guard cell and a 5010 analytical cell, and two Hewlett Packard 3396A programmable integrators (Hewlett Packard, Palo Alto, CA, USA). The potentials applied were +775, +225 and +750 mV for guard cell, E1 (DHBAs) and E2 (salicylate), respectively. An ESA DHBA-250 × 3.2 mm column (ESA, Chelmsford, MA, USA) was used for chromatographic separations. The chromatographic conditions were as follows: column temperature 27°C, flow rate 0.55 ml/min. The mobile phase consisted of 50 mM sodium acetate, 50 mM citric acid, 10% (v/v) methanol and 2.5% (v/v) 2-propanol. The pH was adjusted to 2.5 with phosphoric acid.

4.11 Data analysis and statistics

4.11.1 Determination of IC_{50}, K_i^{app} and K_i values (II, III)

The COMT activities measured in the presence of varying concentrations of entacapone or tolcapone were plotted against the log concentration of the inhibitor and the IC_{50} value was determined by non-linear regression using the built-in sigmoidal dose-response equation. Apparent inhibitor constant (K_i^{app}) and active enzyme concentration (E) were determined by fitting the Morrison equation

$$\frac{v_i}{v_0} = 1 - \frac{\left([E]+[I]+K_i^{app}\right) - \sqrt{\left([E]+[I]+K_i^{app}\right)^2 - 4[E]\times[I]}}{2\times[E]} \qquad \text{(Equation 1)}$$

where v_0 and v_i are the reaction velocities in the absence and presence of the inhibitor I, respectively, to v_i/v_0 vs. inhibitor concentration plot (Copeland 2000). COMT activity was plotted as a function of substrate concentration and K_m and V_{max} of total COMT preparation were determined by non-linear regression using Henri-Michaelis-Menten equation. Calculations were performed with GraphPad Prism 3.02 software (Graph Pad Software, San Diego, CA, USA). The 95% confidence intervals were calculated with the built-in equation. Inhibitor constant (K_i) was calculated from the equation

$$K_i^{app} = K_i\left(1 + \frac{[S]}{K_m}\right) \qquad \text{(Equation 2)}$$

where S is the substrate concentration (Copeland 2000).

4.11.2 Pharmacokinetic and pharmacodynamic studies in rats (I, II)

Area under the effect-time curve (AUE) and area under the concentration-time curve (AUC) values (% × min or % × h and ng × h × ml^{-1} or ng × min × ml^{-1}, respectively) were calculated using the trapezoidal rule. When appropriate, the area from last time point to infinity was extrapolated by dividing the last concentration with the terminal rate constant. For calculation of the $AUC_{0-120\ min}$ of entacapone after intravenous administration (**I**), the initial plasma concentration (C_0) was approximated by fitting the equation $C = C_1e^{-k1t} + C_2e^{-k2t}$ to the data and determining time (t) to zero. The bioavailabilities (F) of different entacapone formulations (**I**) were calculated by equation $F = (AUC_{0-120\ min\ p.o.} / AUC_{0-120\ min\ i.v.}) \times 100$ % and entacapone and tolcapone (**II**) by equation $F = (AUC_{0-\infty\ p.o.} \times D_{i.v.}) / (AUC_{0-\infty\ i.v.} \times D_{p.o.}) \times 100\%$, where D is dose. To determine C_0, $AUC_{0-\infty}$, CL and $t_{1/2\beta}$ of entacapone and tolcapone after intravenous administration (**II**), a two-compartment model ($C = Ae^{-\alpha t} + Be^{-\beta t}$) was fitted to drug concentration-time data by Kinetica 2000 v3.1 software (InnaPhase Corporation, Philadelphia, PA, USA).

To describe the relationship between plasma drug concentration (C) and COMT activity in peripheral tissues (E) after oral administration of entacapone and tolcapone, the inhibitory E_{max} model

$$E = E_0 - \frac{E_{max} \times C}{EC_{50} + C} \qquad \text{(Equation 3)}$$

was used. The specific COMT activities (pmol vanillic acid × min^{-1} × mg $protein^{-1}$) were plotted against corresponding concentration values (ng/ml), the baseline COMT activity (E_0), maximum attainable COMT inhibition (E_{max}) and the concentration producing 50% of the maximum attainable inhibition (EC_{50}) were fitted as free parameters using GraphPad Prism 3.02 software (Graph Pad Software, San Diego, CA, USA).

4.11.3 Microdialysis (III)

The mean of three baseline dialysis samples (−80 to −40 min) was determined as an individual baseline value (=100 %), and the DOPAC and HVA amounts in the samples collected after drug exposure were transformed as a percentage of this value. The $AUC_{0-240\ min}$ values (min × % of baseline) of DOPAC and HVA were calculated with the trapezoid rule (baseline set as 100 %).

4.11.4 Statistical analyses (I – IV)

The statistical significance of differences between COMT activity-time curves (**I, II**) and plasma drug concentration-time curves (**II**) after oral administration of COMT inhibitors were tested using two-way factorial ANOVA. Since a significant time × treatment interaction occurred, further comparisons between treatments (**I**) were performed by pairwise comparisons of time × treatment effect using two-way factorial ANOVA with Bonferroni's adjustment for multiple comparisons. The time required for full recovery of COMT activity in various tissues (**II**) was predicted by linear regression from log-transformed data. Independent samples t-test was applied to test the significance of differences between maximal effects (**II**).

Overall comparison between the effects of different treatments on the extracellular DOPAC and HVA levels (**III**) was performed by means of repeated measures ANOVA (time as a within-subject factor). Since significant time × treatment interaction occurred, further comparisons were performed by pairwise comparisons of time × treatment effect using repeated measures ANOVA with Bonferroni's adjustment for multiple comparisons. The effect of the drug was tested with repeated measures ANOVA, and further comparisons between different fractions and baseline were performed by non-orthogonal contrasts with Bonferroni's adjustment. The statistical significance of differences between the $AUC_{0–240\ min}$ values of DOPAC and HVA (**III**) was tested using one-way ANOVA followed by Tukey's test.

One-way ANOVA was used to test the statistical significance of differences between METH doses, COMT genotypes, genders and LD doses (**IV**). Further comparisons between groups were performed with Tukey's test. Overall comparison between the effects of different METH doses and COMT genotypes on the rectal temperature-time curves was performed with two-way factorial ANOVA. Further comparisons between groups at each time point were performed using one-way ANOVA followed by Tukey's test and Bonferroni's adjustment for multiple comparisons. An independent-samples t-test was used for comparison of dopa oxidase activities of skin tyrosinase between WT and HOM mice (**IV**).

5 RESULTS

5.1 The effect of bioavailability on pharmacodynamic response of entacapone (I)

The improvement of oral bioavailability of entacapone by dosing it as a solution (plain solution, pH 7.4; F=35% or entacapone/HP-β-CD solution, pH 3.0; F=19%) resulted in a significantly ($p<0.001$) higher degree of COMT inhibition in erythrocytes than after administration of entacapone as a suspension (**I; Figure 1**). Also a higher maximal degree of COMT inhibition was reached. The COMT activity-time curves observed after administration of entacapone/HP-β-CD solution and plain solution did not differ significantly. Prodrug A suspension (pH 3.0; F=1%) was significantly ($p<0.001$) less effective than the entacapone suspension (pH 3.0; F=8.9%). It inhibited COMT activity in erythrocytes (≤25%) for less than 30 minutes.

Prodrug B was inactive when administered as a suspension but it was nearly as effective as the entacapone suspension when administered as a solution (pH 7.4; F=4%). The COMT activity-time profiles (**I; Figure 1**) were time dependently different ($p<0.05$) since the maximal degree of inhibition was reached later than after administration of the other oral formulations.

The $AUE_{0\text{-}120\ min}$ after administration of entacapone/HP-β-CD solution was over 2-fold higher than that of entacapone suspension, as was the case in $AUC_{0\text{-}120\ min}$ values (Table 4). No further increase in AUE value was achieved with the plain entacapone solution although it yielded a twofold higher AUC value compared to entacapone/HP-β-CD solution (Table 4, Figure 8). Furthermore, intravenous administration did not produce any increase in AUE. Prodrug B solution produced AUE comparable to entacapone suspension as a result of delayed onset of effect and slow recovery of COMT activity.

Table 4. Pharmacokinetic and pharmacodynamic parameters of entacapone in rats after oral administration of entacapone as different formulations and prodrugs. Values are calculated from group data

Formulation	F	C_{max} [a]	t_{max} [a]	$AUC_{0\ 120\ min}$	Maximal inhibition[c]	$AUE_{0\ 120\ min}$
	%	*ng/ml*	*min*	*ng × min × ml^{-1}*	*% (min)*	*% × min*
Prodrug A susp. (pH 3.0)	1	40.2	30	3070	25 (10)	381
Prodrug B sol. (pH 7.4)	4	466	10	17600	34 (30)	3260
Suspension (pH 3.0)	9	1060	10	39000	51 (10)	2660
HP-β-CD sol. (pH 3.0)	19	1640	10	81100	83 (30)	7460
Solution (pH 7.4)	35	4650	10	152000	75 (20)	7110
Solution i.v. (pH 7.4)	n.a.	55800[b]	n.a.	438000	98[d] (2.5)	6650

F, bioavailability; C_{max}, maximal plasma concentration; t_{max}, time required to reach maximal plasma concentration; $AUC_{0\ 120\ min}$, area under curve; $AUE_{0\ 120\ min}$, area under effect-time curve; n.a., not applicable

[a] Apparent value (first data point 10 min)

[b] C_0

[c] Erythrocyte COMT; time (min) to achieve the maximal degree of COMT inhibition given in the parenthesis

[d] First data point

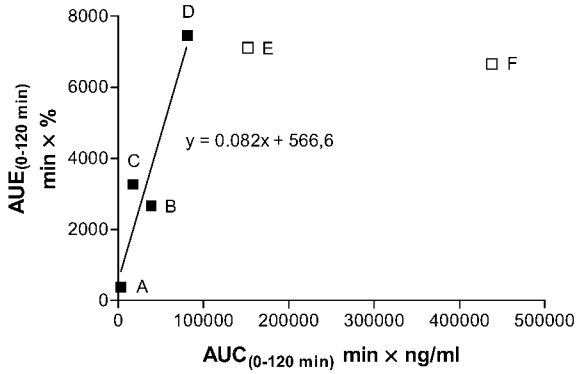


Figure 8. The dependence between area under concentration-time curve (AUC) and area under effect-time curve (AUE) after oral administration of entacapone as different formulations and two prodrugs (**I; Figure 4**). Key: (A) prodrug A suspension, (B) entacapone suspension, (C) prodrug B solution, (D) entacapone/HP-β-CD solution, (E) entacapone solution, (F) entacapone solution (i.v.).

The EC_{50} estimates of entacapone as determined with the inhibitory E_{max} model were within range 124 – 597 ng/ml after admnistration of all formulations, and no significant differences were observed. Therefore, the inhibitory E_{max} model was fitted to data containing all observations after oral administration resulting in an EC_{50} value of 317 ng/ml (95 % confidence interval 191 – 443 ng/ml) (Figure 9).

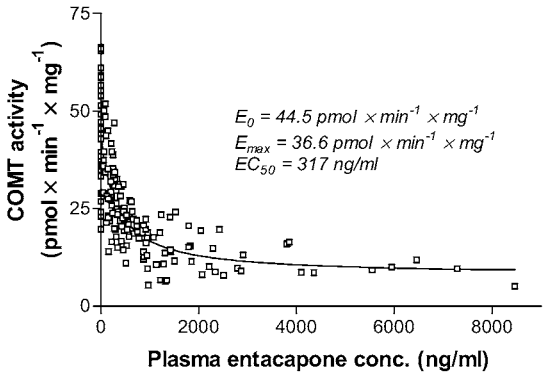


Figure 9. The relationship between COMT activity in erythrocytes and plasma entacapone concentration. The inhibitory E_{max} model $E = E_0 - (E_{max} \times C) / (EC_{50} + C)$ was fitted to the data. The baseline COMT activity (E_0), maximum attainable COMT inhibition (E_{max}) and the concentration producing 50% of the maximum attainable inhibition (EC_{50}) are shown in the inset. Each observation represents a single animal.

5.2 Physicochemical properties of tolcapone (III)

The aqueous solubility and log P_{app} of tolcapone at various pH's are presented in Figure 10. Previously determined entacapone parameters (Savolainen et al. 2000a, b) are included for comparison. The aqueous solubility of tolcapone was low in acidic pH (18.6 ± 2.26 μg/ml at pH 1.2) but increased to 115 ± 12.8 μg/ml (mean ± S.E.M., $n = 3$) at pH 7.4. Correspondingly, the log P_{app} of tolcapone decreased as a function of pH from 2.75 ± 0.02 at pH 5.0 to 1.03 ± 0.02 at pH 7.4 (Figure 10).

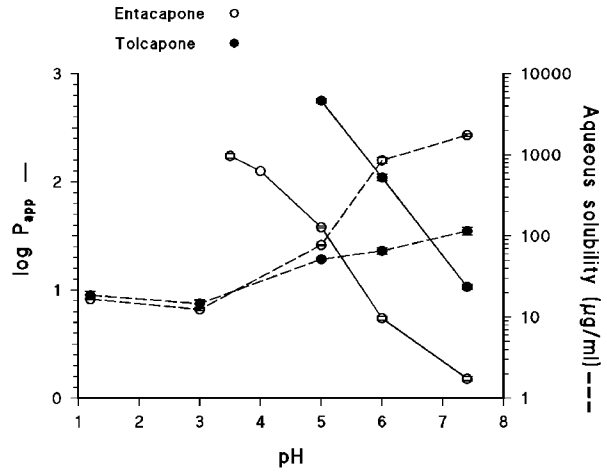


Figure 10. Apparent partition coefficients (log P_{app}) and aqueous solubilities (µg/ml) of entacapone and tolcapone as a function of pH (**III; Figure 1**). Values are mean ± S.E.M. (n = 3). Entacapone data are from Savolainen et al. (2000a, b).

5.3 Pharmacokinetic and pharmacodynamic properties of entacapone and tolcapone (II, III)

5.3.1 *In vitro* potency

The IC_{50}, K_i^{app}, and K_i values of entacapone and tolcapone are summarized in Table 5. Entacapone and tolcapone were equally potent COMT inhibitors both in striatal and liver total COMT. Additionally, the IC_{50} values of entacapone and tolcapone (95% confidence intervals given in parenthesis, n = 3) against liver S-COMT were 14.3 nM (11.1 – 18.5) and 14.8 nM (10.4 – 21.3), respectively. In liver MB-COMT, the IC_{50} values were 73.3 nM (58.9 – 91.2) and 86.5 nM (60.0 – 125), respectively.

Table 5. Inhibitory potencies of entacapone and tolcapone against rat striatal and liver total COMT

	Striatum		Liver	
	Entacapone	Tolcapone	Entacapone	Tolcapone
	nM		*nM*	
IC_{50}	7.4 (6.5 – 8.5)	9.0 (7.8 – 10.3)	20.1 (15.7 – 25.9)	19.7 (15.8 – 24.4)
K_i^{app}	6.4 (5.3 – 7.5)	8.6 (7.1 – 10.1)	27.3 (19.3 – 35.3)	25.4 (19.2 – 31.6)
K_i	1.9 (1.6 – 2.2)	2.5 (2.1 – 2.9)	10.7 (7.6 – 13.9)	10.0 (7.6 – 12.4)

IC_{50} and K_i^{app} values are best least-squares fit values from 4 – 6 independent experiments. The 95% confidence intervals are given in parenthesis. K_i^{app} values were calculated with the Morrison equation (Equation 1) and the K_i values from K_i^{app} values obtained from Equation 2.

The K_m and V_{max} of striatal total COMT were 98.1 μM and 58.4 pmol vanillic acid × min^{-1} × mg $protein^{-1}$, respectively. In liver total COMT, the corresponding values were 156 μM 5210 pmol vanillic acid × min^{-1} × mg $protein^{-1}$. The active enzyme concentration in the reaction medium was 1.0 – 1.1 nM.

5.3.2 Pharmacokinetics after intravenous and oral administration

After intravenous administration, the $AUC_{0-\infty}$ of tolcapone was 3-fold higher than that in the entacapone group (Table 6). The $t_{1/2\beta}$ of tolcapone (2.9 h) was over 3-fold longer than that of entacapone (0.8 h). Total clearance of tolcapone was much lower (Table 6). After administration of a single dose, the plasma tolcapone levels were time-dependently higher ($p<0.05$) than the plasma entacapone levels (**II; Figure 2**) and the $AUC_{0-\infty}$ value of tolcapone was 6-fold higher than that of entacapone (Table 6). The bioavailabilities of entacapone and tolcapone were 19% and 36%, respectively. After the last dose of the 7-day treatment period, the plasma entacapone and tolcapone levels and the AUC values were similar as those detected after a single dose.

Table 6. Pharmacokinetic parameters of entacapone and tolcapone in rats after intravenous and oral administration of a single dose. Values are calculated from group data

	Entacapone	Tolcapone
Intravenous (3 mg/kg)		
C_0 (ng/ml)	22700	29300
$AUC_{0-\infty}$ (ng × h × ml^{-1})	4630	15200
$t_{1/2\beta}$ (h)	0.8	2.9
CL (ml × h^{-1} × kg^{-1})	648	197
Oral (10 mg/kg)		
C_{max} (ng/ml)	808[a]	3830
t_{max} (h)	1[a]	2
$AUC_{0-\infty}$ (ng × h × ml^{-1})	2990	18000
Bioavailability (%)	19	36

C_0, initial plasma concentration; $AUC_{0-\infty}$, area under curve; $t_{1/2\beta}$, elimination half life; CL, total clearance; C_{max}, maximal plasma concentration; t_{max}, time required to achieve maximal plasma concentration

[a] Apparent value (first data point 0.5 h)

The striatum/serum ratios of entacapone and tolcapone were calculated by dividing the drug concentration in the perfused striatum at 15 and 60 min after intravenous administration (3 mg/kg) by the corresponding concentration in serum. Fifteen minutes after intravenous administration of 3 mg/kg entacapone, the plasma entacapone concentration was 5980 ± 885 ng/ml (mean ± S.E.M., n = 5). The entacapone concentration in the striatum was close to the limit of detection (~5 ng/g), and the striatum/serum ratio remained less than 0.1%. Sixty minutes after administration of 10 mg/kg entacapone, serum entacapone concentration was 3170 ± 986 ng/ml (n = 7) and

striatal entacapone concentrations were above the limit of detection. The calculated striatum/serum ratio was 0.27%. In the tolcapone group, the intravenous dose of 3 mg/kg resulted in a plasma concentration of 10300 ± 1930 ng/ml (n = 4) at 15 min and 1580 ± 260 ng/ml (n = 10) at 60 min. The corresponding striatal concentrations were 24.9 ± 2.2 and 14.5 ± 1.6 ng/g, giving striatum/serum ratios of 0.24 and 1%, respectively.

5.3.3 Pharmacodynamics after oral administration

After administration of a single dose, the COMT activity-time curves in the entacapone and tolcapone groups differed time-dependently ($p<0.05$); the maximal degree of inhibition did not differ in the peripheral tissues but the COMT activity recovered faster in entacapone-treated animals than in tolcapone-treated animals (Table 7, Figure 11). As a result, the AUE values were 20 – 43% higher after administration of tolcapone (Table 7). Both entacapone and tolcapone were less effective in the striatum than in the peripheral tissues but tolcapone was generally more effective ($p<0.001$) than entacapone (Figure 11). The AUE value of tolcapone was over 2-fold greater than that of entacapone (Table 7).

Table 7. Pharmacodynamic parameters of entacapone and tolcapone for inhibition of COMT in various rat tissues after oral administration of a single dose and repeated doses twice daily for 7 days (10 mg/kg). Values are calculated from group data

	Entacapone			Tolcapone		
	Maximal inhibition[a]	$AUE_{0\ 8\ h}$	t_{rec}[b]	Maximal inhibition[a]	$AUE_{0\ 8\ h}$	t_{rec}[b]
	%	% × *h*	*h*	%	% × *h*	*h*
Single dose						
Striatum	n.d.	146	n.d.	n.d.	372	n.d.
Duodenum	96.9	466	7.5	103	667	10.3
Kidney	87.6	571	10.5	97.2	686	15.7
Liver	84.2	450	8.6	93.7	601	15.3
b.i.d. 7 days						
Striatum	n.d.	135	n.d.	n.d.	334	n.d.
Duodenum	104	480	8.0	110	676	11.4
Kidney	84.6	550	9.9	95.4	717	19.7
Liver	83.5	388	7.9	89.5	667	15.9

$AUE_{0\ 8\ h}$, area under effect-time curve; t_{rec}, time required to reach maximal inhibition; n.d., not determined

[a] Calculated with equation $E_{max}/E_0 \times 100\%$. E_0 and E_{max} values were estimated with the inhibitory E_{max} model. In some cases, calculated maximal inhibition exceeds 100%.

[b] Estimated by linear regression from log-transformed COMT activity data

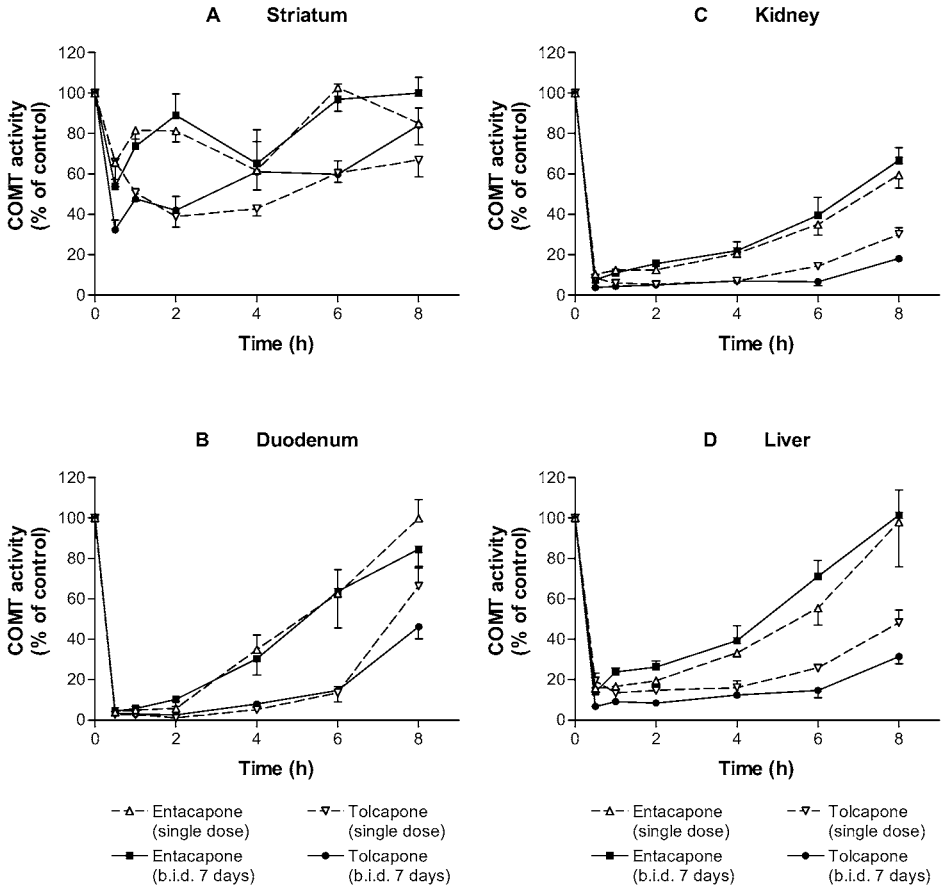


Figure 11. The COMT activity-time curves (% of control) in rat striatum (A), duodenum (B), kidney (C) and liver (D) after oral administration of entacapone and tolcapone (10 mg/kg) as a function of time (**II; Figure 3**). Each point represents mean ± S.E.M. of 5 – 6 animals.

After 7-day treatment, the COMT activity curves were at a significantly ($p<0.001$) lower level in the tolcapone group than the entacapone group (Figure 11). At 0.5 h after the last dose, the degree of COMT inhibition was slightly higher ($p<0.05$) in the liver and kidney of tolcapone-treated animals. The AUE values were 30 – 72% higher in the tolcapone-treated animals than those in entacapone-treated animals (Table 7). In the tolcapone group, the COMT activity (0.5 – 8 h) was time-dependently at a lower level in striatum, kidney and liver ($p<0.05$, duodenum $p=0.08$) than that seen after a single dose. The activity also recovered more slowly than after a single dose.

5.3.4 Pharmacokinetic-pharmacodynamic relationship

The relationship between the plasma drug concentration and the COMT activity in liver is presented in Figure 12. The E_0 and E_{max} estimates were very close to actual values. Therefore, maximal degree of COMT inhibition in various tissues was calculated also by using these estimates (Table 7). The EC_{50} values of entacapone in duodenum, kidney and liver (446, 54.2 and 158 nM, respectively) were apparently lower than those of tolcapone (1170, 260 and 591 nM, respectively) but no significant differences were observed (for details, see original publication **II; Table 1)**. Furthermore, there was a trend to lower EC_{50} values of tolcapone after the 7-day treatment.

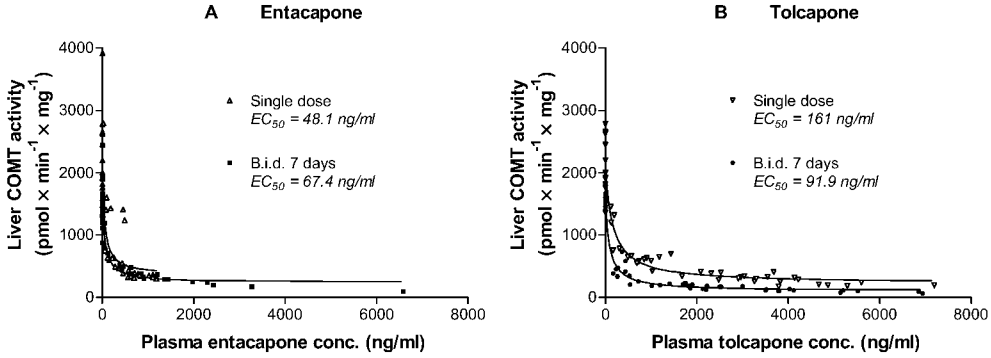


Figure 12. The relationship between COMT activity in liver and plasma drug concentration after oral administration of entacapone (A) and tolcapone (B) at a dose 10 mg/kg (**II; Figure 4**). The inhibitory E_{max} model $E = E_0 - (E_{max} \times C) / (EC_{50} + C)$ was fitted to data. The concentration producing 50% of the maximum attainable inhibition (EC_{50}) is shown in the inset. Each observation represents a single animal.

5.3.5 Efficacy during intrastriatal administration

The perfusion of 10 μM entacapone or tolcapone did not increase significantly striatal extracellular DOPAC levels (**III; Figure 2**). At 100 μM, entacapone increased DOPAC levels significantly at 40 – 200 min ($p<0.05$ vs. baseline) and tolcapone at 80 – 120 min ($p<0.05$ vs. baseline). However, only animals perfused with 100 μM entacapone had overall higher DOPAC levels (0 – 240 min; $p<0.05$) than the control animals. In parallel, the $AUC_{0-240\ min}$ value of DOPAC was significantly ($p<0.01$) increased only after perfusion of 100 μM entacapone (Figure 13).

The perfusion of 10 μM entacapone or tolcapone decreased significantly ($p<0.05$) striatal extracellular HVA levels, entacapone at 20 – 200 min and tolcapone at 20 – 240 min (**III; Figure 2**). Both with entacapone and tolcapone, the HVA levels (0 – 240 min)

were significantly ($p<0.001$) lower than in control group. Also the negative $AUC_{0-240\ min}$ of HVA was significantly ($p<0.001$) higher in both entacapone and tolcapone group than in control animals. At 100 μM, both entacapone and tolcapone decreased significantly HVA levels (0 – 240 min; $p<0.01$) compared to controls but entacapone was time-dependently more effective ($p<0.05$) than tolcapone. Accordingly, the negative $AUC_{0-240\ min}$ value was significantly ($p<0.001$) higher in both entacapone- and tolcapone-perfused animals compared to control animals. After perfusion of entacapone, the $AUC_{0-240\ min}$ of HVA was 77% higher ($p<0.01$) than that after perfusion of tolcapone. Both at 10 and 100 μM, the effect of tolcapone on HVA levels waned more slowly than that of entacapone.

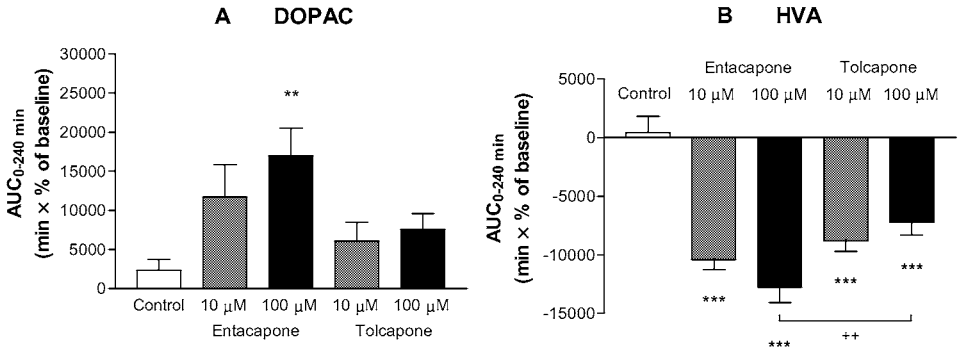


Figure 13. The $AUC_{0\ 240\ min}$ values (mean ± S.E.M., n = 7 – 10) of DOPAC (A) and HVA (B) after intrastriatal administration of entacapone and tolcapone (**III; Figure 3**). To calculate the AUC values, baseline was set at 100 %.
** $p<0.01$, *** $p<0.001$ vs. control group; $^{++}$ $p<0.01$ entacapone vs. tolcapone

5.4 The role of COMT in dopamine-linked oxidative stress (IV)

5.4.1 Methamphetamine toxicity

METH increased body temperature in a time dependent manner in all genotypes (WT and HOM, $p<0.05$; HET $p=0.057$) (Figure 14). The most prominent increase was observed at 3 h after the first METH dose. Homozygotes were more prone to the hyperthermia; a significant increase in rectal temperature (1.4°C, $p<0.05$) was seen already at the lowest METH dose level (2.5 mg/kg) whereas other genotypes showed significant increase at 5 mg/kg (WT, $p=0.052$; HET, $p<0.05$). At the highest dose level (10 mg/kg), METH increased the rectal temperature maximally by 3.2°C ($p<0.05$ vs. control group) in homozygous mice. Furthermore, 1 and 3 h after first dose, the METH-induced hyperthermia was more severe in homozygotes than in heterozygotes and wild type animals ($p<0.05$). This was also the case at 5 mg/kg (at 3 h after the first METH

dose, p<0.01). At 10 mg/kg, 3 out of 8 HOM animals died during the experiment. Therefore, 10 mg/kg × 4 of METH was not used in further studies.

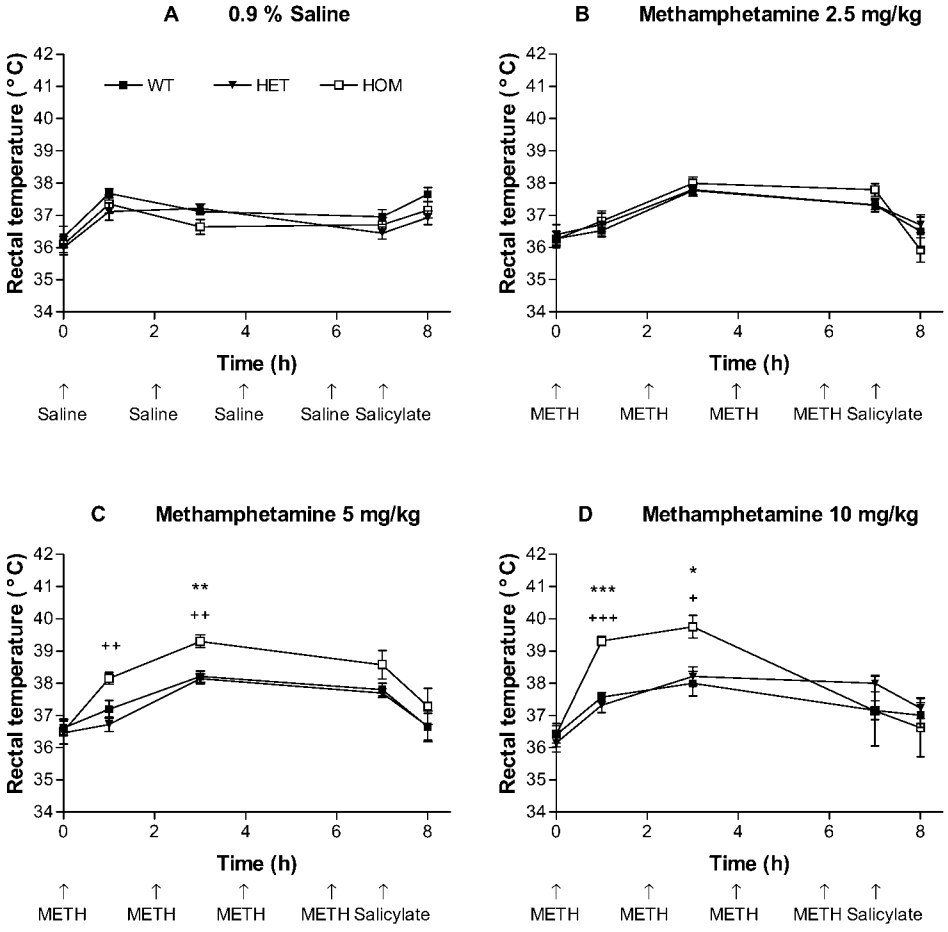


Figure 14. The effect of methamphetamine on rectal temperature in COMT-deficient male mice (A 0.9 % saline, B 2.5 mg/kg, C 5 mg/kg, D 10 mg/kg) as a function of time (**IV; Figure 1**). Four doses were given intraperitoneally with 2 h intervals. 100 mg/kg (i.p.) salicylate was given 1 h prior to sacrifice. Values are mean ± S.E.M. (n = 5 – 8 in each group). WT, wild-type; HET, heterozygote; HOM, homozygote.
* p<0.05, ** p<0.01, *** p<0.001 HOM vs. WT; $^{+}$ p<0.05, $^{++}$ p<0.01, $^{+++}$ p< 0.001 HOM vs. HET

5.4.2 Methamphetamine-induced hydroxyl radical production

METH increased the striatal 2,3-DHBA levels only slightly (<25%, n.s.) at the lowest dose (2.5 mg/kg × 4 i.p.) (Figure 15). A similar trend was observed in cortex and hypothalamus. However, the SAL levels were increased concomitantly by about 20 –

50% ($p<0.05$ HOM and WT, HET only in the cortex) resulting in a decreased 2,3-DHBA/salicylate ratio. The 2,3-DHBA and salicylate levels seen after 5 mg/kg × 4 did did not differ significantly from those observed after 2.5 mg/kg × 4 (except HET in the cortex). No COMT-genotype dependent differences were seen either in 2,3-DHBA levels or in 2,3-DHBA/salicylate ratio. Detailed comparison of 2,3-DHBA and salicylate levels as well as 2,3-DHBA/salicylate ratios is presented in the original publication (**IV; Table 1**).

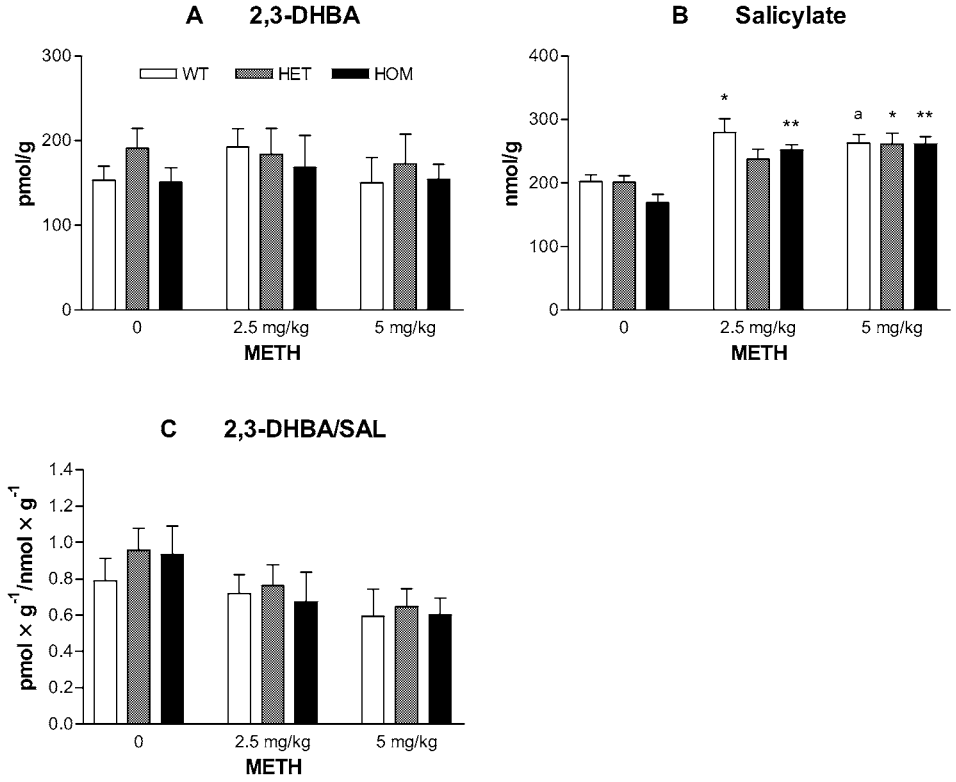


Figure 15. 2,3-Dihydroxybenzoic acid (2,3-DHBA) and salicylate levels and 2,3-DHBA/salicylate ratios in striatum after administration of methamphetamine (four i.p. injections with 2 h intervals) in male COMT-deficient mice treated with 100 mg/kg (i.p.) salicylate 1 h prior to sacrifice. Controls received 0.9% saline. Values are mean ± S.E.M. (n = 5 – 7 in each group).

In all brain regions examined, the 2,5-DHBA levels were 3- to 6-fold higher than those of 2,3-DHBA (**IV; Table 1**). METH treatment had no significant effect on 2,5-DHBA levels but the 2,5-DHBA/salicylate ratio was decreased (significant only in HOM). No genotype-dependent differences were observed in 2,5-DHBA levels or 2,5-DHBA/salicylate ratios at any dose level in any brain area examined.

5.4.3 Enzymatic defense mechanisms

Liver GST activity was about 50% lower ($p<0.001$) in females than males (**IV; Table 2**). In contrast, quinone reductase activity was higher ($p<0.05$) in females (**IV; Table 2**). However, no significant COMT genotype-dependent differences were observed. Liver 7-ethoxyresorufin O-deethylation activities did not differ between genders and COMT genotypes (**IV; Table 2**). The 7-pentoxyresorufin O-dependentylation activity (mean ± S.E.M., n = 11 – 20) was 0.640 ± 0.063, 0.699 ± 0.069 and 0.786 ± 0.127 μmol × min^{-1} × mg^{-1} in WT, HET and HOM males and 0.537 ± 0.0675, 0.602 ± 0.0377 and 0.613 ± 0.0635 μmol × min^{-1} × mg^{-1} in WT, HET and HOM females, respectively, but with no significant COMT genotype- and gender-dependent differences.

LD treatment (30, 50 and 80 mg/kg i.p. twice daily for two days) did not change the activities of liver GST, quinone reductase and 7-ethoxyresorufin O-deethylation activities in female COMT-deficient mice (**IV; Table 3**). In the brain, the activities of GST and quinone reductase were lower than those in the liver. However, LD treatment had no effect on the activity of these enzymes (Figure 16).

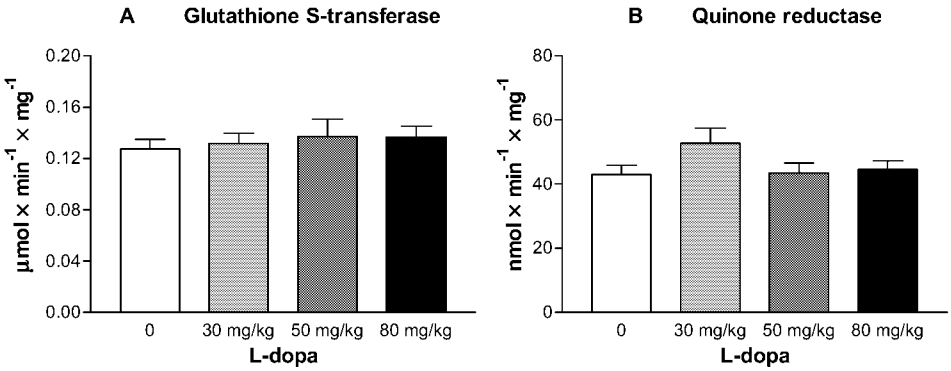


Figure 16. Glutathione S-transferase (A) and quinone reductase (B) activities in the brain of COMT-deficient mice after levodopa challenge. Values are mean ± S.E.M. (n = 11 – 26).

5.4.4 DOPA oxidase activity of skin tyrosinase in mice pups

In adult mice, DOPA oxidase activity of skin tyrosinase was hardly detectable. However, 7 – 8-day old male COMT-deficient (HOM) mice pups had significantly ($p<0.001$) higher activity than wild type (WT) animals (Figure 17). A similar trend was observed in female mice pups but statistical significance was not reached.

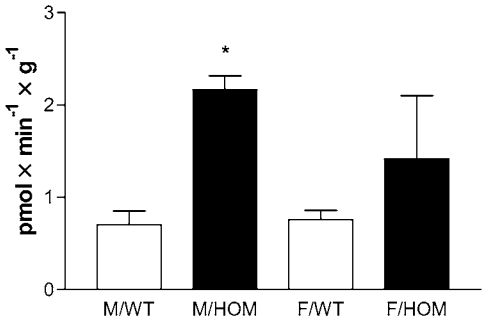


Figure 17. DOPA oxidase activity of skin tyrosinase in hairless mice pups. Values are mean ± S.E.M. ($n = 5 - 8$).

6 DISCUSSION

6.1 The effect of bioavailability on pharmacodynamic response of entacapone (I)

The present results confirm that increased bioavailability of entacapone results in better pharmacodynamic response, i.e. inhibition of erythrocyte COMT activity. Surprisingly, administration of plain entacapone solution resulted in higher AUC values than entacapone/HP-β-CD solution, but the AUE was not increased. This could be explained by the fact that the AUC value was increased mainly as a result of increased C_{max}; the concentrations were equal already at 30 min after administration. Furthermore, the inhibitory E_{max} model (pharmacokinetic-pharmacodynamic modeling) showed that after oral administration, half-maximal inhibition was attained already at 317 ng/ml (1040 nM) and that COMT inhibition near to maximum attainable inhibition (E_{max}) was reached already at plasma concentrations below 2000 ng/ml. Thus, formulations producing high but transient plasma peak levels do not further improve the pharmacodynamic response of entacapone. On the other hand, the AUC (or bioavailability) value as such did not predict the pharmacodynamic response after a certain threshold.

In general, a previous entacapone study in rats indicated that the COMT activity-time profile in erythrocytes does not fully predict the situation in the gut (see Nissinen et al. 1992), where majority of orally administered LD has been suggested to undergo *O*-methylation (Nissinen et al. 1988). Instead, the degree of COMT inhibition and duration of inhibitory action in erythrocytes seem to reflect better COMT inhibition in the liver (Nissinen et al. 1992). The apparently higher EC_{50} value of entacapone in erythrocytes compared to that in duodenum, kidney and liver (see section 6.3.4) suggests that the COMT activity in these tissues may be more easily inhibited than that in the erythrocytes.

The present results also suggest that an increased duration of "effective" plasma levels could prolong the duration of the COMT inhibiting effect of entacapone. However, the treatment strategy of entacapone is to inhibit COMT transiently when LD is present (see Männistö and Kaakkola 1999). Since the pharmacokinetic profiles of LD and entacapone are quite similar at therapeutic oral doses (Kaakkola 2000), prolongation of COMT inhibiting effect of entacapone is not likely to improve its clinical benefit markedly.

In humans, there is large interindividual variation in entacapone concentrations after oral administration (Keränen et al. 1994; Rouru et al. 1999). Thus, improved bioavailability of entacapone could be considered as a means to decrease interindividual variation in absorption and thus to achieve better control in pharmacodynamic response. Furthermore, the dose could be reduced, which might decrease the risk of gastrointestinal adverse effects associated to entacapone treatment (see Kaakkola 2000;

Olanow et al. 2001). It is noteworthy that the present findings do not necessarily reflect the situation in humans. Firstly, administration of the solid dosage form was simulated with the entacapone suspension. Secondly, the anatomy of the gastrointestinal tract differs between humans and rats. Finally, there are species differences in the metabolism of entacapone (Wikberg et al. 1993).

6.2 Physicochemical properties (III)

The present results confirmed the earlier suggestions that tolcapone is clearly more lipophilic than entacapone (see Dingemanse 1997; Männistö and Kaakkola 1999). At acidic pH (1.2 – 3), the aqueous solubilities of entacapone and tolcapone are equally low but entacapone has a 15-fold better aqueous solubility than tolcapone at pH 7.4 (1750 vs. 115 μg/ml, respectively). In contrast, tolcapone has higher log P_{app} than entacapone; at pH 7.4, the log P_{app} values of entacapone and tolcapone are 1.03 and 0.178, respectively. In general, the different lipophilicities seem to explain the different behaviors of entacapone and tolcapone *in vivo*. The role of different physicochemical properties of entacapone and tolcapone on their potency, bioavailability and duration of action is discussed in the relevant sections.

6.3 Pharmacokinetic and pharmacodynamic properties of entacapone and tolcapone (II, III)

6.3.1 *In vitro* potency

The *in vitro* potency of entacapone and tolcapone has been studied only in a few comparative studies. Moreover, the results are controversial. Learmonth et al. (2002) have claimed that tolcapone is more potent than entacapone against both rat brain and liver total COMT although the confidence intervals of the IC_{50} values overlapped. In contrast, De Santi et al. (1998) have reported that entacapone is significantly more potent than tolcapone in human duodenal, liver and kidney COMT. In the study of Lotta et al. (1995) with human recombinant S- and MB-COMT, the K_i values of the two inhibitors were similar (0.3 nM) in S-COMT, but tolcapone had an apparently lower value (0.3 nM) than entacapone (2.0 nM) in MB-COMT. However, the authors did not discuss this apparent difference at all.

Generally, the K_i value should be preferred in comparison of the potencies of inhibitors; the IC_{50} values are applicable only when determined in standardized experimental conditions (Copeland 2000). The IC_{50} of a simple reversible inhibitor is dependent on the K_i of the inhibitor, the substrate concentration and the K_m of substrate (Copeland 2000). For tight binding inhibitors, such as nitrocatechol-structured COMT inhibitors (Schultz and Nissinen 1989; Nissinen et al. 1992; Lotta et al. 1995; Borges et

al. 1997), the IC_{50} value depends also on the total enzyme concentration in the sample. This is due to the fact that in tight binding inhibition, the inhibitor is bound to the enzyme with such a high affinity that the amount of free inhibitor molecules is significantly depleted by the formation of the enzyme-inhibitor complex (Copeland 2000). Viera-Coelho and Soares-da-Silva et al. (1999) have demonstrated the effect of the enzyme concentration on the IC_{50} value of tolcapone. At a fixed amount of COMT enzyme in the assay, tolcapone and reference compound 3,5-dinitrocatechol were equally potent against S- and MB-COMT both in brain and liver and there was no difference in the IC_{50} values between liver and brain. However, when the amount of protein in the COMT activity assay was standardized (i.e. actual enzyme concentration varied), both tolcapone and 3,5-dinitrocatechol were more potent against MB-COMT than S-COMT and the IC_{50} values in liver COMT were significantly higher than those in brain COMT. The difference between liver and brain could be explained by the fact that there is a higher amount of COMT units/mg protein in the liver than in the brain (Vieira-Coelho and Soares-da-Silva 1999) and the relative amount of MB-COMT is lower both in the brain and liver. In contrast, the IC_{50} of tropolone, which is a significantly less potent competitive inhibitor, did not depend on the enzyme concentration.

In the present study, the IC_{50} and K_i values of entacapone and tolcapone were studied using rat total COMT preparations (**II, III**). The K_i values were determined by taking the enzyme concentration in the sample into account. Since both S-COMT and MB-COMT are present in all rat and human tissues (Lundström et al. 1995), determination of *in vitro* potency of COMT inhibitors by using crude organ homogenates with natural S- and MB-COMT distribution may reflect better the situation *in vivo* than enzyme preparations enriched with either S- or MB-COMT (Vieira-Coelho and Soares-da-Silva 1999). The present results indicate that entacapone and tolcapone are equally potent *in vitro*; their K_i values were equal both in striatal and liver total COMT. The K_i values determined in liver total COMT are in line with previously reported values in rat liver COMT preparations (Zürcher et al. 1990a; Nissinen et al. 1992). Quite surprisingly, the K_i values were lower in the striatum than in liver. This may be partly due to the lower K_m of total COMT in striatum than in liver, which in turn reflects different relative amounts of S- and MB-COMT in these tissues (see also section 2.1.4.1) and their different kinetic parameters (see section 2.1.3).

6.3.2 Pharmacokinetics

There is only limited knowledge of pharmacokinetics of entacapone and tolcapone in rats. A few previous studies have suggested that the bioavailability of entacapone is lower and the total clearance higher than that of tolcapone (Funaki et al. 1994, 1995; Savolainen et al. 2000a). The situation is similar in humans (Keränen et al. 1994; Jorga et al. 1998a). The bioavailability of both entacapone and tolcapone seems to increase

with dose, but there is no animal data where both drugs have been given at similar doses.

The present results confirm that entacapone is eliminated faster than tolcapone after intravenous administration (**II**). When the drugs were administered orally at 10 mg/kg, significantly higher plasma levels and AUC values were seen with tolcapone (**II**). It is noteworthy that the observed plasma levels are comparable to those reached after administration a single 200 mg dose in healthy volunteers (Keränen et al. 1994; Dingemanse et al. 1995a; Jorga et al. 1998a). The estimated bioavailability of tolcapone was twice that of entacapone, which is in line with previous rat studies (Funaki et al. 1994, 1995; Savolainen et al. 2000a) and also human studies (Keränen et al. 1994; Jorga et al. 1998a; Heikkinen et al. 2001b). However, the sampling schedule (blood samples were collected only from 0.5 h onward) may slightly have underestimated the bioavailability of entacapone.

The reasons for the low bioavailability of entacapone remain unclear. However, there is some non-clinical evidence that low aqueous solubility and the slow dissolution rate of entacapone may partly explain its low bioavailability (Savolainen et al. 2000a). The fact that the bioavailability of only about 35% was reached by giving entacapone as a solution suggests that also limited transport of entacapone through gastrointestinal epithelium may limit its bioavailability (see Rowland and Tozer 1995). Drugs are transported passively through the intestinal epithelium either paracellularly or transcellularly. The transcellular route, where the lipid matrix of the cell membranes form the main barrier to absorption, is generally considered as being more important (Pagliara et al. 1999). Since entacapone is hydrophilic at neutral pH, it evidently penetrates poorly through the cell membranes in the small intestine (Savolainen et al. 2000a). The increased lipophilicity of entacapone at acidic pH could favor its absorption from stomach and proximal small intestine, but in turn, its low aqueous solubility at acidic pH may hinder absorption from the upper GI tract (Savolainen 2000). One could assume that tolcapone penetrates the membranes more easily and has overall better absorption than entacapone due to its generally higher lipophilicity. As is the case with entacapone, the low aqueous solubility at acidic pH is likely to hinder absorption of tolcapone from the upper GI tract. At neutral pH, the aqueous solubility is improved slightly but log P_{app} is still 1.03. Thus, it could be assumed that major site of tolcapone absorption is small intestine. This is supported by the fact that the t_{max} of tolcapone occurs later than that of entacapone, both in the present rat study (see Table 7) and in studies with healthy volunteers (Keränen et al. 1994; Dingemanse et al. 1995a). On the basis of absorption kinetics in healthy volunteers, it has been suggested that tolcapone may be transported actively via the same carrier mechanism as LD (Jorga et al. 1998a). However, if this is the case, one could assume better bioavailability than 60%. The lower bioavailability of entacapone compared to tolcapone may also be due to higher first-pass metabolism and clearance (Jorga et al. 1998a; Heikkinen et al. 2001b).

Previous COMT inhibition studies in rats have indicated that tolcapone penetrates into the brain whereas entacapone has a mainly peripheral action (Zürcher et al. 1991; Nissinen et al. 1992; Männistö et al. 1992b; Learmonth et al. 2002). In the present study, direct measurement of entacapone and tolcapone concentrations in perfused striatum after intravenous administration confirmed that the brain penetration of tolcapone is about 3 times higher than that of entacapone. In parallel, the degree of COMT inhibition in striatum was clearly higher after oral tolcapone treatment than after entacapone treatment. This is also probably a reflection of the higher lipophilicity of tolcapone, as has been suggested earlier (Dingemanse 1997, 2000; Männistö and Kaakkola 1999). However, in addition to BBB permeability, which is mainly governed by the lipophilicity of the drug, drug transport to the brain is also influenced by binding to plasma proteins i.e. high lipophilicity as such does not guarantee good brain (or tissue) penetration (Bradbury 1989; Greig 1989). It is generally accepted that mainly it is the free fraction in plasma that is transported through biological membranes (Rowland and Tozer 1995). Thus, the different protein binding of entacapone (98% bound, 2% free) and tolcapone (99.9% bound, 0.1% free) (Dingemanse 1997; Männistö and Kaakkola 1999) may partly explain differences both in distribution and elimination of these drugs. However, high protein binding is often a result of high lipophilicity (Greig 1989), which makes it difficult to predict the net effect of lipophilicity on tissue penetration. For example, if the striatum/serum ratios of entacapone and tolcapone (see section 5.3.2) were calculated with estimated free concentrations of entacapone and tolcapone in serum (120 ng/ml and 10.3 ng/ml, respectively), the resulting striatum/serum ratios would be about 4% and 240% instead of <0.1% and 0.24% as calculated with total serum drug concentrations. This clearly demonstrates that there is an equilibrium between bound and free form in plasma; when the free concentration decreases as a result of transport into the tissues, then bound drug is released.

Furthermore, lipophilicity may theoretically affect the preference of a COMT inhibitor between S- and MB-COMT. In fact, Viera-Coelho and Soares-da-Silva et al. (1999) have claimed that tolcapone can discriminate between S- and MB-COMT *in vivo* i.e. tolcapone would be more potent against MB-COMT than against S-COMT. They suggested that this phenomenon may be due to different subcellular location of S- and MB-COMT and the ability of tolcapone to interact with these isoforms. Since tolcapone is a rather lipophilic compound, it may favor MB-COMT as a result of interacting with membrane structures adjacent to MB-COMT (Vieira-Coelho and Soares-da-Silva 1999) as seems to be the case with substrates of COMT (see sections 2.1.3 and 2.4.1). No similar studies have been performed with entacapone. Since both S- and MB-COMT are present in all tissues (see above), the clinical relevance of this proposed discrimination remains unclear.

6.3.3 Pharmacodynamics

The effect of entacapone and tolcapone on total COMT activity was studied *ex vivo* (**II**). After a single dose, the maximal degree of COMT inhibition in peripheral tissues did not differ between entacapone- and tolcapone-treated animals. However, as was expected on the basis of the plasma levels, the COMT activity recovered faster in the peripheral tissues of entacapone-treated animals than was the case in tolcapone-treated animals. In the present study, the estimated time to reach full recovery of COMT activity in kidney and liver after a single tolcapone dose was over 12 h suggesting that some accumulation does occur during repeated administration at 12-h dosing intervals. In fact, a higher degree of COMT inhibition and a slower recovery of COMT activity in the liver and kidney were observed after the last dose of the 7-day treatment than after the single dose. This phenomenon was not observed in entacapone-treated animals. No accumulation of either entacapone or tolcapone was observed in plasma. The present results (single dose) are in agreement with the study of Learmonth et al. (2002) who used an oral dose of 30 mg/kg. Unexpectedly, Napolitano et al. (2003) reported recently that entacapone and tolcapone inhibited liver and duodenal COMT almost equally at 15 mg/kg (p.o.) for 6 h after administration. Duration of action of new nitrocatechol COMT inhibitors, BIA 3-202 and BIA 3-335, is longer than that of entacapone but shorter than that of tolcapone (Learmonth et al. 2002, 2004).

In humans, the degree of COMT inhibition in the body is generally assessed by inhibition of erythrocyte COMT activity. The activity in erythrocytes has been shown to correlate with that in liver and kidney (Weinshilboum 1978; Boudíková et al. 1990). The present animal results in the liver are in line with studies in healthy volunteers: after 100 or 200 mg entacapone, the COMT activity in erythrocytes recovers within about 8 h (Keränen et al. 1994) but after the corresponding tolcapone doses, 13 – 21 h is needed (Dingemanse et al. 1995a, b). No accumulation of either entacapone or tolcapone in plasma has been observed in healthy volunteers during repeated administration at the recommended dosing regimen (200 mg 10 times daily with 2 h intervals and 200 mg t.i.d., respectively) (Dingemanse et al. 1996; Rouru et al. 1999). At higher tolcapone doses, however, some accumulation was observed in plasma (Dingemanse et al. 1996). Furthermore, repeated administration of entacapone for 6 days did not potentiate its erythrocyte COMT inhibiting effect and the COMT activity recovered similarly after discontinuation of the treatment as seen after a single dose (Rouru et al. 1999). In the case of tolcapone, the erythrocyte COMT activity-time profiles and the EC_{50} values were not changed during one week's treatment (Dingemanse et al. 1996). However, COMT is continuously inhibited (by 30 – 80%) with 200 mg t.i.d. regimen from the first day. Most importantly, it is not known whether these drugs accumulate in tissues. The present non-clinical results indicate that the accumulation risk of tolcapone, as assessed by potentiation of COMT inhibition, is higher than that of entacapone. This is of particular importance since tolcapone seems to possess a greater toxicity potential

than entacapone (Haasio et al. 2002b). Impaired liver function (i.e. decreased metabolism of tolcapone) may further increase the risk of tolcapone accumulation (Borroni et al. 2001; Haasio et al. 2002a). Thus, serious hepatotoxicity could be manifested in some tolcapone-treated patient having already impaired liver function (Acuna et al. 2002). On the other hand, PD patients could be particularly prone to the oxidative phosphorylation uncoupling effect of tolcapone, because they may already have genetic deficits in mitochondrial respiratory function (Haasio 2003; see also section 2.3.2.1). As a result, ROS production may be synergistically increased.

In general, current evidence suggests that there is no potential safety concerns related to inhibition of COMT as such. However, the consequences of total lack of COMT or continuous COMT inhibition are still poorly characterized. Since COMT is widely distributed throughout the body and has important physiological roles (see sections 2.1.4 and 2.1.5), continuous inhibition of COMT activity can theoretically have diverse effects. For example, continuous COMT inhibition may alter the metabolism of catecholestrogens, which may in turn have detrimental effects (see section 2.1.4.1). Lavigne et al. (2001) have reported that inhibition of COMT increases the 2-hydroxy-estrogen levels and oxidative DNA damage in MCF-7 cells. On the other hand, Haasio et al. (2003) found no pathological changes in mammary gland or uterine tissues of COMT-deficent mice at the age of 12 month. Furthermore, no signs of relevant abnormalities were detected in liver and kidney.

The differences in duration of action have led to different treatment strategies for entacapone and tolcapone (see section 2.4.3.2). Since entacapone is taken with each LD dose, the degree of COMT inhibition at the time of LD administration is reproducible (Dingemanse 2000). With the tolcapone regimen, despite its longer duration of action, the degree of COMT inhibition varies throughout each day (Dingemanse et al. 1996; Dingemanse 2000). Thus, the entacapone treatment strategy may result in better response control. Since the duration of LD benefit is shorter than that of tolcapone, tolcapone or COMT inhibitors with a significantly longer duration of action than entacapone, such as BIA 3-202 (see section 2.5.1) may not be suitable for concomitant administration with each LD dose in frequent dosing (i.e. entacapone strategy) since they may accumulate. On the basis of pharmacokinetic studies in healthy volunteers (Ahtila et al. 1995; Jorga et al. 1998b), it could be hypothesized that tolcapone could produce slightly more stable LD levels than entacapone when given simultaneously with a controlled-release LD-DDC inhibitor preparation.

In the present study, the pharmacodynamics of entacapone and tolcapone in the brain were studied also by administering drugs directly into the striatum via the microdialysis probe (**III**). This experiment was designed to evaluate the role of physicochemical properties on their pharmacodynamic effect in striatum when the influence of systemic pharmacokinetic processes (absorption, distribution) on drug delivery into the brain is excluded by administering drugs directly into the striatum.

This special experimental setup gives indirect information of tissue and brain penetration of COMT inhibitors.

After systemic administration, tolcapone (1, 10, 30 mg/kg) inhibits central COMT and decreases striatal extracellular HVA levels with a simultaneous increase in extracellular DOPAC (Kaakkola and Wurtman 1992). Also entacapone has similar effects but this occurs at higher doses. Since entacapone and tolcapone are equally potent when they reach the COMT enzyme (see section 6.3.1), these results point to better brain penetration of tolcapone and thus to a higher amount of drug in the brain tissue. However, it was a surprise that intrastriatal perfusion of entacapone and tolcapone in the present study led to the opposite results. At 100 μM, entacapone was more effective than tolcapone as judged by HVA $AUC_{0-240\ min}$. In parallel, there was also a trend to higher DOPAC levels with entacapone. Finally, entacapone seemed to act more rapidly.

The observed differences between the effects of entacapone and tolcapone after local intrastriatal administration could be explained by different local distribution as a result of different penetration into the cells surrounding the microdialysis probe. The more lipophilic tolcapone evidently penetrates better into the brain tissue surrounding the probe than entacapone and is thus able to diffuse further away from the microdialysis probe, even out of the brain. Thus, the tolcapone concentration in the vicinity of the probe apparently decreases faster than that of entacapone and this results in a lower degree of inhibition of local COMT activity. Theoretically, the lower local tolcapone concentration could also be due to higher degree of binding to proteins present in the extracellular fluid around the probe. However, the protein content of extracellular and cerebrospinal fluid is low compared to plasma (Davson and Segal 1996; Abbott 2004). In parallel, the results of Männistö et al. (1992a) indicate that the distribution of nitecapone (a peripherally acting COMT inhibitor) in the brain was very limited compared to more the lipophilic clorgyline (MAO-A inhibitor) after intracerebroventricular administration. Generally, de Lange et al. (1995) have reported that lipophilicity affects the spatial distribution of drugs in the brain. This was evidenced by the demonstration that the extracellular concentration gradient was steeper for the more lipophilic acetaminophen than for the less lipophilic atenolol. It should also be emphasized that drug delivery and sample collection via microdialysis probe gives information about very localized effects. The results of Westerink and De Vries (2001) indicate that millimolar drug concentrations in perfusate are needed to reach a substantial brain concentration at a distance of 1 mm from the probe.

6.3.4 Pharmacokinetic-pharmacodynamic relationship

The EC_{50} values of entacapone and tolcapone indicate similar *in vivo* potency (**II**). It is noteworthy that the EC_{50} values of both entacapone and tolcapone seem to vary between tissues indicating that there may be differences in the availability of entacapone

and tolcapone. The inhibitory E_{max} model also showed that a level near to the maximum attainable COMT inhibition (E_{max}) is achieved at plasma concentrations below 2000 ng/ml, both with entacapone and tolcapone (**II, III**). Thus, tolcapone plasma concentrations seen shortly after administration of 10 mg/kg are higher than those needed to reach maximal achieveable COMT inhibition in the peripheral tissues. This is also supported by the fact that the maximal degree of COMT inhibition did not differ significantly between entacapone- and tolcapone-treated animals. Additionally, a further increase in tolcapone dose (100 mg/kg) resulted in a similar COMT activity-time profile as in the present study (Zürcher et al. 1991). The pharmacokinetic-pharmacodynamic relationship of tolcapone has also been studied in humans (Dingemanse et al. 1995a, b) and it seems that concentrations above 2000 – 3000 ng/ml cause only marginally higher COMT inhibition in erythrocytes.

There seems to be almost the same order of magnitude difference between IC_{50} and EC_{50} values of both entacapone and tolcapone in the liver (**II, III**). Since COMT is an intracellular enzyme, this gradient may reflect the generally poor ability of these COMT inhibitors to reach the target i.e. poor transport from circulation to extracellular space and from extracellular space to the intracellular compartment (see also section 6.3.2). One explanation is their high degree of binding to plasma proteins, which limits at least to some extent the efficacy of tolcapone. In fact, Haasio et al. (2002a) have demonstrated that addition of serum to the reaction medium increases the IC_{50} value of entcapone by only 2-fold whereas there is a ten-fold increase in the corresponding tolcapone value.

6.4 The role of COMT in dopamine-linked oxidative stress (IV)

6.4.1 Methamphetamine-induced hydroxyl radical production

It has been suggested that COMT is an important part of the antioxidant defense in the brain (see section 2.3.2.3). Thus, COMT-deficiency (or total inhibition of COMT) as such could theoretically increase oxidative metabolism of DA and lead to increased production of ROS. LD has been shown to possess low potency to induce $OH^{\bullet}$ production (Smith t al. 1994; Camp et al. 2000; Gerlach et al. 2001). Thus, METH was used to induce oxidative stress in the present study. It is known that the neurotoxicity of METH is mediated at least in part via the formation of ROS; METH enters dopaminergic neurons via the dopamine transporter and displaces intracellular DA, which in turn can be oxidized to ROS via MAO or auto-oxidation (see Davidson et al. 2001). Thus, lack of COMT could potentiate the neurotoxicity of METH.

In the present study, $OH^{\bullet}$ production in the mouse brain was assessed by a salicylate trapping method, which is based on quantitation of tissue levels of 2,3- and 2,5-DHBA formed in a reaction of $OH^{\bullet}$ with salicylate (Kondo et al. 1994; Giovanni et al. 1995; Fleckenstein et al. 1997; McCabe et al. 1997). It is generally accepted that 2,3-DHBA

levels mirror better formation of $OH^{\bullet}$ since salicylate is known to undergo 5-hydroxylation with the formation of 2,5-DHBA by liver CYP isoforms (Ingelman-Sundberg et al. 1991; Dupont et al. 1999). No COMT-genotype dependent differences were seen in 2,3-DHBA levels without METH-treatment. This was not a surprise, since COMT-deficiency does not increase basal DA levels in the brain (Huotari et al. 2002a). The lowest METH dose increased only slightly (n.s.) the 2,3-DHBA level accompanied by a concomitant increase in the salicylate levels. Most importantly, no COMT-genotype dependent changes were seen. In a previous mouse study, METH (5 mg/kg × 4 i.p.) increased striatal 2,3-DHBA levels twofold in C57BL6 mice but salicylate levels were not reported (Kondo et al. 1994). In contrast, Giovanni et al. (1995) did not find any significant increase in the neostriatal content of 2,3-DHBA in rats after METH (15 mg/kg × 4 i.p.). However, salicylate levels were increased.

In mice, the susceptibility to METH-induced neurotoxicity seems to be strain-dependent. Kita et al. (1998) have shown that BALB and DBA mice are more sensitive to METH-induced hyperthermia and subsequent striatal DA depletion than C57BL mice ($OH^{\bullet}$ production was not monitored). Though the COMT mouse strain has been regularly enriched over several generations with C57BL/6J mice, it has originally a 129Sv/C57BL/6J genetic background. This may explain to some extent why the present results differ from those of Kondo et al. (1994). Additionally, the COMT mouse strain is evidently quite resistant to METH-induced $OH^{\bullet}$ production i.e. existing defense mechanisms are able to clear any $OH^{\bullet}$ formed. However, it remains to be determined whether the effect of METH on $OH^{\bullet}$ production is similar in female COMT-deficient mice. It could be speculated that lower GST activity could make females more prone to the toxic effects of DA, since it has been shown that GST M2-2 is involved in the metabolism of oxidized catecholamines (Baez et al. 1997; Segura-Aguilar et al. 1997).

It is known that METH-induced hyperthermia contributes to the neurotoxic effects of METH (Bowyer et al. 1994; Albers and Sonsalla 1995); prevention of hyperthermia attenuates METH neurotoxicity (Bowyer et al. 1993; Xie et al. 2000). The interrelationship between hyperthermia and ROS production is not completely understood but it has been suggested that hyperthermia facilitates the formation of ROS (Fleckenstein et al. 1997). In the present study, a moderate hyperthermia and a slight increase in $OH^{\bullet}$ production occurred equally in all COMT genotypes at the lowest METH dose (2.5 mg/kg × 4). At higher doses, METH increased rectal temperature significantly more in homozygous animals than in heterozygous and wild type animals but there was no COMT genotype-dependent difference in $OH^{\bullet}$ production. This suggests that METH-induced hyperthermia and ROS production were not causally associated in COMT-deficient mice.

6.4.2 Enzymatic defenses

Several enzymatic defenses could be activated as a response to oxidative stress (Duffy et al. 1998; Morel and Barouki 1999; Raza et al. 2002). In contrast, the activity of endogenous ROS-generating systems such as liver cytochrome P450 enzymes may be repressed. In this study, the activities of quinone reductase and GST were determined to assess whether the COMT deficiency and/or LD challenge activate these defenses.

Female mice had lower GST activity and higher quinone reductase activity in the liver than male mice. The reason for is unclear, but it is possible that estrogen regulates the expression of these enzymes, as seems to be the case with COMT (Xie et al. 1999; Huotari et al. 2002a). COMT-deficient mice have shown also other sexually dimorphic effects (Gogos et al. 1998; Huotari et al. 2002a, b, 2004). Most importantly, COMT-deficiency did not change the basal activities of either GST and quinone reductase or 7-ethoxyresorufin O-deethylation (CYP 1A) and 7-pentoxyresorufin O-depentylation (CYP 2B) in the liver. Since high-dose LD treatment did not induce any marked changes either in the activity of these enzymes in the liver or GST activity and quinone reductase in the brain of female COMT-deficient mice, it is tempting to suggest that the normal capacity of these mechanisms is adequate to clear ROS formed from LD/DA and even to counteract some of the increases.

Taken together, the present preliminary results suggest that COMT does not play an important role in DA-linked oxidative stress. However, it should be emphasized that in animals lacking COMT, antioxidant mechanisms other than those examined in the present study may be compensatorily activated. Moreover, such changes may be restricted to only in DA-rich brain areas. Thus, additional studies are still needed to confirm the role of COMT in DA-linked oxidative stress.

6.4.3 DOPA oxidase activity of skin tyrosinase in mice pups

Some LD is metabolized via oxidation, transamination and sulphoconjugation (see Nutt and Fellman 1984; Männistö et al. 1992c). When major pathways of LD metabolism, i.e. DDC and COMT, are inhibited, minor pathways such as oxidation by tyrosinase may become activated. This could be one contributing factor to the LD toxicity since tyrosinase converts LD to the potentially cytotoxic DOPA quinone (see Asanuma et al. 2003). In hairless COMT-deficient male pups, we found that the dopa oxidase activity of skin tyrosinase acivity was significantly higher than in their wild-type counterparts but not in adult mice. However, this result is the only indication of possible redirection of LD metabolism when COMT is extensively inhibited.

7 SUMMARY AND CONCLUSIONS

In general, the present non-clinical results confirm the earlier suggestions about different behavior of entacapone and tolcapone. The present results also provide further evidence that the reasons for higher potency of tolcapone *in vivo* are pharmacokinetic rather than pharmacodynamic. Different physicochemical properties explain partly the differences between entacapone and tolcapone. This non-clinical study, together with other recent findings, provides comparative new information about entacapone and tolcapone, which may help to understand the potential safety risks associated with these drugs and to develop new COMT inhibitors to achieve an optimal outcome as an adjunct to LD-DDC inhibitor therapy. Firstly, improving bioavailability could further improve the clinical benefit of entacapone. A marked prolongation of its duration of action cannot be considered as an unambiguous goal, since this would lead to continuous COMT inhibition in frequent adminstration. Secondly, tolcapone-associated adverse effects may be partly related to its lipophilicity and long duration of action (i.e. potential to accumulate in the tissues).

The preliminary studies on the role of COMT in DA-linked oxidative stress did not reveal any potential concerns associated with COMT-deficiency. The present results, however, do not fully exclude the possibility that lack of COMT may increase DA-dependent formation of ROS. Further studies are also needed to assess the effect of COMT-deficiency on the activity of other antioxidant mechanisms such as glutathione peroxidase and catalase. The correlation of METH-induced hyperthermia with COMT genotype in mice supports earlier findings that under certain conditions, COMT-deficiency may lead to some alterations in neurotransmitter turnover.

Based on the results of the experimental section (**I – IV**), the following specific conclusions can be drawn:

1. The results confirm that the pharmacodynamic response of entacapone (i.e. area under the erythrocyte COMT inhibition-time curve, AUE) could be increased by improving its bioavailability. Above certain threshold, however, higher AUC value provides no further increase in AUE. Thus, a longer duration of "effective" plasma levels is needed to improve further the overall pharmacodynamic response.
2. Entacapone and tolcapone are equally potent in rat liver and striatal total COMT *in vitro* (i.e. when the drugs reach easily the COMT enzyme).
3. The oral bioavailability of tolcapone is better than that of entacapone in rats. Furthermore, tolcapone is eliminated more slowly than entacapone. As a result, tolcapone has a longer duration of COMT inhibiting action in rat tissues than entacapone. With a 12 h dosing interval, the COMT activity in the liver is continuously inhibited in tolcapone-treated animals and some accumulation in the liver and kidney occurred. Direct measurement of drug concentrations as well as

COMT inhibition studies confirm the earlier hypothesis that tolcapone penetrates better into the brain than entacapone. A probable explanation for the better tissue and brain penetration of tolcapone is that it has higher lipophilicity than entacapone.

4. Entacapone and tolcapone seem to have similar potency *in vivo*. Pharmacokinetic-pharmacodynamic modeling also showed that concentrations above about 2000 ng/ml produce only a minor further increase in the degree of COMT inhibition. This suggests that peak tolcapone concentrations reached in clinical practice are higher than needed to reach maximal degree of COMT inhibition in the periphery.
5. Intrastriatal administration of entacapone and tolcapone led to opposite results compared to those reported in the brain after systemic administration; entacapone appeared to inhibit COMT more effectively than tolcapone. Different physicochemical properties of entacapone and tolcapone are likely explanation for their different local effects.
6. Though there was potentiation of the hyperthermic response of METH, COMT-deficiency did not increase METH-induced hydroxyl radical production. Furthermore, COMT-deficiency did not change significantly the activities of GST and quinone reductase.

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ORIGINAL PUBLICATIONS

This doctoral dissertation is based on the following publications, referred to in the text by Roman numerals **I – IV**.

I Markus Forsberg, Jouko Savolainen, Tomi Järvinen, Jukka Leppänen, Jukka Gynther, Pekka T. Männistö: Pharmacodynamic response of entacapone in rats after administration of entacapone formulations and prodrugs with varying bioavailabilities.
Pharmacology & Toxicology 90: 327-332, 2002

II Markus Forsberg, Marko Lehtonen, Minna Heikkinen, Jouko Savolainen, Tomi Järvinen, Pekka T. Männistö: Pharmacokinetics and pharmacodynamics of entacapone and tolcapone after acute and repeated administration: a comparative study in the rat.
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A 68. Korhonen, Ossi. Starch acetate as a novel tablet excipient for extended oral drug delivery.
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