Comparison of the hepatic safety of catechol-O-methyltransferase inhibitors entacapone and tolcapone with special reference to uncoupling of oxidative phosphorylation

Kristiina Haasio

ACADEMIC DISSERTATION

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Anything is possible if you wish hard enough
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1. Abstract

Entacapone and tolcapone are novel catechol-O-methyltransferase (COMT) inhibitors developed for adjunctive treatment with levodopa in Parkinson’s disease (PD). In clinical use, tolcapone has induced severe hepatic dysfunction, including three fatal cases. Tolcapone, but not entacapone, has also been shown to be an uncoupler of oxidative phosphorylation in vitro at low micromolar concentrations.

The toxicity of entacapone and tolcapone was compared in animal studies in vivo and in vitro. 2,4-dinitrophenol (DNP), a known uncoupling agent, was used as a reference substance. In the in vivo studies, rats were treated with high repeated oral doses. Clinical symptoms, mortality and rectal body temperature were recorded. Furthermore, clinical chemistry and haematological parameters were measured, and at necropsy, tissue samples for histological examination and biochemical determinations were extracted. At cellular level, mitochondrial energy status was determined after oral treatment in rats. In vitro, mitochondrial membrane potential in isolated rat liver mitochondria was determined to obtain data on the direct effects of these agents on uncoupling of oxidative phosphorylation. To evaluate the role of total COMT inhibition in the toxicity of COMT inhibitors, mice lacking the Comt-gene were used to assess COMT inhibition-induced pathology in organs expressing high COMT activity in otherwise healthy animals.

Entacapone was well tolerated at high oral repeated doses by rats. Tolcapone induced clinical signs and increased mortality at liver concentrations that caused no adverse effects with entacapone. Rectal body temperature increased with tolcapone and DNP treatments. Rigor mortis occurred instantly after death in tolcapone-treated rats. The mitochondrial ATP/ADP ratio decreased after tolcapone and DNP treatments. Hepatotoxicity was expressed as an increase in liver enzymes as well as histopathological changes in tolcapone-treated and to a lesser degree in DNP-treated animals. No pathological findings in tissues were observed in mice lacking COMT activity.

The study showed that tolcapone has potential hepatotoxic properties at high doses in animals. The findings in vivo, at the cellular level and in vitro are consistent with those observed after treatment with DNP, suggesting that hepatotoxic properties of tolcapone may be due to uncoupling of oxidative phosphorylation. Entacapone was well tolerated and did not express any signs related to uncoupling. Total depletion of COMT activity did not induce any liver problems in the knock-out mice, indicating that hepatotoxicity is not a class effect of these COMT inhibitors.
2. Original publications

This thesis is based on the following original papers referred to in the text by their Roman numerals:


### 3. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>adrenaline</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ALAT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>APHOS</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ASAT</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>area under curve</td>
</tr>
<tr>
<td>BBM</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>peak plasma concentration</td>
</tr>
<tr>
<td>C&lt;sub&gt;last&lt;/sub&gt;</td>
<td>last measurable plasma concentration</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyl transferase</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DDC</td>
<td>dopa decarboxylase</td>
</tr>
<tr>
<td>DHPG</td>
<td>3,4-dihydroxyphenylglycol</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-dinitrophenol</td>
</tr>
<tr>
<td>DOPA</td>
<td>3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenyl acetic acid</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>median effective dose</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HPLC-EC</td>
<td>HPLC with colourimetric electrochemical detection</td>
</tr>
<tr>
<td>HVA</td>
<td>homovanillic acid</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>median inhibitory concentration</td>
</tr>
<tr>
<td>levodopa</td>
<td>L-dihydroxyphenylalanine, L-dopa</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamino oxidase</td>
</tr>
<tr>
<td>MB-COMT</td>
<td>membrane-bound COMT</td>
</tr>
<tr>
<td>N</td>
<td>number of observations/animals</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
</tr>
<tr>
<td>NAD</td>
<td>no abnormalities detected</td>
</tr>
<tr>
<td>OFF time</td>
<td>sudden loss of effectiveness with abrupt onset of akinesia (Fahn 1974)</td>
</tr>
<tr>
<td>3-OMD</td>
<td>3-O-methyl dopamine</td>
</tr>
<tr>
<td>ON time</td>
<td>sudden return of effectiveness, may be accompanied by hyperkinasia (Fahn 1974)</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PST</td>
<td>phenolsulphotransferase</td>
</tr>
<tr>
<td>RCR</td>
<td>respiratory control ratio</td>
</tr>
<tr>
<td>= (oxygen consumption rate with ADP)/(oxygen consumption rate without ADP)</td>
<td></td>
</tr>
<tr>
<td>S-COMT</td>
<td>soluble COMT</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDH</td>
<td>sorbitol dehydrogenase</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>ULN</td>
<td>upper limit of normal</td>
</tr>
</tbody>
</table>
The catechol-\(O\)-methyltransferase (COMT; EC 2.1.1.6) enzyme was characterized by Axelrod and Tomchick in 1958 (Axelrod and Tomchick 1958; Axelrod and LaRoche 1959; Guldberg and Marsden 1975; Männistö and Kaakkola 1999). COMT is present in most mammalian tissues, with the highest activities occurring in the liver and kidneys (Guldberg and Marsden 1975; Nissinen et al. 1988; Karhunen et al. 1994). COMT catalyses the inactivation of catecholamines by methylation of the 3-hydroxyl group of the catechol ring both in the periphery and the central nervous system (CNS) (Figure 1, page 14). Besides catecholamines, levodopa, the cornerstone in treatment of Parkinson’s disease (PD), is one of the substrates of COMT. The gradual destruction of dopaminergic neurons in the brain leads to a dopamine (DA) deficiency and the onset of symptoms of PD (Schipira 2001; Orth and Schapira 2002). Levodopa is administered together with dopa decarboxylase (DDC) inhibitors, which diminish the peripheral metabolism of levodopa and increase its bioavailability (Teräväinen et al. 2001). To induce further increases of DA in the brain, COMT inhibitors were designed to be used in the treatment of PD as adjuncts to levodopa and DDC inhibitors.

The first generation of COMT inhibitors was developed during the 1960s. Pyrogallol was reported to be a potent COMT inhibitor already in 1959 by Axelrod and LaRoche, and it was later used as a standard inhibitor in the development of new agents (Ross and Haljasmaa 1964a). Several of the first-generation inhibitors were based on catechol structure (Ross and Haljasmaa 1964a; 1964b; Baldessarini and Greiner 1973; Gugler and Dengler 1973). Gallates, catechols, tropolone and its derivatives and pyrogallol were all tested \textit{in vitro} and \textit{in vivo} and appeared to be effective but were usually extremely short-acting (Belleau and Burba 1961; Ross and Haljasmaa 1964a; Dorris and Dill 1977). They were also toxic, inducing convulsions in experimental animals. In clinical trials with e.g. N-butylgallate and U-0521, they were established to be non-effective in the treatment of PD, offering no advantage when compared with dopa decarboxylase (DDC) inhibitors administered together with levodopa (Ericsson 1971). None of the first-generation COMT inhibitors was taken into routine clinical use.

Second-generation COMT inhibitors were developed simultaneously by three laboratories during the late 1980s (Bäckström et al. 1989; Borglyua et al. 1989; Waldmeier et al. 1990). Approximately ten years later, a long-acting inhibitor, BIA 3-202, with limited access to the brain, was reported to have beneficial effects on brain DA metabolism in the rat (Parada et al. 2001). All of the second-generation COMT inhibitors are selective and orally active, and all except one are structurally nitrocatechols. Two of them were taken into clinical use in the late 1990s. Entacapone mainly acts peripherally, while tolcapone crosses the blood-brain barrier (BBB) and acts in both the periphery and CNS (Männistö and Kaakkola 1999). Both
are designed to be administered concomitantly with levodopa, tolcapone three times a day, and entacapone up to ten times a day with each levodopa dose (Dingemanse 1997; Männistö and Kaakkola 1999). Although they are relatively similar structurally, their kinetics and metabolic pathways differ. In non-clinical studies, both inhibitors proved to be safe after undergoing extensive toxicity testing in several animal species (Schläppi et al. 1996a; 1996b; 1996c; Tasmar Product Monograph 1997; Entacapone Product Monograph 1999). In clinical studies, they were well tolerated, with the main side effects being gastro-intestinal and dopaminergic (Männistö and Kaakkola 1999).

After being on the market for about one year and when 60 000 patients had been exposed to tolcapone, the compound was found to induce liver toxicity and three fatal cases were reported within a short time span (Assal et al. 1998; Mayoral et al. 1999; Olanow 2000; Spahr et al. 2000; Watkins 2000). In 1998, the European Medicines Evaluation Agency recommended suspension of the marketing authorization for tolcapone in the European Union (European Medicine Evaluation Agency 1998). At the same time, the European Medicines Evaluation Agency evaluated the clinical data for entacapone and found no reason to restrict its use.

Since entacapone and tolcapone are structurally related nitrocatechols, concern has arisen regarding hepatotoxicity of this class of COMT inhibitors. As no signs of liver toxicity in non-clinical studies or in clinical trials of these inhibitors have been observed, comparison of these agents at high doses is the next logical step in evaluating possible hepatotoxic properties and mechanisms.

Introduction
5. Review of the literature

5.1. COMT and catecholamine metabolism

Catechol-O-methyltransferase (COMT) is an intracellular enzyme that catalyses the O-methylation of catechol-structured compounds, such as catecholamine neurotransmitters, catechol hormones and xenobiotic catechols, in a variety of tissues in mammals (Figure 1, page 14) (Axelrod and Tomchick 1958; Guldberg and Marsden 1975; Bonifati and Meco 1999). The COMT enzyme was initially characterized in the 1950s, when both adrenaline (A) and noradrenaline (NA) were found to undergo an O-methylation reaction (Axelrod 1957). Adrenaline was metabolized in the rat liver fraction in the presence of S-adenosylmethionine and magnesium ions to metanephrine. This led to the finding that endogenous amines, including NA, A and dopamine (DA), are O-methylated to normetanephrine, metanephrine and 3-methoxytyramine, respectively, when S-adenosylmethionine donates its methyl group to one of the hydroxy groups of catecholamines (Axelrod and Tomchick 1958). Later it has been found that besides A, NA and DA, the physiological substrates of COMT also include 3,4-dihydroxyphenylalanine (DOPA) and catecholestrogens (Guldberg and Marsden 1975; Männistö and Kaakkola 1999). These amines are inactivated by methylation of the 3-hydroxyl group of the catechol ring, and COMT catalyses this reaction using S-adenosyl-L-methionine as the methyl donor. In the 1970s, the presence of two distinct forms of COMT in rat erythrocytes was demonstrated (Assicot and Bohuon 1971); one fraction was in soluble form (S-COMT), and the other was bound to membranes (MB-COMT) (Assicot and Bohuon 1971; Borchardt et al. 1974).

Cellular and tissue distribution of COMT

The distribution and cellular localization of COMT have been evaluated in man and in several animal species (Guldberg and Marsden 1975; Nissinen et al. 1992; Karhunen et al. 1994). The highest activity of COMT is found in the liver and kidney, but activity has also been detected in several other organs, including the brain, lung, spleen, mammary gland, uterus, stomach, intestines and adrenals (Guldberg and Marsden 1975; Nissinen et al. 1988; Karhunen et al. 1994; De Santi et al. 1998). Axelrod and Tomchick compared liver COMT activities in several mammalian species and found the highest activity in the rat, followed by the mouse and guinea pig, while the activity of COMT in human liver was about one-tenth of that in the rat liver (Axelrod and Tomchick 1958; Guldberg and Marsden 1975). Although MB-COMT represents only 2-10% of total COMT activity in the rat liver and brain, it has a 3- to 100-fold higher affinity for catechol substrates than S-COMT, thus being responsible for O-methylation at physiologically relevant concentrations of neurotransmitters (Assicot and Bohuon 1971; Aprille and Malamud 1975; Rivett et al. 1983; Jeffery and Roth 1984; Roth 1992). The activity of S-COMT pre-
dominates when MB-COMT is saturated (Roth 1992; Vieira-Coelho and Soares-da-Silva 1999). The subcellular localization of MB-COMT using rat liver preparations has been postulated to be on mitochondrial or plasma membranes (Grossman et al. 1985; Tilgmann et al. 1992). Ulmanen et al. (1997) have shown by immunocytochemistry in cell culture studies using both mammalian cell lines and rat primary neurons that MB-COMT is located on intracellular membranes like the rough endoplasmic reticulum rather than on plasma membranes, while S-COMT is expressed both in the nuclei and cytoplasm.

In the mouse liver, most of the COMT activity is associated with membranes (Aprille and Malamud 1975). The liver membranes contain 70-90% of the total COMT activity in homogenate. The specific activity in mouse membranes is 8 to 9-fold higher than that in rat membranes, whereas the activity of S-COMT is comparable in the mouse and rat (Aprille and Malamud 1975).

S-COMT is the predominant form of the enzyme expressed in the periphery of most rat tissues (Karhunen et al. 1994; Lotta et al. 1995). Rat liver shows highest activity for S-COMT, while the lowest activity of both S-COMT and MB-COMT is found in the heart (Ellingson et al. 1999). MB-COMT is detected mainly in the liver and kidneys and in smaller amounts in the brain and intestines (Karhunen et al. 1994). The cellular localization of S-COMT in rat liver is limited to hepatocytes, and Kupffer cells show no activity (Karhunen et al. 1994). Using immunohistochemical staining methods, S-COMT has been found to be the major form in the rat brain with non-neuronal localization (Karhunen et al. 1994). The most intensely stained areas were lateral, third and fourth ventricles, followed by cells of the choroid plexus (Karhunen et al. 1994).

In the human liver, the activity is significantly higher in men than in women, and it increases with age (Agathopoulos et al. 1971; De Santi et al. 1998). MB-COMT is distributed among all tissues, with the highest levels being found in the liver (Roth 1992). In the human brain, both isoforms of COMT are present, but MB-COMT is the functionally significant form (Roth 1992; Karhunen et al. 1994; Hong et al. 1998). Since the highest ratio of MB-COMT to S-COMT is found in brain tissue, this suggests that MB-COMT may be localized in neuronal cells of the CNS in man (Roth 1992; Tenhunen et al. 1994). In human breast tissue, COMT, but no other methyltransferase enzyme, has been reported to be present (Assicot et al. 1977). An increase in COMT activity has been observed in the majority of primary mammary carcinoma samples of high malignancy (Assicot et al. 1977; Hoffman et al. 1979). Using immunocytochemical staining methods, S-COMT has been localized in the cytoplasm of epithelial cells of secretory tubules in both human and rodent mammary glands; there is a strong reaction in malignant cells, particularly in tumours (Amin et al. 1983; Tenhunen et al. 1999). In human mammary epithelial cells, nuclear COMT has been observed in normal cells as well as cancer cells (Weisz et al. 2000).
5.2. Other catecholamine-metabolizing enzymes

Other metabolic pathways in inactivating catecholamines include oxidative removal of their amino group by monoamine oxidase (MAO) and sulphoconjugation by phenolsulphotransferase (PST) (Kopin 1994) (Figure 1). MAO metabolizes catecholamines to 3-methoxy-4-hydroxyphenylglycol. There are two isozymes of MAO, A and B, which have slightly different substrates; e.g. NA is preferentially metabolized by MAO-A, whereas DA is a substrate of both types in most species (O’Carroll et al. 1983; Kopin 1994). In the human brain, approximately 70% of total MAO activity is of type B, which oxidizes most of the DA in man (Riederer et al. 1986). However, in rodents, DA is oxidized mainly by MAO-A (Johnston 1968).

Cellular and tissue localization of MAO

In the periphery, MAO is expressed in several mammalian tissues, the highest concentrations being in the liver and kidney (Kalaria and Harik 1987; Kopin 1994). Some tissues, e.g. bovine liver and kidney, contain mainly MAO-B, whereas rat skeletal muscle contains only minor amounts of both forms (Shih et al. 1999b).

In the brain, the distribution of MAO-A and -B shows little species variation. MAO-A is predominantly found in catecholaminergic neurons, MAO-B is most abundant in serotonergic and histaminergic neurons and glial cells, being the dominant isozyme in the human brain (Kopin 1985; Riederer et al. 1986; Kalaria and Harik 1987; Shih et al. 1999b). Both MAO isozymes are located on the outer mitochondrial membrane.

Role of PST

Sulphation of catecholamines is performed by several types of PST (Kopin 1985). The highest PST concentrations are observed in the rat brain and liver, but enzyme activity is also found in the adrenals, lung, kidneys and testes (Foldes and Meek 1974). In the rat, sulphoconjugation of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) plays a more important role than in e.g. dogs and primates (Kopin 1985).

Gene-manipulated mice deficient in catecholamine-metabolizing enzymes

The effects of total COMT or MAO inhibition in the brain has been evaluated in COMT- or MAO-deficient mice (Gogos et al. 1998; Shih et al. 1999a; Huotari et al. 2002). In COMT knock-out mice, striatal, hypothalamic and cortical MAO-dependent metabolite DOPAC and 3,4-dihydroxyphenylglycol (DHPG) levels were increased, and COMT-dependent metabolites HVA and 3-methoxy-4-hydroxyphenylglycol were absent in mice lacking the Comt-gene. A trend for DA and NA to increase in striatal and hypothalamic brain homogenates of COMT knock-out mice has also been found (Huotari et al. 2002). Gogos et al. (1998) reported slightly aggressive behaviour of male heterozygous mice. Mice lacking MAO-A or MAO-B have been observed express different changes in behaviour (Shih et al. 1999b). Since MAO-A is reported to be responsible for 5-hydroxytryptamine, NA and DA metabolism, mice deficient in MAO-A have elevated brain levels of 5-hydroxytryptamine and thus show more aggressive behaviour. MAO-B is responsible...
for phenylethylamine metabolism and knock-out animals do not exhibit aggression (Shih et al. 1999b).

In humans, due to the role of MAO in the metabolism of catecholamines in the brain, both MAO-A and MAO-B inhibitors have been taken into clinical use. Selective inhibition of MAO-B by e.g. selegiline is used in the treatment of PD to increase the concentration of DA in the human brain independently of levodopa (Deleu et al. 2002).

5.3. COMT inhibitors in the treatment of Parkinson’s disease

5.3.1. Rationale for the use of COMT inhibitors

In Parkinson’s disease, dopaminergic neurons in the brain are gradually destroyed, leading to a DA deficiency. When clinical symptoms appear, ca. 70-80% of the dopaminergic neurons have already been lost (Schapira 2001; Teräväinen et al. 2001; Orth and Schapira 2002). The decrease in DA

![Figure 1. Biosynthesis and catabolism of catecholamines. DOPA, 3,4-dihydroxyphenylalanine; DA, dopamine; NA, noradrenaline; A, adrenaline; NM, normetanephrine; DOMA, 3,4-dihydroxymandelic acid; MN, metanephrine; VMA, vanillylmandelic acid; 3-MT, 3-methoxytyramine; DOPAC, 3,4-dihydroxyphenylacetic acid; DHPG, 3,4-dihydroxyphenylglycol; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; 3-OMD, 3-O-methyldopa.](image-url)
levels in the striatum provokes the typical symptoms of PD (Schapira 2001). The aetiology of neuronal destruction is unknown. More than one aetiological factor has been postulated to cause PD, including genetic and environmental factors (Orth and Schapira 2002). Levodopa, the most effective symptomatic treatment available for PD, has been in clinical use since the 1960s (Birkmayer and Hornykiewicz 1961). Levodopa is a DA precursor, that can cross the BBB and is further decarboxylated to form DA. In the periphery, after oral administration, approximately 70% of levodopa is metabolized to DA by dopa decarboxylase (DDC). As a COMT substrate, a smaller amount of levodopa is metabolized by COMT through O-methylation to form 3-O-methyldopa (3-OMD) (Figure 2). Peripherally acting selective DDC inhibitors, such as carbidopa and benserazide, have been used as adjuncts to levodopa treatment in PD patients (Bartholini and Pletscher 1975; Rivest et al. 1999). DDC inhibitors increase the bioavailability of levodopa in the brain, but due to the increased metabolism by the COMT pathway, less than 10% of the levodopa dose reaches the brain (Nutt and Fellman 1984; Männistö and Kaakkola 1990). The plasma concentration of 3-OMD increases due to the long half-life of

![Figure 2. Effect of COMT inhibitors on levodopa metabolism. DA, dopamine; 3-OMD, 3-O-methyldopa; DDC, dopa decarboxylase; BBB, blood-brain barrier.](image_url)
this metabolite and remains high during chronic therapy (Kuruma et al. 1971). The methylated product may further reduce the utilization of levodopa by competing with it on the BBB level (Nutt and Fellman 1984; Reches and Fahn 1984; Männistö and Kaakkola 1990). By blocking the O-methylation pathway of levodopa, COMT inhibitors increase its bioavailability and reduce the formation of 3-OMD in PD patients (Männistö 1994). With concomitant use of COMT inhibitors and levodopa, daily doses of levodopa can be reduced while retaining the clinical benefit (Nutt et al. 1994; Kurth et al. 1997). The administration of COMT inhibitors to PD patients slows the elimination of levodopa from the plasma, which increases the ON time in these patients (ON time, sudden return of effectiveness that may be accompanied by hyperkinesia) (Fahn 1974; Nutt et al. 1994; Dingemanse 1997). In the brain, reuptake of catecholamines into presynaptic neurons and metabolism by MAO are the most important routes in eliminating released catecholamines; COMT plays only a minor role. COMT inhibition is reported not to alter catecholamine levels in plasma noticeably (Illi et al. 1994; Li et al. 1998; Männistö and Kaakkola 1999; Rojo et al. 2001).

5.3.2. First-generation COMT inhibitors

Following description and partial characterization of the COMT enzyme in the late 1950s and the reported occurrence of 3-O-methylated metabolites of catecholamines in mammals, the development of first-generation COMT inhibitors began (Axelrod and Tomchick 1958). These inhibitors include e.g. pyrogallol and its derivatives, tropolones, 3,4-dihydroxy-2-methylpropiophenone (U-0521) and catechol. The COMT inhibition activity of all of these compounds was weak or non-selective, they had poor bioavailability and they tended to be short-acting. None of the first-generation COMT inhibitors was therefore taken into routine clinical use (Ericsson 1971; Reches and Fahn 1984).

Pyrogallol (1,2,3-trihydroxybenzene) (Figure 3) was shown to be a potent COMT inhibitor in vitro and in vivo by Axelrod and LaRoche in 1959. It was a good substrate of COMT, acting as a competitive inhibitor, and had a short duration in vivo (Belleau and Burba 1961), but it induced toxic effects due to properties other than COMT inhibition (Wylie et al. 1960; Angel and Rodgers 1968). Pyrogallol acts both in the CNS and periphery (Angel and Rodgers 1968), but with regular use for two weeks, COMT activity in the rat heart and brain showed no major changes after a subcutaneous daily dose of 50 mg/kg (Maitre 1966). While pyrogallol is not suitable for clinical use because of these properties, it has been used as a tool in studies on COMT inhibition.

Gallates are effective COMT inhibitors in vitro and in vivo but have an extremely short duration of action in the rat, only 15-30 minutes after an intraperitoneal injection (Dorris and Dill 1977). A few clinical trials performed with N-butyl gallate (Figure 3) failed to reveal any advantage over DDC inhibitors administered together with levodopa (Ericsson 1971). When ten parkinsonian patients in whom the maximum dosage of levodopa had been reached were treated with an initial dose of
N-butyl gallate 250 mg, followed by increasing doses of up to 750 mg daily, the symptoms were alleviated. However, the clinical improvement was not as effective as that obtained with DDC inhibitors (Ericsson 1971).

Catechol (1,2-dihydroxybenzene) and several of its derivatives are COMT inhibitors in vitro and in vivo (Ross and Haljasmaa 1964a; Baldessarini and Greiner 1973), but they are toxic in vivo because of properties unrelated to COMT inhibition (Angel and Rodgers 1968; Bakke 1970). Some of them, e.g. catechol-configurated flavonoids, are more potent inhibitors of COMT in vitro than pyrogallol (Gugler and Dengler 1973). Catechol itself is highly toxic, inducing convulsions in the rat after an oral dose of 100 mg/kg (Angel and Rodgers 1968; Bakke 1970). U-0521 (3, 4-dihydroxy-2-methylpropionophenone) (Figure 3) was reported to effectively diminish plasma and brain 3-O-methyldopa (3-OMD), enhancing the accumulation of both DOPA and DA in rat striatum after an intraperitoneal injection of 100 mg/kg or more (Fahn et al. 1979; Reches and Fahn 1984). However, it was not effective when administered orally to rats or in clinical use with parkinsonian patients (Fahn et al. 1979; Reches and Fahn 1984).

Tropolone (2-hydroxycycloheptatrienone) (Figure 3) and its derivatives inhibit both peripheral and central COMT in vitro and in vivo but are short-acting (Belleau and Burba 1961; 1963; Ross and Haljasmaa 1964a). The inhibitory effects are comparable with those of pyrogallol in the periphery; however, in the brain, tropolone shows much less activity than pyrogallol (Ross and Haljasmaa 1964a; 1964b). Tropolone 100 mg/kg administered intraperitoneally caused an increase in DA and DOPAC concentrations in rat striatum, while simultaneously reducing the concentration of HVA (Broch 1972). However, the central COMT inhibition was quite modest and was not correlated with the concentration of tropolone. Moreover, tropolones have been reported to be toxic in mice, rats and rabbits at doses inducing convulsions (Ri 1951).

Since the majority of first-generation COMT inhibitors had metal-chelating properties, a chelation mechanism was proposed for complex formation between catecholamines and the COMT enzyme (Senoh et al. 1959). Metal chelators of various structures were tested in vitro in mouse brain homogenates for their capacity to inhibit the COMT enzyme. Some of these compounds, e.g. 8-hydroxyquinoline, were more potent COMT inhibitors than pyrogallol in vitro, but the majority failed to express any COMT inhibitory effects in mouse brain extract in vitro, indicating that the metal-chelating effect alone was insufficient to produce COMT inhibition (Ross and Haljasmaa 1964a).

5.3.3. Second-generation COMT inhibitors entacapone and tolcapone

A new generation of COMT inhibitors was discovered in the 1980s, and these compounds were shown to inhibit COMT and to alter levodopa metabolism in vivo. Entacapone, tolcapone, nitecapone and the recently described BIA 3-202 and 3-335 are all structurally nitrocatechols, while CGP 28014 is a pyridine derivative (Figure 3) (Nissinen et al. 1988; Borglyua et al. 1989; Waldmeier et al. 1990; Parada et al. 2001;
Figure 3. Structure of first-generation COMT inhibitors (pyrogallol, tropolone, n-Butyl gallate, U-0521), second-generation inhibitors (entacapone, tolcapone, nitecapone, BIA 3-202, CGP 28014) and the uncoupling agent 2,4-dinitrophenol.
Bonifacio et al. 2002). The nitrocatechol-structured COMT inhibitors entacapone and tolcapone are known as tight-binding inhibitors, but the binding to COMT is reversible (Nissinen et al. 1992; Lotta et al. 1995). They are highly selective inhibitors with virtually no action on tyrosine hydroxylase, dopamine β-hydroxylase, DDC or MAO-A and MAO-B (Bäckström et al. 1989; Zürcher et al. 1990b).

Entacapone acts mainly peripherally, whereas tolcapone crosses the BBB (Zürcher et al. 1990a; Nissinen et al. 1992; Dingemanse 1997).

**Pharmacology in vitro and in vivo**

Entacapone selectively inhibits rat S-COMT from the duodenum, liver, brain and erythrocytes at low nanomolar concentrations *in vitro* (Nissinen et al. 1992). After an oral dose of 10 mg/kg entacapone S-COMT activity is greatly reduced in rat duodenum, liver and erythrocytes. The longest duration of inhibition is in the duodenum, but striatal COMT activity is suppressed only transiently (Nissinen et al. 1992). Tolcapone, by contrast, markedly inhibits both peripheral and central COMT (Zürcher et al. 1990b). In several human peripheral organs (liver, kidneys, duodenum, lungs), both entacapone and tolcapone have been shown to be potent COMT inhibitors *ex vivo* (De Santi et al. 1998). Hepatic COMT activity of human liver *ex vivo* was inhibited by entacapone at an IC$_{50}$ 151 nmol/l, while that for tolcapone was 773 nmol/l; the results for other organs were similar (De Santi et al. 1998).

Oral entacapone administered together with levodopa and carbidopa to rats or cynomolgus monkeys reduced the formation of 3-OMD and elevated serum levodopa levels (Cederbaum et al. 1991; Nissinen et al. 1992). The increases in levodopa, DA and DOPAC concentrations were dose-dependent, and the lowest effective dose in the rat was 3 mg/kg, which also reduced 3-OMD concentrations (Nissinen et al. 1992). However, even at a dose of 30 mg/kg, the HVA concentration in the rat was not decreased, indicating that central COMT activity is not inhibited by entacapone (Nissinen et al. 1992). Tolcapone, in contrast, induced about 90% decrease in the levels of HVA and 3-MT in the rat brain at the dose of 30 mg/kg p.o. (Zürcher et al. 1990b). After oral administration to rats, tolcapone 0.2 mmol (54.6 mg/kg) markedly reduced the peripheral metabolism of exogenous DOPA (100 mg/kg p.o.) to 3-OMD when given together with benserazide (50 mg/kg p.o.), thus increasing the bioavailability of DOPA in the brain (Zürcher et al. 1990b).

Tolcapone inhibits brain COMT activity in the rat at single doses of 3-10 mg/kg, while 30 mg/kg or more of entacapone is needed for temporary suppression of COMT activity (Zürcher et al. 1990a; Männistö et al. 1992a; 1992b). Extracellular DOPAC tends to increase and HVA to decrease in the rat striatum after a dose of 10 mg/kg tolcapone intraperitoneally, whereas the respective dose of entacapone has no effect (Kaakkola and Wurtman 1993). At an intraperitoneal dose of 30 mg/kg, neither entacapone nor tolcapone has any effect on extracellular levels of free catecholamines in the brain of anaesthetized rats, although tolcapone does reduce
COMT-dependent metabolites (Li et al. 1998). In rabbits, 30 mg/kg of tolcapone intravenously increases catecholamine baseline levels in plasma ca. 300% (Garrido et al. 1994).

**In vivo Parkinson animal models**

Proof of principle has been shown in animal models of PD for both entacapone and tolcapone. When neurological deficits resembling those seen in PD were induced in monkeys by administration of neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, co-administration of entacapone 12.5 mg/kg p.o. or tolcapone 15 or 30 mg/kg p.o. prolonged the antiparkinsonian activity of levodopa/carbidopa (Smith et al. 1997; Tasmor Product Monograph 1997). Unilateral destruction of dopaminergic neurons in the substantia nigra of rats by neurotoxin 6-hydroxydopamine led to contralateral circling behaviour when rats were treated with levodopa/carbidopa. Entacapone 3 or 10 mg/kg and tolcapone 30 mg/kg prolonged the circling response to levodopa/carbidopa in these rats (Da Prada et al. 1993; Törnwall and Männistö 1993).

Entacapone and tolcapone inhibit both S- and MB-COMT, but tolcapone inhibits MB-COMT 10 times more effectively than entacapone (Lotta et al. 1995). Tolcapone is very potent in inhibiting MB-COMT in rat liver and brain tissue in vivo after oral administration (Vieira-Coelho and Soares-da-Silva 1999). However, the expression of MB-COMT in tissues is far less than that of S-COMT, although the former possesses higher affinity for catecholamines than the S-COMT (Guldberg and Marsden 1975; Lotta et al. 1995).

Acute toxicity of both entacapone and tolcapone is low; oral LD$_{50}$ values in the rat are higher than 2 g/kg, and toxicity is not modified by the combined administration of levodopa and carbidopa (Borgulya et al. 1991; Törnwall and Männistö 1991; Kaakkola et al. 1994). The metabolic routes of tolcapone are more complicated than those of entacapone. The main metabolic pathway for entacapone and tolcapone is glucuronidation, but tolcapone is also metabolized through methylation, reduction and hydroxylation (Wikberg et al. 1993; Dingemanse et al. 1995).

**Clinical use**

The symptoms of PD are alleviated by administration of levodopa. It improves the disability of patients with PD more than any other drug. Since levodopa is actively metabolized, it is given concomitantly with DDC inhibitors, which inhibit the conversion of levodopa to DA, thus increasing its bioavailability. However, most of the exogenous levodopa is methylated to 3-OMD by COMT. The administration of entacapone or tolcapone together with levodopa and a DDC inhibitor increases plasma concentrations of levodopa dose-dependently without significantly affecting the peak plasma concentration of levodopa (Nutt et al. 1994; Fahn 1998; Terasvainen et al. 2001). The concentrations of DOPAC and HVA, the main metabolites of DA, are increased after treatment with entacapone, while after tolcapone DOPAC is increased and HVA decreased. Although the central actions of entacapone and tolcapone differ, systemic administration leads to an increased level of DA in the brain. Both inhibitors are eliminated rapidly after an oral dose. The
plasma elimination half-life after entacapone is about 1-2 hours, whereas the half-life of tolcapone is approximately 2-3 hours. The bioavailability of entacapone (35%) is less than that of tolcapone (65%). Entacapone is 98% and tolcapone 99.9% bound to plasma proteins. Since entacapone is metabolized more rapidly than tolcapone, it is administered in clinical use up to 10 times a day with each dose of levodopa, while tolcapone is administered three times a day. Both inhibitors provide benefits to parkinsonian patients in concomitant dosing with levodopa (Keränen et al. 1994; Dingemanse 1997; Männistö and Kaakkola 1999). One to two months’ treatment with entacapone or tolcapone at a clinical dose of 200 mg (entacapone with each levodopa dose, tolcapone three times daily) inhibits the formation of 3-OMD by 50% and 80%, respectively (Kaakkola 2000). Both entacapone and tolcapone significantly increase the ON time and decrease the OFF time in patients with advanced PD (Kaakkola 2000). The increase in daily ON time varies from 1 to 2 hours with entacapone and from 0 to 2.5 hours with tolcapone (Kaakkola 2000).

The plasma levels of DA, NA, A and total catecholamines were reported to increase in seven out of eight PD patients at a mean tolcapone dose of ca. 270 mg/day (Rojo et al. 2001). One patient simultaneously had elevated liver enzymes and a 513% increase in catecholamine levels. Entacapone has not been reported to change plasma catecholamine levels in healthy volunteers (Illi et al. 1994; Scheinin et al. 1998).

5.4. Drug induced hepatotoxicity

Although most hepatotoxic compounds are detected during non-clinical testing and are not developed further, hepatotoxicity remains the leading cause of withdrawal of drugs from the market (Lasser et al. 2002; Thomas 2002). This is because drug-induced hepatotoxicity is generally only seen after a drug has been marketed and used by tens of thousands of patients, as opposed to phase II clinical trials with limited numbers of participants (Batt and Ferari 1995; Lasser et al. 2002).

5.4.1. Mechanisms, clinical symptoms and signs of hepatotoxicity

Drug-induced hepatotoxicity is typically observed as a hepatocellular or cholestatic injury (Watkins 2000). Cholestatic injury is reflected as a selective interference of the drug with the liver’s ability to make and secrete bile (Watkins 2000). In humans, it is characterized by an elevation in serum alkaline phosphatase (APHOS) and bilirubin. In liver tests, cholestatic injury is suspected when the value of APHOS is two times the upper limit of normal (ULN) or the ratio of alanine transaminase (ALAT) to APHOS is less than two (Benichou 1990; Kaplowitz 2001). However, in the rat, APHOS is predominantly of intestinal origin, and thus, it cannot be used as a biomarker of cholestatic injury as such but should be used together with γ-glutamyl transferase and bilirubin (Loeb and Quimby 1999). In hepatocellular injury, liver cells are damaged, and the toxic signs manifest as a rise in clinical serum chemistry parameters (Benichou 1990; Kaplowitz 2001). In man, alanine aminotransferase (ALAT),
as a relatively liver-specific enzyme, is the most useful marker of hepatocellular injury in patients. In animals, especially in mice, rats and dogs, hepatic ALAT activity is 3-10 times higher than in any other tissue (Loeb and Quimby 1999). ALAT is localized both the cytoplasm and mitochondria of cells, but the activity is several times higher in the former. It is released to serum when liver cells are dying, thus elevating the normal levels of serum ALAT. In patients, a serum ALAT level two- or threefold the ULN or an ALAT/APHOS ratio of five or more is considered clinically significant and a sign of hepatocellular liver injury (Benichou 1990; Dossing and Sonne 1993; Watkins 2000; Kaplowitz 2001). In non-clinical toxicity studies, ALAT is used as a marker of hepatocellular injury, with activity values of treated and untreated control animals being compared. In addition to ALAT activity, increased sorbitol dehydrogenase (SDH) activity appears early in the course of liver injury in animals. SDH is a cytosolic enzyme with the highest activity in the liver and kidneys. A positive correlation between increased ALAT and SDH activities and hepatocellular changes in the rat has been reported (Travlos et al. 1996). ALAT activity is cleared in about four days, and SDH activity returns to normal even faster (Mizrahi et al. 1987; Loeb and Quimby 1999). Although both these enzymes are located periportally in the liver, the main target for the injury is the centrilobular area due to diminished oxygen during the passage through periportal areas to the centrilobular area (Haschek et al. 2002). A few reports are available on histological hepatic necrosis in connection with the elevation of serum ALAT in rats and dogs (Balazs et al. 1961; Loeb and Quimby 1999). Balazs et al. (1961) have reported a distinct correlation between ALAT activity and the degree of necrosis, although in other studies the correlation is less clear (VanVleet and Alberts 1968). It has been postulated that due to changed permeability of the cell membranes ALAT and SDH can leak into serum before cell death and that histological necrosis is seen variably (Loeb and Quimby 1999).

The lack of specific clinical or histological features of drug-induced liver lesions is one of the reasons behind the sporadic incidence of liver injury since injuries produced by drugs may be indistinguishable from those due to other reasons (Dossing and Sonne 1993; Watkins 2000). Some drugs cause liver toxicity in a dose-related manner with a reaction arising only after a sufficient amount of the drug is taken. Dose-independent reactions occur as a rare complication of therapeutic doses of the drug (Dossing and Sonne 1993).

Hepatocellular necrosis can occur within minutes after a toxic insult and is usually associated with severe metabolic disturbances. Cell necrosis is a typical consequence of acute hepatic injury. The onset of cell death is characterized by breakdown of the plasma membrane permeability barrier, which leads to loss of metabolic intermediates, leakage of enzymes and collapse of all electrical and ionic gradients across the plasma membrane (Mehendale et al. 1994). Fountoulakis et al. (2002) reported a threefold elevation of ALAT six hours after carbon tetrachloride dosing of rats, and at 24 hours, the increase in ALAT values was extremely pronounced.

Typical centrilobular hepatocellular

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**Review of the literature**
necrosis induced by carbon tetrachloride is reported to be associated with catecholamine release (Schwetz and Plaa 1969; Roberts et al. 1997). A and NA can potentiate the sensitivity of the liver to carbon tetrachloride, but administered alone they fail to induce any toxicity. The toxicity is characterized by increased serum aminotransferase activities and hepatocellular necrosis (Schwetz and Plaa 1969). Endogenous A and NA are also capable of potentiating the toxicity induced by carbon tetrachloride, as well as several other adrenergic drugs, in the rat (Roberts et al. 1997). The mechanism behind the adrenergic agents’ increased toxicity is suggested to be related to induced hypoxia within the liver cells (Zempel et al. 1983).

5.4.2. Hepatotoxicity induced by COMT inhibitors

The first-generation COMT inhibitors have been studied in relation to hepatotoxic properties. Tropolone has been reported to induce non-specific hepatitis in mice in vivo after a single dose of 400 mg/kg; this is also the oral LD₅₀ for mice (Table 1) (Ri 1951; Nakagawa and Tayama 1998). In isolated rat hepatocytes, tropolones induced a dose- and time-dependent loss of cell viability, which is associated with a decrease of intracellular adenosine triphosphate (ATP) (Ri 1951; Nakagawa and Tayama 1998). Tropolone is also reported to suppress the growth of murine hepatocytes, with the IC₅₀ being ca. 1.23 µg/ml (Inamori et al. 1993).

Gallates induced a concentration-dependent cell death in rat hepatocytes in vitro, which was preceded by the depletion of ATP (Table 1) (Nakagawa and Tayama 1995). Since propyl gallate also induced an increase in state 4 oxygen consumption in isolated rat liver mitochondria, the cytotoxicity is linked to uncoupling of oxidative phosphorylation (Nakagawa and Tayama 1995).

The non-clinical toxicity studies performed for the marketing applications of second-generation COMT inhibitors entacapone and tolcapone revealed no signs of hepatotoxicity (Eckhardt et al. 1996; Schläppi et al. 1996a; 1996b; 1996c; Tasmar Product Monograph 1997; Entacapone Product Monograph 1999). In non-clinical studies with tolcapone in mice, only slight to moderate hepatocellular hypertrophy was observed after 18 months’ treatment (dose not given) (Olanow 2000).

In vivo, COMT inhibition alone has not been reported to induce hepatotoxicity. COMT knock-out mice, developed by means of homologous recombination in embryonic stem cells leading to a mouse strain in which the gene encoding the COMT enzyme is disrupted, have shown no signs of liver injury (Gogos et al. 1998; Huotari et al. 2002). The mice have been viable and fertile, with only minor changes in catecholamine metabolism (Huotari et al. 2002).

The first-generation COMT inhibitors were not taken into clinical use because of properties connected to short duration of action, toxicity or inefficiency in clinical studies (Ericsson 1971; Reches and Fahn 1984). Hepatotoxic signs were observed in clinical use up to 12 weeks with N-butyl gallate, when markedly abnormal ALAT activities (values not given) were reported in four out of 11 patients receiving dosages above 2250 mg daily (Simpson and Varga 1972).
The use of entacapone has been reported to be associated with hepatotoxicity in one patient after three weeks’ treatment (Fisher et al. 2002). Two other cases were earlier reported to the Australian Adverse Drug Reaction Advisory Committee (ADRAIC). However, in two of these three cases, the patients had concomitant medications with hepatotoxic potential, and the third case had a history of long-standing alcohol abuse and alcohol-induced liver cirrhosis (Beck et al. 2002). In none of these cases was hepatotoxicity hepatocellular, instead being cholestatic injury in terms of ALAT activity or the ALAT/APHOS ratio (Watkins 2000; Kaplowitz 2001).

In clinical use of tolcapone, severe hepatotoxicity has been observed in several PD patients (Table 1). A total of nine cases of abnormal hepatic reactions have been reported, three with a fatal outcome (Assal et al. 1998; Bonifati and Meco 1999; Colossimo 1999; Olanow 2000). In November 1998, marketing authorization for tolcapone was suspended in the European Union because of increasing concern about reports of severe hepatotoxicity (European Medicines Evaluation Agency 1998). Findings of severe centrilobular hepatic necrosis with inflammatory infiltrates (plasma cells, eosinophils) due to tolcapone treatment were reported in three parkinsonian patients (Olanow 2000). In one patient who died after tolcapone treatment, fulminant hepatitis was verified histologically (McCaul et al. 1986; Spahr et al. 2000). In all three tolcapone-treated patients, ALAT was increased to at least three times the ULN (Olanow 2000). The incidence of hepatic failure was 10-100 times higher than in the general population (Mayoral et al. 1999). In clinical trials, 3.5% of tolcapone-treated patients had elevated liver enzymes three times higher than the ULN compared with only 1% of placebo-treated patients.

Table 1. Hepatotoxic properties of COMT inhibitors.

<table>
<thead>
<tr>
<th>COMT inhibitor</th>
<th>Toxicity to hepatocytes</th>
<th>In clinical use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vitro</td>
<td>In vivo</td>
<td>Serum aminases</td>
</tr>
<tr>
<td>Tropolone</td>
<td>yes</td>
<td>hepatitis</td>
<td>not in clinical use</td>
</tr>
<tr>
<td>Gallates</td>
<td>yes</td>
<td>no data</td>
<td>↑</td>
</tr>
<tr>
<td>U-0521</td>
<td>no data</td>
<td>no data</td>
<td>NAD</td>
</tr>
<tr>
<td>Entacapone</td>
<td>no data</td>
<td>NAD</td>
<td>NAD</td>
</tr>
<tr>
<td>Tolcapone</td>
<td>no data</td>
<td>necrosis</td>
<td>↑</td>
</tr>
</tbody>
</table>

NAD = no abnormalities detected
In addition, a clear dose-response effect was evident.

5.5. Uncoupling as a phenomenon

In healthy cells, ATP-driven Ca\(^{2+}\) pumps maintain a Ca\(^{2+}\) gradient across the plasma membrane. Since calcium precipitates are prominent in necrotic tissue, an increase in free Ca\(^{2+}\) may be one of the first responses to cell injury. However, in at least some models of drug-induced liver injury caused by hypoxia, Ca\(^{2+}\) does not rise before the toxic lesion (Harman et al. 1992; Mehendale et al. 1994). Uncouplers of oxidative phosphorylation, such as Br-A23187, can cause bioenergetic cell death rather than Ca\(^{2+}\)-mediated cell killing (Mehendale et al. 1994). Thus, cell killing in models of hypoxic injury to hepatocytes is not dependent on Ca\(^{2+}\), but mitochondria are important targets of hepatocellular injury by toxicants that causes oxidative stress, leading to mitochondrial depolarization, uncoupling of oxidative phosphorylation, ATP depletion and cell death (Mehendale et al. 1994).

5.5.1. Uncoupling at cellular level

Oxidative phosphorylation is the major mechanism by which aerobic cells produce ATP using a respiratory assembly located in the inner mitochondrial membrane (Berg et al. 2002). In oxidative phosphorylation, ATP synthesis is coupled to the flow of electrons towards oxygen by a proton gradient across the inner mitochondrial membrane (Figure 4). Electron flow results in pumping of protons out of the mitochondrial matrix and generation of membrane potential. The membrane potential increases as the electrons flow down the respiratory chain to oxygen. The mitochondrial membrane potential across the inner membrane is the driving force for phosphorylation since ATP is synthesized when protons flow back to the matrix (Fromenty et al. 1990; Brown 1992). The

![Figure 4.](image)

*Figure 4. In mitochondria, oxidation and ATP synthesis are coupled by transmembrane proton fluxes (A). When an uncoupling agent is present (B), a protonated form of e.g. DNP transports protons across the inner mitochondrial membrane, neutralizing the proton gradient.*
tight coupling of electron transport and phosphorylation in mitochondria can be disrupted by uncoupling agents, which act as proton carriers that allow protons to flow into the mitochondrial matrix without passing through the transmembrane protein complex that synthesizes ATP from adenosine diphosphate (ADP) (Terada 1990; Krähenbühl 2001). In the presence of an uncoupling agent, electron transport proceeds in the normal fashion, but ATP is not formed since the proton-motive force across the inner membrane is dissipated. The rate of oxygen consumption in the absence of ADP in respiratory substrate-supplemented mitochondria is low. With the addition of an uncoupler, the respiratory rate increases suddenly in a dose-dependent manner, and at the same time, the mitochondrial membrane potential decreases (Terada 1990). The loss of respiratory control leads to increased oxygen consumption and to decreased ATP production as a compensatory function of the cell. The energy is liberated as heat, which results in a rise in body temperature (Tainter and Wood 1934; Kaiser 1964; Terada 1990; Brown 1992).

The five different metabolic states of mitochondria have been determined experimentally by Chance and Williams (1955) (Table 2) to clarify steady-state levels during passage of electrons from the substrate to oxygen. In state 1, mitochondria display a slow respiration rate (Chance and Williams 1955; Hackenbrock 1966). State 2 is characterized by high concentrations of ADP and respiration is close to zero. State 3 is the active state of respiration and phosphorylation (Chance and Williams 1955). In aerobic state 4, ADP is totally phosphorylated and the respiration rate is again low. In state 5, oxygen is lacking and the ADP level is high. The adding of uncoupling agents converts this state to state 2 (Chance and Williams 1956). In all states, mitochondria show the typical morphological features determined by Hackenbrock (1966). The level of respiration in vitro is expressed as a respiratory

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Table 2. Metabolic states of mitochondria according to Chance and Williams (1955).

<table>
<thead>
<tr>
<th>State</th>
<th>ADP level</th>
<th>Respiration rate</th>
<th>Rate-limiting substance</th>
<th>Ultrastructural findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>low</td>
<td>slow</td>
<td>ADP</td>
<td>condensed formation, intramitochondrial granules</td>
</tr>
<tr>
<td>2</td>
<td>high</td>
<td>slow</td>
<td>substrate</td>
<td>highly condensed matrix</td>
</tr>
<tr>
<td>3</td>
<td>high</td>
<td>fast</td>
<td>respiratory chain</td>
<td>matrix ↓, intracristal space ↑, small granules in matrix, inner membrane foldings</td>
</tr>
<tr>
<td>4</td>
<td>low</td>
<td>slow</td>
<td>ADP</td>
<td>condensed formation, intramitochondrial granules</td>
</tr>
<tr>
<td>5</td>
<td>high</td>
<td>0</td>
<td>oxygen</td>
<td></td>
</tr>
</tbody>
</table>

---

Review of the literature
control ratio (RCR), which is the relationship between the respiration rate in state 3 and the respiration rate in state 4. If the respiration rate in state 4 increases, the mitochondrial membrane potential decreases, no ATP is formed and electron transport and phosphorylation is uncoupled (Berg et al. 2002).

Various compounds are known to be uncouplers, but weakly acidic uncoupling agents are the most potent uncouplers, speculated to produce uncoupling by their protonophoric action on the mitochondrial membrane. Typical examples of uncoupling agents include salicylic acids, non-steroidal inflammatory drugs and some local anaesthetics (Krähenbuhl 2001). As a weak acid, the uncoupling effect of DNP is attributable to its protonophoric nature (Terada 1990). Entacapone and tolcapone are both structurally related to the known uncoupling agent DNP. Nissinen et al. (1997) have shown that tolcapone interacts with mitochondrial oxygen consumption, being a more potent uncoupler than DNP in vitro.

5.5.2. Signs of uncoupling in vivo
Uncoupling agents inducing disruption between electron transport and phosphorylation are generally weakly acidic, lipid-soluble substances, which enables them to carry protons across the inner mitochondrial membrane (Terada 1990). DNP, a known uncoupling agent of oxidative phosphorylation, was used in the 1930s for weight reduction in humans, until it was found to be a toxic compound (Figure 3). Horner et al. (1942) reported that more than 4500 patients used “one capsule three times a day after meals”. Among these patients, gastro-intestinal symptoms, such as nausea, vomiting and loss of appetite, were common. With long-term use, cutaneous lesions were frequent and were complicated by polyneuritis, otitis media and cataracts. As several patients developed cataracts and some died due to overdose, the treatment of obesity with DNP was discontinued in the 1940s (Horner 1942; Simon 1953). Most fatal cases have, however, been among workers in the agricultural industry since DNP was used as a pesticide and herbicide (Bidstrup and Payne 1951).

The symptoms of intoxication in man consist of body temperature exceeding 40ºC, extreme fatigue, profuse sweating and dehydration, laboured respiration and rapid onset of rigor mortis after death (Figure 5) (Perkins 1919; Cutting and Tainter 1933; Poole and Haining 1934; Horner 1942). Fatal doses of DNP in the treatment of obesity have been between 2.66 mg/kg/day for 14 days and 46 mg twice 7 days apart (Masserman and Goldsmith 1934; Poole and Haining 1934). In rats and dogs, oral treatment with DNP has induced increased heart rate and abnormal electrocardiographic readings, elevation of body temperature, increased oxygen consumption and increased metabolic rate (Tainter and Cutting 1933b; Kaiser 1964; Bakke and Laurence 1965). The cause of death in acute animal studies is generally considered to be a result of the pyretic effect of DNP, produced by an increase in metabolic rate. Spencer et al. (1948) indicated that rats treated once by gavage either died within 1-2 hours or recovered completely. Since uncoupling of oxidative phosphorylation induces a reduction in mitochondrial energy production, leading to usage of glycogen in
the liver and muscles, instantly occurring rigor mortis expresses very rapid consumption of ATP and glycogen from the skeletal muscle after death (Gracey 1981). In acute DNP poisoning, the glycogen content in muscles and liver is diminished (Stoner et al. 1952). Rigor mortis has been reported to either occur within 10 minutes of death due to DNP treatment (Figure 5) (Tainter and Wood 1934) or already be complete at the time of death (Barnes 1969; Parker 1973).

5.5.3. Uncoupling induced by COMT inhibitors
Several of the first-generation COMT inhibitors have been shown to possess uncoupling properties in vitro. None of them has been taken into long-term clinical use. The second-generation COMT inhibitors have also been reported to express uncoupling properties in vitro, but in clinical use, they have not induced any symptoms related to uncoupling in parkinsonian patients.

Pyrogallol, which structurally resembles DNP, is reported to be a 4-5 times weaker uncoupling agent than DNP in vitro (Ross and Haljasmaa 1964a; Conyers et al. 1968). It uncouples oxidative phosphorylation in vitro in isolated mitochondria of both rat kidney and beef heart (Table 3) (Conyers et al. 1968). Complete uncoupling was achieved at a concentration of 1 µM expressed as the ratio of phosphate esterified to oxygen consumed. In the same experiment, complete uncoupling by DNP was achieved at a concentration of 0.2 µM (Conyers et al. 1968).

Tropolone and its derivatives reduced intracellular ATP levels in isolated rat hepatocytes, and this reduction is associated with the loss of cell viability (Nakagawa and Tayama 1998). In isolated rat liver mitochondria, tropolones also caused a concentration-dependent increase in the rate

Figure 5. Biological effects of uncoupling of oxidative phosphorylation in mitochondria. RCR, respiratory control ratio.
of state 4 oxygen consumption, thus having uncoupling properties of oxidative phosphorylation (Table 3) (Nakagawa and Tayama 1998). This indicates that the cytotoxicity induced by tropolones is associated with ATP depletion via mitochondrial dysfunction related to oxidative phosphorylation (Nakagawa and Tayama 1998).

Propyl gallate and related gallates have been reported to express uncoupling effects on mitochondria isolated from rat liver (Table 3) (Nakagawa and Tayama 1995). As a sign of uncoupling of oxidative phosphorylation, propyl gallate induced an increase in the state 4 oxygen consumption in rat liver mitochondria as well as a depletion of ATP in rat hepatocytes (Nakagawa and Tayama 1995).

Tolcapone has proven to be a potent uncoupler of oxidative phosphorylation \textit{in vitro} at low micromolar concentrations (2.6 µM), while entacapone is a weak uncoupler at high concentrations only (Table 3) (Nissinen et al. 1997). Borroni et al. (2001) reported uncoupling properties of tolcapone at a similar concentration range (3 µM), and tolcapone also shows cytotoxic properties at the same concentration in rat hepatocytes.

5.5.4. COMT inhibition and uncoupling in relation to protein binding

Entacapone is 98% and tolcapone 99.9% bound to plasma proteins (Keränen et al. 1994; Dingemanse et al. 1995). The strong protein binding is postulated to diminish the pharmacologically active concentration, thus also affecting the uncoupling properties of the drug (Borroni et al. 2001). In practice, the plasma concentration needed for clinical response to most of the drugs is less than the saturated concentration, and therefore, an equilibrium exists between the

<table>
<thead>
<tr>
<th>COMT inhibitor</th>
<th>Uncoupling effects</th>
<th>Liver toxicity</th>
<th>Central effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entacapone</td>
<td>( \text{EC}_{50} ) 58.0 µM</td>
<td>no</td>
<td>no</td>
<td>Nissinen et al., 1992, 1997; Vaalavirta et al., 1998</td>
</tr>
<tr>
<td>Tropolone</td>
<td>intracellular ATP ↓, state 4 respiration ↑</td>
<td>yes</td>
<td>( \leq 0 )</td>
<td>Ri 1951; Nakagawa and Tayama 1995, 1998</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>1 mM (complete)</td>
<td>no data</td>
<td>yes</td>
<td>Wylie et al. 1960; Ross and Haljasmaa 1964a; Conyers et al. 1968</td>
</tr>
<tr>
<td>Gallates</td>
<td>intracellular ATP ↓, state 4 respiration ↑</td>
<td>yes</td>
<td>yes</td>
<td>Wylie et al. 1960; Ross and Haljasmaa 1964a; Simpson and Varga 1972; Nakagawa and Tayama 1995</td>
</tr>
<tr>
<td>Tolcapone</td>
<td>( \text{EC}_{50} ) 2.6 µM</td>
<td>yes</td>
<td>yes</td>
<td>Nissinen et al. 1997; Vaalavirta et al. 1998; Männistö and Kaakkola, 1999</td>
</tr>
</tbody>
</table>

* \( \text{EC}_{50} \) is the concentration for half of the maximal stimulation of succinate-supported mitochondrial respiration.
free and bound drug in plasma. The protein-bound fraction is stored in the albumin and the free fraction penetrates such tissues as the liver, where the drug accumulates if it is not efficiently metabolized by, e.g. glucuronidation or sulphation. Despite extensive protein binding, both inhibitors have proved to be potent COMT inhibitors in clinical use (Kaakkola 2000). The concentration that produces COMT inhibition is about equal for both inhibitors (Table 4), but tolcapone expresses uncoupling properties at a much lower concentration than entacapone (Nissinen et al. 1997; Borroni et al. 1999; 2001).

5.6. Pathological findings in tissues in connection with uncoupling of oxidative phosphorylation

5.6.1. Histological findings
Histological findings due to uncoupling of oxidative phosphorylation in the tissues are rare. Since the uncoupling of oxidative phosphorylation is behind the toxic mechanism of DNP, it has been extensively used as an experimental example of the lesions induced by uncoupling. Most of the experimental studies have been performed in isolated hepatocytes in vitro or on rat skeletal muscle in vivo (Hackenbrock 1966;Buffa et al. 1970; Melmed et al. 1975; Sahgal et al. 1979; Shah et al. 1982; 1985; Kawahara et al. 1991; Nakagawa and Tayama 1995). Only in a few in vivo studies with DNP have possible histological lesions been sought; liver tissue in particular has been studied. After an oral single-dose treatment of DNP to rats, lesions in the kidney, liver and spleen have been observed (Spencer et al. 1948; Arnold et al. 1976). In the kidneys, tubular necrosis or degeneration is typically present, in the spleen congestion and haemosiderosis, and in the liver cloudy swelling and slight congestion. However, the liver of dogs treated with single doses of DNP revealed no histological findings (doses not given) (Tainter and Cutting 1933b). In skeletal muscle, the only light microscopy findings, reported in experimental rat studies with DNP, consist of subsarcolemmal accumulations of mitochondria seen as ragged red fibres after modified Gomori trichrome staining (Melmed et al. 1975; Sahgal et al. 1979). The red fibres were confined to oxidative type I muscle fibres. Large subsarcolemmal aggregates were verified to be mitochondria in transmission electron microscopy (TEM) studies (Sahgal et al. 1979).

In humans, most of the histological lesions concentrate in the heart and lungs, and a few liver findings have been reported due to DNP poisoning. Poole and Haining (1934) found degeneration of liver cells and pyknotic nuclei after five days of DNP treatment at 7 mg/kg. Necrosis of hepatocells...
cytes was also identified in a woman who received DNP for one week (dose not given) (Lattimore 1934), and “an acute degenerative hepatitis” with early necrosis and fatty change was reported in a case of a man dying in a chemical plant where DNP was being manufactured (Warthin 1918). However, the latest histological reports on humans date back to the 1940s, and in most cases, no autopsy or histological evaluation was performed. The liver does not appear to be a sensitive organ for DNP toxicity in humans or animals exposed orally, and no consistent light microscopy liver findings have been associated with uncoupling of oxidative phosphorylation induced by DNP (Research Triangle Institute 1995).

5.6.2. Electron microscopy findings
The ultrastructure of mitochondria in different experimental metabolic states was determined in mouse liver by Hackenbrock in 1966 (Table 5). In state 1, at a slow respiration rate, the ultrastructure is characterized by condensed formation with large intramitochondrial granules (Chance and Williams 1955; Hackenbrock 1966). The mitochondria in this state are indistinguishable from those in state 4. In state 2, the mitochondria show highly condensed formation. The volume of their outer compartment seems to have more volume than the inner compartment (Hackenbrock 1966). When oxidative phosphorylation is uncoupled, the mitochondria show a decrease in density and an increase in the number of cristae, which may be responsible for the increase in rate of respiration (Oldham et al. 1967).

Table 5. Histological findings in liver and skeletal muscle and TEM findings in mitochondria after treatment with DNP as an uncoupling agent.

<table>
<thead>
<tr>
<th>Liver findings</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td>Lattimore 1934; Poole and Haining 1934; Spencer et al. 1948</td>
</tr>
<tr>
<td>necrosis of hepatocytes</td>
<td></td>
</tr>
<tr>
<td>cloudy swelling, congestion</td>
<td></td>
</tr>
<tr>
<td>degeneration of liver cells</td>
<td></td>
</tr>
<tr>
<td>TEM, isolated mitochondria in vitro</td>
<td>Buffa et al. 1970</td>
</tr>
<tr>
<td>contraction of the inner membrane; reduction of the total volume</td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle findings</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td>Melmed et al. 1975</td>
</tr>
<tr>
<td>ragged red fibres</td>
<td></td>
</tr>
<tr>
<td>accumulations of mitochondria; swelling; abnormal cristae; rough endoplasmic reticulum disappeared</td>
<td>Kawahara et al. 1991</td>
</tr>
<tr>
<td>accumulations of mitochondria; inclusions; concentric laminar bodies; vacuoles, dilated sarcotubules</td>
<td>Sahgal et al. 1979</td>
</tr>
<tr>
<td>increase in number; swelling; inclusions; concentric laminar bodies; unfolded cristae; vacuoles, dilated sarcotubules</td>
<td>Shah et al. 1982, 1985</td>
</tr>
<tr>
<td>unfolded cristae</td>
<td>Melmed et al. 1975</td>
</tr>
</tbody>
</table>
phosphorylation is activated (state 3), the volume of mitochondrial matrix decreases, simultaneously with an increase in the volumes of the outer compartment and intracristal space. The matrix is associated with small granules and the inner membrane forms irregular foldings (Hackenbrock 1966). When uncoupling agents are added, the morphology shows some similarities with that of state 2 (Chance and Williams 1956). Although histological findings may be lacking, typical mitochondrial changes in TEM studies of isolated rat liver mitochondria or skeletal muscle cells have been observed after the treatment with DNP (Table 5). Buffa et al. (1970) reported contracted conformation of the inner mitochondrial sac formed by the inner membrane and matrix in isolated rat liver mitochondria. Mitochondrial swelling with reduced density in the matrix and loss of cristae have also been observed (Melmed et al. 1975; Sahgal et al. 1979; Shah et al. 1985; Kawahara et al. 1991). In several cases, the number of mitochondria was increased, especially in skeletal muscle under the sarcolemma, whereas in the perinuclear or intermyofibrillar region the number of mitochondria was unaffected (Melmed et al. 1975; Sahgal et al. 1979; DiMauro et al. 1985; Shah et al. 1985; Kawahara et al. 1991). Large aggregates of mitochondria were also observed in light microscopy (Melmed et al. 1975; Sahgal et al. 1979).

Isolated rat liver mitochondria after incubation with pentachlorophenol, another uncoupling agent, showed an increased inner mitochondrial space and a matrix containing constricted or indistinct cristae (Weinbach et al. 1967). Mitochondria with a ruptured outer membrane were also seen. However, when rats were treated intraperitoneally with 60 mg/kg of pentachlorophenol, the mitochondria in liver sections did not differ from those of control animals (Weinbach et al. 1967).
Entacapone and tolcapone, two nitrocatechol-structured COMT inhibitors, have been used as adjuncts to levodopa in the treatment of Parkinson’s disease for several years. Tolcapone has been shown to induce liver toxicity in clinical use, and three fatal cases have been reported. Since these two COMT inhibitors are structurally related to each other, exploring differences in their toxicity profiles was warranted. Furthermore, as some data existed on tolcapone being an uncoupler of oxidative phosphorylation, a possible connection between hepatotoxicity and uncoupling by COMT inhibition was sought.

These studies were performed to examine:
1. Comparative toxicity profiles of entacapone and tolcapone in vivo (I, II).
2. Role of uncoupling of oxidative phosphorylation in these toxicity profiles in vivo and in vitro (II, III, IV).
3. Effects of total COMT inhibition in mice in vivo (V).

6. Aims
7. Materials and methods

7.1. Animals and treatment

In comparative toxicity studies *in vivo*, (I, II, III), outbred Crl:CD<sup>®</sup>BR rats (Sprague-Dawley origin, supplied by Charles River Wiga, Sulzfeld, Germany) were used. The dosing for these studies was conducted in a semi-barrired, limited-access animal room. The test suspensions were administered orally for eight days (I) or two weeks (I, II, III) to each rat at 24-hour intervals by gavage using a flexible plastic catheter and a syringe. In the first study, the doses of entacapone and tolcapone were 200 and 400 mg/kg/day, and in the second study, 300 and 500 mg/kg/day, with DNP 20 mg/kg/day serving as a positive reference substance. Entacapone doses were based on previous toxicological studies (Entacapone Product Monograph 1999). Tolcapone doses were selected according to non-clinical and clinical exposure data (Tasmar Product Monograph 1997). The dose of DNP was selected to be the highest dose not inducing mortality (Spencer et al. 1948). The control animals received the vehicle 0.5% methyl cellulose. The commercially available rodent SDS diet RM1 (E) SQC (Special Diet Services Ltd, Witham, Essex, England) and tap water from the public supply were available ad libitum. Animal care was performed according to the regulations of the Council of Europe (1990) and the National Research Council, USA (1996). The study protocols were approved by the Provincial State Office of Southern Finland or by the Animal Ethics Committee of Orion Corporation, Finland.

7.2. Test substances

Entacapone and tolcapone were synthesized at Orion Pharma, Espoo, Finland (Bäckström et al. 1989; Borgylua et al. 1989). The drug substances were identified by comparing the substances to standards using infra-red spectra. The impurities in both entacapone and tolcapone, determined by high-performance liquid chromatography (HPLC), were under 0.1%. In comparative toxicity studies, DNP served as a positive reference compound. All of these test substances were suspended in autoclaved 0.5% methyl cellulose, and the dosing suspensions were prepared daily immediately before the oral dosing.
7.3. Methods in in vivo studies

7.3.1. Clinical signs, mortality, body weight and organ weights (I, II, V)

The animals were inspected at least once a day to monitor clinical signs or reactions to the treatment and mortality. Individual body weight and body weight gain as well as body-related organ weights (II, liver; V, liver and kidneys) were recorded.

7.3.2. Behavioural studies (V)

The activity of all three genotypes was determined using the ten-channel IRS Actometer System (custom-designed by L. Yaviz and E. Koivisto at the University of Kuopio) for determining the effects of COMT deficiency on the circadian rhythm of locomotor activity and on diurnal rhythm. The measurements were taken at 30-minute consecutive intervals for a total of 72 hours. An elevated plus-maze test was performed in mice as described by Handley and Mithani (1994) with some modifications (Vasar et al. 1993). During a three-minute observation session the following measurements were taken: latency period of first open part entry, number of attempts to enter the central square, number of line crossings in open arms and in the central square, total time spent in the open part and open arms, and number of visits to open and closed arms of plus-maze. Immediately after the elevated plus-maze test, general motor activity of each mouse was tested for two minutes in a dimly illuminated round box by counting the number of line crossings and rearings. A forced swimming test was performed as described by Porsolt et al. (1977), and active swimming and floating times were measured.

7.3.3. Body temperature measurements (I, II, IV)

Rectal body temperature was measured twice prior to the dosing period to adapt the animals to this measurement. During the study the rectal temperature was measured on several days, once in the morning immediately before dosing and one or two hours after the dosing of test substances or vehicle. A digital thermometer with a plastic-covered stainless probe was used.

7.3.4. Haematology and clinical chemistry (I, II, V)

Blood samples were taken from fasted animals on the day of terminal necropsy for haematological (I, V) or clinical chemistry (I, II, V) analyses. The haematological analyses were performed using a Sysmex F-800 Microcellcounter (Toa Medical Electronics Co. Ltd., Kobe, Japan). In clinical chemistry determinations, the main target was the effect of the treatment on liver function. The analyses were carried out using a BM/Hitachi 911 E automatic analyzer (Boehringer Mannheim, Mannheim, Germany).

7.3.5. Determination of catecholamines from plasma (V)

Blood samples were collected into tubes with ethylenediaminetetraacetic acid as an anticoagulant. The concentrations of NA, A and DHPG in plasma were determined at the Clinical Research Services, Turku University, Turku, Finland, using high-performance liquid chromatography with colorimetric electrochemical detection (HPLC-EC), as
Materials and methods

described previously (Scheinin et al. 1991).

7.3.6. Determination of MAO-A and MAO-B in liver homogenates (V)
The enzyme activity of MAO-A and MAO-B was determined from mouse liver homogenates (V) using an enzymatic radiochemical method. The MAO-A assay was performed according to Young et al. (1986), using \(^{14}\)C-hydroxytryptamine creatinine sulphate as the substrate. MAO-B activity was determined using \(^{14}\)C-phenylethylamine hydrochloride as the substrate, with some modifications to the two methods described by Keller & al (1987) and Koulu & al (1989). The results were expressed as pmol of deaminated metabolite, 5-hydroxyindoleacetic acid (for MAO-A) or phenylacetic acid (for MAO-B), formed per min mg protein. In both determinations, the protein content was measured according to Peterson (1977), with bovine serum as the standard.

7.3.7. Analysis of drug concentrations in plasma and liver (I, II)
Exposure of the animals to entacapone and tolcapone was monitored by determining the plasma and liver concentrations of entacapone and tolcapone during the dosing period.

For plasma concentration analysis (I, II), samples were drawn at several time points after the dosing. Blood was collected into lithium-heparinized tubes and kept in ice until centrifuged. The plasma was separated and the concentrations of entacapone and tolcapone were determined using validated HPLC methods. The limit of detection was 25 ng/ml for both analytes.

For systemic exposure, the area under the mean plasma concentration (AUC) of entacapone and tolcapone was calculated compartment-model independently using a linear trapezoidal approximation of up to 12 hours (\(C_{\text{last}}\)). The area prior to the first sampling point (10 minutes) was estimated with no lag time in absorption. The area beyond the time \(C_{\text{last}}\) was approximated using a triangular area up to the next sampling point. The proportion of the approximated area of the area for the 24-hour dosing interval (\(\text{AUC}_{0-24h}\)) was a maximum of 8%.

For liver tissue sampling, the animals were sacrificed by inhaled carbon dioxide two hours after the dosing (II). The medial lobe of the liver was removed, and liver concentrations of entacapone and tolcapone were determined by validated HPLC methods (Timm and Erdin 1992; Wikberg et al. 1993). The limit of quantification was 50 ng/g of fresh liver tissue.

7.3.8. Histopathology (I, II, III, V)
Tissue samples from the liver were preserved in 4% buffered formaldehyde. The tissues were embedded in paraffin wax, sections cut into 4 µm and stained with haematoxylin and eosin (H & E). Tissues were examined under a light microscope.

7.3.9. Electron microscopy (III)
Samples for transmission electron microscopy were taken from the liver and skeletal muscle (M. gastrocnemius). Tissue samples of a maximum size of 1 mm\(^3\) were prefixed in phosphate-buffered (pH 7.2) 2.5% glutaraldehyde. Postfixation was performed with phosphate-buffered 1% osmium tetroxide. The thin sections were studied after dehydration with ethanol,
embedding in epoxy resin, thin-sectioning with an ultramicrotome and post-staining with uranyl acetate and lead citrate.

7.4. Material and methods in studies at cellular level

7.4.1. Adenosine nucleotides from liver mitochondria and liver tissue (II)

Mitochondria were isolated immediately from fresh liver samples of rats by homogenization and differential centrifugation and used for determination of adenosine nucleotides. The liver tissue samples were prepared according to Faupel et al. (1972).

The adenosine nucleotide [adenosine monophosphate (AMP), ADP, ATP] concentrations in mitochondria and liver homogenates were determined by HPLC using ultraviolet detection as described by Carter and Muller (1990). The levels of adenosine nucleotides were expressed as an ATP/ADP ratio. The energy status of the cell was also expressed as an energy charge [= (½ ADP + ATP) / (AMP + ADP + ATP)].

7.4.2. Mitochondrial oxygen consumption (II)

The oxygen consumption of isolated mitochondria was assayed with an oxygen electrode connected to an amplifier and a chart recorder (Trounce et al. 1996; Nissinen et al. 1997) using 5 mM succinate as the respiration substrate. The rate of oxygen consumption before (state 4) and after (state 3) adding 2 µl of ADP was recorded and finally the mitochondria were maximally uncoupled by adding a known uncoupler, carbonyl cyanide chlorophenylhydrozone. The respiratory control ratio [= (oxygen consumption rate with ADP, state 3)/(oxygen consumption rate without ADP, state 4)] (RCR) was calculated. The protein content of the samples was estimated by the method of Lowry (1951).

7.4.3. Mitochondrial membrane potential studies (IV)

Mitochondria from rat liver (IV) were isolated with differential centrifugation and prepared as reported earlier (Nissinen et al. 1997). Mitochondrial membrane potential was measured using a fluorometric method originally described by Åkerman and Wikström (1976), and slightly modified by Kauppinen and Hassinen (1984) and Fromenty et al. (1990).

Mitochondrial membrane potential was presented as percentage of membrane potential in fully energized mitochondria using the following formula:

\[
\text{Membrane potential} \% = \left( \frac{F_{\text{max}} - F_n}{F_{\text{max}} - F_{\text{min}}} \right) \cdot 100,
\]

where \( F_{\text{max}} \) = Fluorescence maximum (zero membrane potential). The reading was taken at the start of the measurement before addition of succinate. \( F_{\text{min}} \) = Fluorescence minimum (maximal membrane potential). The reading was taken after addition of succinate. \( F_n \) = Fluorescence after the addition of the test compound.

7.4.4. Measurement of COMT activity in protein-binding studies (IV)

COMT activity in vitro was determined with and without serum proteins using a soluble COMT preparation from rat liver as described earlier (Nissinen et al. 1992) and 3,4-dihydroxybenzoic acid as the...
substrate. The results were expressed as IC$_{50}$ values ($\mu$M) of the test substances to induce half of the maximal inhibition of COMT (%).

7.5. Statistics

Biochemical parameters, clinical chemistry and body weight related organ weights were analysed using one-way analysis of variance in Studies I and II. Log transformation was applied to assure the assumptions of the model (normality of residuals). For the overall tests, a p-value (two-sided) of less than 0.05 was considered statistically significant. If statistically significant results were found, Bonferroni-corrected contrasts were applied to characterize these results in more detail. Statistical evaluation of the body temperature was performed using the analysis of covariance for unbalanced repeated measures with one between-factor (dose groups) and one within-factor (time points: 0, 2, 26, 50, 74, 98, 122 hours). The values at the commencement of treatment (0 hours) were used as a covariate.

In Study V, body weight, organ weights, body weight related organ weights, behavioural data, biochemical determinations and clinical chemistry values of the different COMT knock-out genotypes were evaluated using two-way analysis of variance with two between-factors (genotype and sex). Tukey’s test was used for further individual comparison in behavioural studies. Before statistical analysis, all clinical chemistry parameters were log-transformed to assure the assumption of normality. For the overall tests, a p-value of less than 0.05 was considered statistically significant. If the main effects of genotype or the interactions between genotype and sex were statistically significant, pair-wise comparisons were used to characterize these results in more detail.

Statistical analyses were performed with SAS® statistical software (version 6.12).
8. Results

8.1. Comparative toxicity of entacapone and tolcapone in vivo

8.1.1. Clinical signs, mortality, body weight and organ weights (I, II, V)

Entacapone was well tolerated in rats; even at a dose of 600 mg/kg/day up to 15 days, no clinical signs of toxicity or deaths occurred. Tolcapone, in turn, induced clinical symptoms, including tachypnea, laboured breathing, decreased spontaneous motor activity and drowsiness, on the fourth day after treatment of 400 mg/kg/day. Mortality due to tolcapone treatment was high, and the tolcapone-treated animals were terminated because of poor condition at the latest on the eighth day of treatment. At necropsy, all of the deaths were considered to be treatment-related. The reference substance, DNP, induced no clinical signs at the dose of 20 mg/kg/day during the 15 days of treatment. Sudden onset of rigor mortis was observed after tolcapone treatment already at a dose of 300 mg/kg/day and DNP of 20 mg/kg/day.

No mortality or clinical signs due to total COMT inhibition were present in COMT-deficient mice aged up to one year. The motor activity of homozygous females was significantly higher than that of wild-type mice (p<0.05). In Porsolt’s forced swimming test, male homozygous mice had a shorter floating time (p<0.05) (less depression) than their heterozygous counterparts, while both heterozygous and homozygous females had longer floating times than wild-type females (p<0.05).

8.1.2. Haematology and clinical chemistry (I, II, V)

Treatment with entacapone 600 mg/kg/day or tolcapone 200 mg/kg for 14 days in the rat induced no changes in haematological parameters. In COMT knock-out mice, the parameters did not vary between homozygous, heterozygous and wild-type animals.

Clinical chemistry parameters were unaffected by entacapone treatment (Table 6). However, tolcapone caused a significant decrease (p<0.001) in protein and globulin concentrations in serum even at the lower dose of 300 mg/kg/day. In addition, serum albumin was decreased (p<0.001) in animals treated with tolcapone 500 mg/kg/day. ALAT was significantly higher in this dose group than in the control group (p<0.001). There was also an increase in SDH for DNP 20 mg/kg/day (p<0.01), and a marginal increase for tolcapone 300 mg/
kg/day (p=0.09). The serum glucose concentration was elevated in the tolcapone 500 mg/kg/day group and in the DNP group (p<0.001).

In the 12-month-old homozygous COMT knock-out mice, clinical chemistry values for APHOS, ALAT, calcium, total proteins (Prot), albumin (Alb), urea and glucose (Gluc) were higher than in the wild-type mouse (V).

8.1.3. Catecholamine plasma levels (V)
The plasma levels of A, NA or DHPG did not vary between homozygous and wild-type mice of the COMT knock-out strain.

8.1.4. Activity of MAO-A and MAO-B in the liver (V)
No statistically significant differences in the enzyme activities of MAO-A or MAO-B in the liver tissue were present in different genotypes of the COMT knock-out mice.

8.1.5. Plasma and liver tissue concentrations (I, II)
The exposure factors (exposure in animal/maximum clinical exposure in man) for entacapone and tolcapone and AUC$^{0-24h}$ values from mean plasma concentrations were calculated in relation to the AUC$^{0-24h}$ reached in humans after maximum clinical doses of entacapone (10 x 200 mg daily) or tolcapone (3 x 200 mg daily) (Tasmar Product Monograph 1997; Entacapone Product Monograph 1999). The exposure factor achieved at the highest entacapone dose level (600 mg/kg/day) was 21. With tolcapone, an exposure factor of 14 was achieved at a dose of 400 mg/kg/day.

The concentrations of entacapone and tolcapone found in liver tissue two hours after dosing of 300 and 500 mg/kg/day were comparable (Figure 6). The mean plasma concentrations of tolcapone were higher than those achieved after entacapone treatment in the respective dose groups (300 and 500 mg/kg/day).

Table 6. Clinical chemistry values (mean ± SD) of the rats treated with entacapone (Enta), tolcapone (Tolca) or 2,4-dinitrophenol (DNP). N=6, except in Enta groups N=12 and in Tolca 300 group N=11 (II).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>S-Prot (g/l)</th>
<th>S-Alb (g/l)</th>
<th>S-Glob (g/l)</th>
<th>S-ALAT (U/l)</th>
<th>SDH (U/l)</th>
<th>S-Gluc (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>66 ± 1.9</td>
<td>33 ± 1.4</td>
<td>34 ± 0.8</td>
<td>57 ± 12.9</td>
<td>15.9 ± 1.5</td>
<td>6.7 ± 1.3</td>
</tr>
<tr>
<td>Enta 300</td>
<td>65 ± 3.2</td>
<td>33 ± 1.3</td>
<td>32 ± 2.5</td>
<td>53 ± 10.0</td>
<td>15.5 ± 5.4</td>
<td>6.2 ± 1.0</td>
</tr>
<tr>
<td>Enta 500</td>
<td>64 ± 3.7</td>
<td>33 ± 1.3</td>
<td>31 ± 2.9</td>
<td>56 ± 8.1</td>
<td>18.2 ± 5.5</td>
<td>6.9 ± 0.9</td>
</tr>
<tr>
<td>Tolca 300</td>
<td>59 ± 2.2***</td>
<td>31 ± 0.9</td>
<td>28 ± 1.9***</td>
<td>56 ± 6.8</td>
<td>21.8 ± 5.3</td>
<td>7.1 ± 0.7</td>
</tr>
<tr>
<td>Tolca 500</td>
<td>54 ± 3.4***</td>
<td>28 ± 1.5***</td>
<td>26 ± 1.9***</td>
<td>109 ± 9.1***</td>
<td>16.1 ± 9.4</td>
<td>11.2 ± 2.3***</td>
</tr>
<tr>
<td>DNP 20</td>
<td>63 ± 4.2</td>
<td>32 ± 2.1</td>
<td>32 ± 2.3</td>
<td>60 ± 16.2</td>
<td>27.4 ± 10.4*</td>
<td>9.6 ± 1.0***</td>
</tr>
</tbody>
</table>

** p<0.01, *** p<0.001
S-Prot, serum proteins; S-Alb, serum albumin; S-Glob, serum globulin; S-ALAT, serum ALAT; S-Gluc, serum glucose
8.1.6. Pathology induced by treatment with entacapone and tolcapone

8.1.6.1. Histopathological findings (I, II, III, V)

Histological examination of liver tissue from entacapone-treated rats at any dose level did not reveal treatment-related changes (Figure 7). As a sign of hepatotoxicity, diffuse centrilobular necrosis at tolcapone 400 mg/kg/day was observed in one out of five animals (Figure 7). In the rats treated with the high dose of tolcapone (600 mg/kg/day), necrotic foci or single cell necrosis was seen. Centrilobular hypertrophy was observed in the groups treated with tolcapone 500 mg/kg/day or DNP 20 mg/kg/day. Minor necrotic foci or single cell necrosis was also found in the liver tissue of rats treated with DNP. All other findings were considered to be normal for this strain of rats and thus were of no toxicological significance.

In the COMT knock out-mice, special emphasis was given to liver, kidneys and mammary glands in the homozygous and wild-type animals. The only finding was decreased midzonal or centrilobular vacuolization of the liver in homozygous male mice as compared with the wild-type animals. Anisocaryosis was observed in both genotypes. All other findings in different organs were distributed evenly amongst the different genotype groups, thus being normal background data.

8.1.6.2. Electron microscopy findings (III)

Changes occurred in the shape and internal structure of mitochondria of liver cells in DNP- and tolcapone-treated rats compared...
with entacapone-treated animals (Figure 8). The DNP-induced changes consisted of swollen mitochondria, deformed or broken cristae and reduced matrix density. With tolcapone, the matrix density was also reduced and mitochondria were swollen. In addition, intracellular edema was observed in the liver cells of DNP- and tolcapone-treated animals. The mitochondria were similar to each other in the entacapone and control groups.

In the skeletal muscle, intermyofibrillar edema and swelling of transverse (T)-tubules were seen in both the tolcapone and DNP groups but more prominently in the tolcapone group. Contracted sarcomeres, visualized as shortened I-bands, were also seen. Mitochondrial swelling and decreased matrix density were less prominent in skeletal muscle than in liver tissue, but the changes were still identifiable in the DNP- and tolcapone-treated animals. In the entacapone and control groups, no apparent findings were made.

8.2. Uncoupling effects related to entacapone and tolcapone

8.2.1. Signs of uncoupling in vivo

8.2.1.1. Body temperature (I, II, IV)

Entacapone had no effect on body temperature even at a dose of 600 mg/kg/day. By contrast, after each dose of tolcapone (300 mg/kg/day) body temperature increased within one or two hours from dosing about one centigrade, the highest increase being 1.1°C (p<0.01) (Figure 9). DNP 20 mg/kg/day also induced a significant increase in rectal body temperature compared with controls (1.3°C, p<0.001). The increased temperatures decreased to basal level within 24 hours. A more marked rise in body temperature up to 41°C preceded the deaths of six rats treated with tolcapone 600 mg/kg/day (Figure 10). The combination of carbidopa and levodopa did not induce any

Figure 7. Liver of entacapone (left) and tolcapone (right) treated rat. Centrilobular necrosis is seen in the tolcapone-treated rat liver. Magnification 100x, H&E staining (I).
changes in body temperature. When entacapone (400 mg/kg) was added to the treatment, the temperature decreased slightly (p<0.05). Tolcapone increased body temperature at a dose of 50 mg/kg even in the rats treated with carbidopa/levodopa (p<0.01).

8.2.2. Signs of uncoupling at cellular level

8.2.2.1. Adenosine nucleotide concentrations in liver mitochondria and liver tissue (II)

Treatment with entacapone did not interfere with oxidative mitochondrial energy production (Table 7). Nor did entacapone induce changes in whole liver ATP synthesis. Tolcapone treatment of 500 mg/kg/day reduced both mitochondrial ATP synthesis and whole liver ATP concentration (p<0.001). Mitochondrial ATP production was diminished, as seen in the ATP/ADP ratio (p<0.01). The energy charge in the mitochondria and liver tissue (p<0.01) was also significantly reduced. DNP 20 mg/kg/day decreased the mitochondrial ATP concentration, which was reflected as a reduction in the whole liver ATP production (p<0.01), and thus, the ATP/ADP ratio.
ADP ratio was decreased in the mitochondria as well as in the whole liver of DNP-treated rats. There was also a significant reduction of energy charge in DNP-treated mitochondria (p<0.001).

**8.2.2.2. Mitochondrial oxygen consumption (II)**

Treatment with tolcapone produced a mild effect on mitochondrial energy production, reflected in the marginal decrease in the respiratory control rate (RCR) (p = 0.08). The RCR ratio of DNP-treated rats was lowered by only a few percentage points (Table 7).

**8.2.2.3. Mitochondrial membrane potential (IV)**

Entacapone had no effect on mitochondrial membrane potential when the cumulative final concentration of entacapone remained under 100 μM (Figure 11). Tolcapone added in increasing concentrations gradually disrupted the membrane potential. The concentration required to decrease the membrane potential by 50% was 3.6 μM. DNP caused a concentration-dependent decrease of mitochondrial membrane potential at slightly lower concentrations than tolcapone. The concentration required to decrease the membrane potential by 50% was 1.7 μM. At concentrations of 5 μM or above, tolcapone and DNP further suppressed the membrane potential (Figure 11).

**8.2.3. COMT activity in relation to protein binding (IV)**

Entacapone and tolcapone inhibited COMT at equal concentrations (IC50 values of 0.25 μM and 0.24 μM, respectively; Figure 12), as measured by COMT activity without preincubation in rat serum. However, when the compounds were preincubated in serum for 30 minutes, the COMT inhibitory activity of entacapone was somewhat reduced (IC50 value 0.54 μM), while that of tolcapone was shifted to micromolar level (IC50 value 2.62 μM).

---

**Figure 9. Mean change in rectal body temperature of rats measured one or two hours after entacapone (Enta), tolcapone (Tolca) and DNP treatment at doses of 300–600 mg/kg of entacapone and tolcapone and 20 mg/kg of DNP. Bars represent SD, ** p<0.01, *** p<0.001.**
Figure 10. Rectal body temperature after tolcapone treatment of individual rats that died at various time points during the study. The temperature was measured just before death. Doses were tolcapone 400 or 600 mg/kg/day tolcapone. † = time of death (I).

Table 7. Adenosine nucleotide ratios; mitochondrial and liver tissue values (mean ± SD) of the rats treated with entacapone (Enta), tolcapone (Tolca) or 2,4-dinitrophenol (DNP). N=6 (II).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mitochondria</th>
<th>Liver tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP/ADP</td>
<td>EC&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control 0</td>
<td>3.12 ± 0.36</td>
<td>0.83 ± 0.02</td>
</tr>
<tr>
<td>Enta 500</td>
<td>2.84 ± 0.44</td>
<td>0.81 ± 0.04</td>
</tr>
<tr>
<td>DNP 20</td>
<td>1.46 ± 0.35***</td>
<td>0.63 ± 0.07***</td>
</tr>
<tr>
<td>Control&lt;sup&gt;3&lt;/sup&gt; 0</td>
<td>3.55 ± 0.73</td>
<td>0.85 ± 0.04</td>
</tr>
<tr>
<td>Tolca 500</td>
<td>1.88 ± 0.69**</td>
<td>0.69 ± 0.10**</td>
</tr>
</tbody>
</table>

<sup>1</sup> EC = energy charge
<sup>2</sup> RCR = Respiratory Control Ratio
<sup>3</sup> Control for the group treated with tolcapone 500 mg/kg/day
** p<0.01, when compared with the control group); *** p<0.001
§ p = 0.08
**Figure 11.** Concentration dependency for the effect of entacapone, tolcapone or 2,4-dinitrophenol on mitochondrial membrane potential (% of maximum). Mitochondria were isolated from rat liver. \(N=1-5\). \(EC_{50}\) is the concentration which induces a 50% inhibition of membrane potential (IV).

**Figure 12.** Effect of addition of rat serum on COMT inhibition activity (%) of entacapone and tolcapone using soluble COMT from rat liver. \(N=3\). \(IC_{50}\) indicates the concentration at which the COMT inhibition is 50% of the maximum (IV).
8.3. Summary of the results in vivo and in vitro

Table 8 summarizes the results of in vivo and in vitro studies.

<table>
<thead>
<tr>
<th>Finding</th>
<th>Entacapone</th>
<th>Tolcapone</th>
<th>DNP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortality</td>
<td>—</td>
<td>↑</td>
<td>—</td>
</tr>
<tr>
<td>Relative liver weight</td>
<td>—</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Liver enzymes</td>
<td>—</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Serum proteins</td>
<td>—</td>
<td>↓</td>
<td>—</td>
</tr>
<tr>
<td>Body temperature</td>
<td>—</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Onset of rigor mortis</td>
<td>—</td>
<td>&lt; 10 min</td>
<td>&lt; 10 min</td>
</tr>
<tr>
<td>Liver histology</td>
<td>—</td>
<td>necrosis</td>
<td>necrotic foci</td>
</tr>
<tr>
<td>Liver mitochondria</td>
<td>—</td>
<td>swelling; deformed cristae; matrix density ↓</td>
<td>swelling; deformed cristae; matrix density ↓</td>
</tr>
<tr>
<td>Skeletal muscle ultrastructure</td>
<td>—</td>
<td>intermyofibrillar oedema contracted sarcomeres</td>
<td>intermyofibrillar oedema contracted sarcomeres</td>
</tr>
<tr>
<td>Skeletal muscle mitochondria</td>
<td>—</td>
<td>swelling; matrix density ↓</td>
<td>swelling; matrix density ↓</td>
</tr>
<tr>
<td><strong>At cellular level</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP/ADP mitochondria</td>
<td>—</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>EC¹ liver tissue</td>
<td>—</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>RCR² mitochondria</td>
<td>—</td>
<td>(↑)</td>
<td>—</td>
</tr>
<tr>
<td>Mitochondrial membrane potential</td>
<td>—</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

1 EC = energy charge  
2 RCR = respiratory control ratio  
(↑/↓) marginal increase/decrease  
— not affected

---

Table 8. Summary of the signs in relation to hepatotoxicity and oxidative phosphorylation in vivo and in vitro in rats after treatment with entacapone, tolcapone or 2,4-dinitrophenol (DNP).
9. Discussion

9.1. Comparative toxicology of entacapone and tolcapone in vivo

9.1.1. Clinical signs and mortality
COMT inhibition has been postulated to be toxic, inducing hepatotoxicity and leading to further complications in clinical use (Rivest et al. 1999; Olanow 2000). Since entacapone and tolcapone are structurally related COMT inhibitors, they have been speculated to have similar toxic effects. Both have undergone extensive non-clinical toxicity testing in several animal species, and no toxicity has been reported, even with high-dose treatment (Tasmar Product Monograph 1997; Entacapone Product Monograph 1999). Despite the safety studies and clinical trials, several cases of hepatotoxicity due to clinical treatment with tolcapone have emerged (Assal et al. 1998; Mayoral et al. 1999; Olanow 2000).

The toxicity of entacapone and tolcapone proved to be different in rats receiving high oral doses (I, II). Entacapone was well tolerated up to 15 days at high oral doses with no signs of toxicity. However, the first signs of toxicity after tolcapone treatment were expressed on the third day, and mortality increased during the next eight days of treatment such that the remaining animals in the tolcapone-treated groups were euthanized. The clinical symptoms in rats consisted of laboured breathing, decreased spontaneous motor activity and drowsiness. The symptoms usually faded away after two to three hours from dosing, although some of the rats died quite suddenly. The symptoms after tolcapone treatment are consistent with those in Spencer et al. (1948), who reported that rats after oral dosing of 30 mg/kg or more of DNP either died within one to two hours or recovered completely. This is also in accordance with a mouse study on DNP, where peak concentrations of DNP were reported to be reached within one hour in plasma and within two hours in liver tissue after oral dosing (Robert 1986). In Study II, the selected dose of DNP (20 mg/kg/day) was approximated to be the highest dose not to induce any mortality or clinical symptoms (Spencer et al. 1948). The doses of entacapone were selected according to previous toxicity studies (Entacapone Product Monograph 1999), while tolcapone doses were based on non-clinical and clinical exposure data (Tasmar Product Monograph 1997). In these earlier toxicological profile studies, tolcapone had proved to be well tolerated (Eckhardt et al. 1996; Schläppi et al. 1996a; 1996b; Tasmar Product Monograph 1997), with only a few clinical symptoms, consisting of respiratory difficulties in connection with premature deaths or some female rats lying on the side for several minutes after the dosing (300 mg/kg/day), reported (Tasmar Product Monograph 1997). In the four-week oral toxicity study in rats, three animals died within 15 to 60 minutes after dosing (Schläppi et al. 1996a). In one case, the cause of death was speculated to be due to exaggerated pharmacological activity or hypoxia (Schläppi et al. 1996a). However, the conditions in non-clinical long-term
toxicity studies were different from those in Studies I and II since in the former studies tolcapone had been administered to rats as a feed mixture, and in dietary studies, the exposure remains lower and no high peak plasma concentrations occur. Thus, the high mortality following tolcapone treatment in Studies I and II is considered to be related to the treatment.

In the behavioural studies with COMT knock-out mice, the differences between the genotypes were marginal and always less than differences between the sexes. Thus, reduced or total lack of COMT enzyme activity does not appear to be associated with any clinical symptoms or changed behaviour.

9.1.2. Hepatotoxic properties of entacapone and tolcapone

Drug-induced hepatotoxicity is the leading reason for withdrawal of a drug from the market (Lee 1995; Lasser et al. 2002; Thomas 2002). Hepatotoxicity is also the most serious problem in tolcapone-induced toxicity in clinical use (Assal et al. 1998; Mayoral et al. 1999; Olanow 2000; Watkins 2000). Three fatal cases due to tolcapone treatment have been reported, and two of them were characterized by hepatic necrosis (Assal et al. 1998; Olanow 2000).

The first indication of drug-induced hepatotoxicity is the rise in clinical chemistry parameters e.g. ALAT (Watkins 2000). In laboratory animals, SDH is the second choice marker of hepatotoxicity (Loeb and Quimby 1999). Tolcapone induced a twofold elevation in rat ALAT values as compared with the control group, indicating a mild hepatocellular disturbance (II). In addition, the serum protein values were decreased significantly in tolcapone-treated rats, signalling the diminished capacity of the liver to synthesize proteins. In DNP treatment, SDH was significantly increased. Robert (1986) has reported treatment of mice with a single dose of DNP 22.5 mg/kg, where plasma ALAT activity was not significantly different from the control levels; SDH was not measured. A positive correlation between hepatocellular damage and an increase of serum ALAT and SDH activities in the rat has been observed earlier (Travlos et al. 1996), with ALAT values also reflecting the severity of hepatocellular necrosis (Balazs et al. 1961). Liver cell necrosis and hypertrophy in animal studies are reported to be associated with increased serum concentrations of either SDH or ALAT in about 40-58% of the cases (Travlos et al. 1996). The elevations in ALAT and SDH activities were reflected in liver histology as necrotic foci and single cell necrosis in both tolcapone- and DNP-treated rats (I, II). Hepatocellular hypertrophy was also observed, with increased liver weight of about 15% (II). Electron microscopy examination of liver cells of tolcapone- and DNP-treated rats revealed swelling of mitochondria, deformation of cristae and reduced matrix density (III). These findings are consistent with those in a tolcapone-treated patient dying from fulminant drug-induced hepatitis (Assal et al. 1998; Spahr et al. 2000). Electron microscopy findings showed mitochondrial swelling with reduced density in the matrix and loss of cristae (McCaul et al. 1986; Spahr et al. 2000).

Tolcapone is reported to induce slight to moderate hepatocellular hypertrophy in mice after 18 months of treatment (Olanow...
2000), but in a four-week study in the rat, while liver weight increased 10%, no hypertrophy was observed (Schläppi et al. 1996a). Hypertrophy is usually regarded as an adaptive response to drug-induced microsomal enzyme induction in the liver, although in some cases, this enhanced metabolism may give rise to long-term toxicity (Greaves 1990).

Centrilobular necrosis was observed in one rat treated with tolcapone for three days. Since the peak serum enzyme rise usually occurs at about 24 hours after the dosing, and induced liver damage, depending on the hepatotoxic agent, the enzyme value and the degree of histological necrosis do not correlate with each other to the same extent later (Dixon et al. 1975). By two to three weeks from the beginning of treatment, serum SDH is more predictive in treatment-related rat liver histopathological lesions than serum ALAT (Travlos et al. 1996). In non-clinical toxicity testing of tolcapone, no elevations of serum enzyme levels in rats or dogs have been reported (Schläppi et al. 1996a; 1996b; 1996c; Tasmar Product Monograph 1997). However, total protein values in female rats were significantly decreased after four weeks’ oral treatment (400/300 mg/kg/day) (Schläppi et al. 1996a), which is consistent with the findings in Study II. By contrast, entacapone did not induce elevation in the liver enzymes or protein values in rats, even at the high doses used.

Centrilobular necrosis in liver tissue is the most frequent form of drug-induced hepatocytic necrosis (Haschek et al. 2002). This was also observed in a liver biopsy of a tolcapone-treated patient (Assal et al. 1998). The patient died of hepatic failure, and histological findings in the liver supported treatment-related hepatotoxicity. Centrilobular necrosis follows circulatory shock, as oxygen is depleted by the passage of blood through the periportal areas. Since centrilobular hepatocytes have a much higher content of drug-metabolizing enzymes than periportal hepatocytes, the toxic response takes place in the centrilobular area. Necrosis can occur within minutes of a toxic insult and is usually associated with severe metabolic disturbances. The onset of cell death is characterized by breakdown of the plasma membrane permeability barrier, which leads to loss of metabolic intermediates and leakage of liver enzymes into the circulation (Mehendale et al. 1994). When the metabolic system is disrupted, the concentration of toxic substances increases in centrilobular areas, leading to degeneration and further necrosis of hepatocytes. Even if destruction of tissue is extensive, inflammatory cell infiltration may still be limited. The necrosis begins to regenerate within 24 hours, and within one week can be totally healed (Haschek et al. 2002).

The specific mechanism of drug-induced hepatotoxicity remains unknown. It might be an idiosyncratic reaction of partly genetic background or may be associated with mitochondrial toxicity (Dossing and Sonne 1993; Acuna et al. 2001). With few exceptions, hepatotoxic reactions induced by drugs are not a dose-related phenomenon (Lee 1995; Watkins 2000). However, in clinical use, tolcapone did induce a clear dose-response effect with respect to liver enzyme elevation (Mayoral et al. 1999; Olanow 2000; Watkins 2000). Gasser and Smit (2001) have also reported that 5.7%
of patients (N=3848) receiving 200 mg three times daily of tolcapone experienced liver function abnormalities. Moreover, in all three fatal cases due to hepatotoxicity after tolcapone treatment clinically, serum ALAT was raised to more than three times the ULN (Assal et al. 1998; Mayoral et al. 1999; Olanow 2000). Entacapone, by contrast, has not induced severe hepatotoxicity in clinical use (Watkins 2000). The mechanism behind tolcapone-induced hepatotoxicity may be associated with the lipid solubility and the metabolism of tolcapone, which differ from those of entacapone (Nissinen et al. 1992; Wikberg et al. 1993; Dingemanse et al. 1995; Dingemanse 1997). If a drug is not efficiently metabolized, e.g. by glucuronidation or sulphation after dissociating from serum proteins, it can accumulate in such tissues as the liver. Thus, intracellular concentrations of tolcapone might be increased in the liver tissue of patients whose glucuronidation rate is poor, which is reflected in liver function abnormalities. Some parkinsonian patients, for instance, have a deficiency in mitochondrial respiratory chain function that affects the first enzyme complex involved in oxidative phosphorylation (Schapira 1994; Schapira 2001). This genetically determined complex I defect may be exacerbated by DA, which increases free radical formation and may damage the mitochondrial respiratory chain function. The dysfunction can reduce ATP synthesis, leaving the cell with inadequate energy (Schapira 2001). If treatment with a COMT inhibitor results in uncoupling of oxidative phosphorylation, these patients are then more vulnerable to toxic effects (Schapira 2001; Orth and Schapira 2002).

Homozygous COMT knock-out mice showed slightly higher levels of certain clinical chemistry values as well as hyperglycemia associated with lower body weight than wild-type mice (V). It could be assumed that in these homozygous mice the levels of catecholamines are higher than in wild-type mice, leading to a mild catabolic condition reflected as hypermetabolism, hyperglycemia and a lower body weight. Since endogenous A and NA are capable of potentiating the hepatotoxicity induced by several drugs in the rat (Roberts et al. 1997), the total COMT inhibition with elevated catecholamines in plasma could be expressed as liver toxicity in the COMT knock-out mouse. However, a complete lack of COMT activity in mice did not cause significant liver problems (V). Experimentally, in rabbits as well as in clinical use, tolcapone has been shown to increase catecholamine plasma levels, and one patient was reported to simultaneously have had elevated liver enzymes (Garrido et al. 1994; Rojo et al. 2001). However, the levels of catecholamines in plasma of knock-out mice were not significantly increased despite high DHPG levels, nor did the activities of the two MAO isoenzymes in the liver (V) differ between the genotypes. There were no increases in general motor activity or in the levels of anxiety and depression in the mice lacking COMT, supporting the view that there was no enhanced catecholaminergic tone in these animals. To be a class effect of COMT inhibitors or COMT inhibition, liver toxicity should be induced by both entacapone and tolcapone in clinical use due to the close structural relationship of these agents. Liver toxicity should also be expressed as histopathology
in COMT-deficient mice (Watkins 2000). Since no toxicity as histopathology or elevated liver enzyme values were noted after entacapone treatment or in homozygous COMT knock-out mice, COMT inhibition as such does not appear to cause significant liver toxicity.

9.1.3. Exposure of animals to entacapone and tolcapone
The plasma concentration of tolcapone two hours after the dosing was clearly higher than that of entacapone (I, II). Moreover, in clinical use, a peak concentration of approximately 6 µg/ml is reached after an oral tolcapone dose of 200 mg, while after an equal dose of entacapone the peak concentration is 1.8 µg/ml (Table 9) (Keränen et al. 1994; Dingemanse et al. 1995). Thus, the AUC of tolcapone at the maximal recommended dosage in man is much higher than that of entacapone (for tolcapone 75 h•µg/ml, dosage 3 x 200 mg/day; for entacapone 20 h•µg/ml, dosage 10 x 200 mg/day). However, the calculated exposure factor in entacapone- and tolcapone-treated animals was 21 and 14, respectively. At the same time point, the concentrations of entacapone and tolcapone were equally high in liver tissue, indicating that both compounds can penetrate the liver similarly although highly bound to plasma proteins (Dingemanse 1997). At comparable liver concentrations, entacapone caused no toxic effects in rats, while tolcapone did induce toxic signs in vivo.

9.2. Uncoupling effects related to entacapone and tolcapone

9.2.1. Signs of uncoupling in vivo
The effect of entacapone and tolcapone on the body temperature of rats measured one or two hours after the dosing was clearly different (I, II). Entacapone caused no elevation, while both tolcapone and DNP treatments induced a significant increase in body temperature of rats. Furthermore,

Table 9. In vitro concentrations of entacapone and tolcapone inducing 50% COMT inhibition (IC50) and in vivo (clinical) data on peak plasma concentrations (C max) after a single dose of 200 mg of entacapone or tolcapone, and the concentrations in the liver tissue in rats in vivo (dose 500 mg/kg/day).

<table>
<thead>
<tr>
<th>Compound</th>
<th>COMT inhibition in vitro</th>
<th>Clinical data</th>
<th>Liver tissue concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (µM)</td>
<td>IC50 (µM)</td>
<td>C max (µg/ml)</td>
</tr>
<tr>
<td>Entacapone</td>
<td>0.24</td>
<td>0.54</td>
<td>1.8</td>
</tr>
<tr>
<td>Tolcapone</td>
<td>0.25</td>
<td>2.62</td>
<td>6.3</td>
</tr>
</tbody>
</table>

*calculated from the clinical data as unbound to serum proteins (Keränen et al. 1994; Dingemanse et al. 1995)
some of the premature deaths of animals after tolcapone treatment were preceded by body temperature increasing up to 41ºC. In several cases of DNP poisoning in clinical use, body temperature has been shown to be markedly increased (Horner 1942). In the presence of levodopa and carbidopa (IV), tolcapone induced an elevation of body temperature after a single oral dose, while entacapone had a slight decreasing effect on temperature. Dopamine agonists are known to induce a decrease in body temperature through stimulation of dopamine D2 receptors (Cox and Tha 1975; Faunt and Crocker 1987). Since concomitant dosing of COMT inhibitors with DDC inhibitors increases the bioavailability of levodopa, the decrease in body temperature after entacapone treatment may be due to a higher DA concentration in the brain. As levodopa treatment has been reported not to interfere with mitochondrial respiration in rat skeletal muscle (Dagani et al. 1991), the mechanism by which tolcapone induces a rise in body temperature is apparently unrelated to DA concentration but may be a result of uncoupling of oxidative phosphorylation.

Rigor mortis was not observed in entacapone-treated animals during the 20- to 30 minute necropsy; however, it was seen immediately after death in tolcapone- and DNP-treated rats (I, II). The rapid onset of rigor mortis has also been reported in previous non-clinical testing of tolcapone and toxicity studies with DNP in the rat (Spencer et al. 1948; Eckhardt et al. 1996; Schläppi et al. 1996b). Instantly occurring rigor mortis indicates very rapid usage of ATP and glycogen from the skeletal muscle cells upon the death (Gracey 1981). After administering uncoupling agents, the amount of ATP is already low before death, and therefore, rigor mortis should occur at the time of death or directly after it. In skeletal muscles, intermyofibrillar oedema together with prominent contraction of sarcomeres in the tolcapone groups resembles findings during the early changes of rigor mortis (Collan and Salmenperä 1976; Kobayashi et al. 1999). Melmed et al. (1975) have postulated that gross morphological changes in mitochondria only occur when the concentration of an uncoupler in the cell is very high. The situation in vivo differs from that in vitro since in vivo studies the concentration of an uncoupling agent in the liver or skeletal muscle cell after one dose may not reach the same concentrations as in isolated mitochondria in an incubation medium in vitro. To induce alterations in the mitochondrial morphology in vivo, the test substance should be administered repeatedly and over a longer period of time to allow it to accumulate in the tissue and cell. During the several days of dosing in Study III the mitochondrial changes due to uncoupling should also have become apparent. In the liver cells, swelling of mitochondria, deformation of cristae and reduced matrix density were observed in DNP- and tolcapone-treated rats, and these findings are comparable with those reported by Spahr et al. (2000) in a clinical case report after tolcapone treatment.

9.2.2. Signs of uncoupling at cellular level

The liver mitochondrial ATP/ADP ratio was unaffected by treatment with high oral doses of entacapone (II). The ratio decreased
significantly after treatment with tolcapone or DNP, indicating uncoupling of oxidative phosphorylation at the cellular level (Cutting and Tainter 1933; Kaiser 1964; Bakke and Laurence 1965). Both tolcapone and DNP have earlier been shown to be potent uncouplers in vitro in isolated rat liver mitochondria (Loomis and Lipmann 1948; Terada 1990; Nissinen et al. 1997). Study II demonstrated that tolcapone has the potential to interfere with oxidative energy metabolism in the liver in vivo. The hampered oxidative ATP production was also seen in the lowered energy charge in the tolcapone-treated rat liver mitochondria and at the level of the whole liver tissue. Decreased ATP production leads to increased oxygen consumption as a compensatory function of the cell and cell death may occur. In isolated rat liver mitochondria, both tolcapone and DNP had also a direct disruptive effect on mitochondrial membrane potential at the same concentration range, EC₅₀ being lower than 5 µM (IV). Entacapone did not have any effect on membrane potential at concentrations lower than 100 µM. Thus, entacapone has no influence on cellular respiration or mitochondrial oxygen consumption until concentrations are beyond the physiological, and does not uncouple oxidative phosphorylation by disrupting the mitochondrial membrane potential. EC₅₀ for stimulation of succinate-supported mitochondrial respiration of tolcapone has earlier been reported to be 2.6 µM, which is in the same concentration range as the concentration for disruption of membrane potential in Study IV (Nissinen et al. 1997). These results are also consistent with those of Borroni et al. (2001), who reported that the lowest effective dose of tolcapone to induce a concentration-dependent increase in respiration rate is 1 µM. At a concentration range of 1 to 300 µM, tolcapone further reduced the mitochondrial membrane potential in a dose-dependent fashion. When uncoupling of oxidative phosphorylation decreases the amount of ATP, the synthesis of ATP and ADP from glucose in the liver is diminished and energy is generated mainly from glycolysis. Increased liver pyruvate induces gluconeogenesis, which is reflected as an elevation of short duration in serum glucose concentration (Berg et al. 2002), as was also shown in the DNP- and tolcapone-treated rats (II). The reason for the greater potential of tolcapone than entacapone to cause uncoupling might relate to tolcapone’s lipid solubility (Dingemanse 1997). Since tolcapone is a more lipophilic compound, it more easily penetrates the cell membrane and crosses the mitochondrial bilayer than the less lipid-soluble entacapone, inducing a disruption in membrane potential. Borroni et al. (2001) have speculated about the discrepancy between in vitro and in vivo findings regarding uncoupling. They assumed that because the free fraction of a drug is diminished by extensive protein binding in vivo, the uncoupling effect of both tolcapone and DNP is counteracted (Borroni et al. 1999; 2001). As entacapone is 98% and tolcapone 99.9% bound to plasma proteins in vitro, the effective free concentrations would be 2% and 0.1%, respectively (Dingemanse 1997). However, entacapone and tolcapone are equally potent COMT inhibitors in vitro (IV), with an IC₅₀ value of ca. 0.25 µM, when a rat liver soluble COMT preparation without added
rat serum is used. After adding the serum to the incubation mixture, a tenfold concentration of tolcapone is needed to achieve a similar IC₅₀ value of COMT inhibition, whereas only twice as much entacapone is needed for a 50% COMT inhibition in the presence of serum (Table 9). However, in clinical use, both inhibitors have proved to be potent COMT inhibitors despite extensive protein binding (Kaakkola 2000). If protein binding noticeably decreases the pharmacological activity of the compounds in vivo, the unbound concentration of entacapone and tolcapone in clinical use should be twice and ten times as high, respectively, to achieve effective COMT inhibition (Table 8), which contradicts the results of Borroni et al. (2001).

Findings in vitro and in vivo are not directly comparable since in vitro the reactions are carried out over a few minutes, whereas in vivo the drug is present for hours. Due to the equilibrium between the protein-bound and free fractions of a drug in plasma, the free fraction penetrates tissues and can act as a potential protonophoric uncoupler if it is not effectively further metabolized. However, the liver concentrations of both tolcapone and entacapone are equally high after oral dosing to rats (II), indicating that both compounds can penetrate the liver despite being highly bound to plasma proteins. Thus, the extensive binding of a drug to serum proteins does not directly affect tissue concentrations or prevent uncoupling of oxidative phosphorylation in vivo.

The dissimilarities in the hepatic safety of entacapone and tolcapone may be due to different metabolic routes as well as the lipophilicity of the two compounds. As a more lipophilic compound, tolcapone readily crosses mitochondrial membranes. The only significant metabolic route for entacapone is glucuronidation, whereas tolcapone also undergoes oxidation and methylation. Uncoupling of oxidative phosphorylation may have a role in the hepatotoxicity caused by tolcapone treatment, as uncoupling properties comparable with those of DNP are expressed by tolcapone both in vivo and in vitro.
10. Conclusions

The non-clinical safety of entacapone and tolcapone, two nitrocatechol-structured COMT inhibitors designed as adjuncts to levodopa treatment of Parkinson’s disease, was compared in vivo and in vitro.

After repeated high oral doses to rats, entacapone did not cause toxicity, while equal doses of tolcapone induced severe toxic signs. These signs were comparable with those induced by DNP, an uncoupler of oxidative phosphorylation. Histopathological findings after tolcapone treatment were of the same type as in DNP-treated rats, including changes to the liver and the ultrastructure of liver and skeletal muscle mitochondria. Moreover, tolcapone was shown to cause uncoupling of respiration on mitochondrial membranes in vitro. Taken together, uncoupling of oxidative phosphorylation may have a role in the hepatotoxicity caused by tolcapone.

COMT inhibition as such did not induce toxic signs in mice lacking COMT activity, nor did it cause any liver problems. The results indicate that toxicity induced by one COMT inhibitor cannot be generalized to all agents in the same class.
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