INTRODUCTION

Tolcapone is a potent catechol-O-methyl transferase (COMT) inhibitor that has successfully been used as an adjunct to levodopa and decarboxylase inhibitors (e.g., Madopar and Sinemet) to treat the symptoms of Parkinson’s disease. By inhibiting COMT, tolcapone effectively reduces the conversion of levodopa into 3-O-methyl-DOPA, a metabolite of levodopa that cannot be readily converted into dopamine, and thus increases the amount of levodopa available to the brain for the biosynthesis of dopamine (1,2). Pharmacokinetic studies in humans have shown that co-administration of levodopa with tolcapone almost doubles the plasma half-life of levodopa with minimal effect on the plasma peak concentration (Cmax) of levodopa (3). Various clinical trials have shown that co-administration of tolcapone with Madopar or Sinemet significantly reduces off-time and increases on-time in patients that experience end-of-dose motor fluctuation and improves daily living activities in patients who have a stable response to levodopa (4-6).

In 1998, tolcapone was suspended in the countries of the European Community and in Canada due to the occurrence of rare but severe
cases of liver injury. In none of these cases was liver function monitored as recommended in the prescribing information. The safety data collected post-suspension suggest that in closely monitored circumstances tolcapone can be used safely.

A series of preclinical studies has been initiated in an attempt to elucidate the mechanism by which tolcapone may induce hepatotoxicity. This review considers various hypothetical mechanisms of toxicity and presents a brief summary of the results obtained in experiments designed to test these hypotheses. More precisely we investigated whether tolcapone could:
1) uncouple mitochondrial respiration and thus impair the energy metabolism of the cell;
2) induce the formation of reactive species and/or radicals capable of reacting with cellular components and thus impair cellular function;
3) be converted in the body into toxic metabolites.

Whenever possible, we compared the concentration of tolcapone showing an effect in vitro with those measured in the plasma of patients treated at therapeutic doses of tolcapone (100 and 200 mg t.i.d.) in order to determine the relevance of these findings to the safety profile of tolcapone. Pharmacokinetic studies have shown that patients treated with tolcapone at the dose regimen of 100 mg t.i.d. exhibit maximal plasma concentrations (Cmax) of 3 µg/ml, whereas those receiving 200 mg t.i.d. showed plasma Cmax of 6 µg/ml (3). Thus, these values were used for the comparison.

DOES TOLCAPONE UNCOUPLE MITOCHONDRIAL RESPIRATION?

Uncouplers of mitochondrial respiration are compounds able to dissipate the proton electrochemical gradient across the inner membrane of mitochondria. These compounds, usually lipophilic weak acids, act as protonophores by shuttling across the inner membrane, thereby catalysing the net uniport of protons and increasing the proton conductance of the membrane. Since the mitochondrial proton gradient is the force that drives the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP), its partial or complete dissipation results in a partial or complete reduction of the synthesis of ATP. Increasing the rate of mitochondrial oxidative phosphorylation can compensate for mild uncoupling whereas marked uncoupling leads to a strong reduction in the synthesis of ATP and eventually to cell death.

Previous experiments carried out with mitochondria isolated from rat liver and incubated in a protein-free buffer, have shown that nitrocatecholic compounds such as tolcapone and, to a lesser extent, entacapone can act as uncouplers of mitochondrial respiration at high concentrations (7). This finding raised the possibility that the rare cases of hepatotoxicity observed with tolcapone may be related to a toxic effect of tolcapone on mitochondrial function.

The long-term toxicological studies carried out for the registration of tolcapone indicated that tolcapone is well tolerated in various animal species even at doses that produce tolcapone plasma concentrations much higher (20-30-fold in terms of Cmax) than those measured in patients treated at therapeutic doses, thus providing evidence that tolcapone does not affect mitochondrial function in vivo even at high doses. Nonetheless, we further investigated the effect of tolcapone on mitochondrial respiration in order to evaluate more precisely the difference between the therapeutic concentrations of tolcapone and those affecting mitochondria.

The effect of tolcapone on the rate of respiration (oxygen consumption) of mitochondria isolated from rat liver is shown in Figure 1. In a protein-free buffer, tolcapone induced a concentration-dependent increase in the respiration rate. The lowest concentration of tolcapone that affected respiration was 1 µM (equivalent to 270 ng/ml), whereas the maximal effect was observed at 30 µM. Similar effects where observed by monitoring, semi-quantitatively, mitochondrial
membrane potential (ΔΨ) using the lipophilic cation TPP⁺ as a potentiometric probe. In the concentration range of 1-300 µM, tolcapone dose-dependently reduced the mitochondrial membrane potential as demonstrated by its ability to reduce uptake of [³H]TPP (Fig. 2, see over). A dose-dependent increase in the respiration rate and in the membrane potential was also observed with the well known uncoupler 2,4 dinitrophenol (DNP; data not shown). These results are in agreement with those of Nissinen et al. (7) and indicate that tolcapone can act as an uncoupler of mitochondrial respiration in vitro. Furthermore, they provide a quantitative evaluation of the concentrations of tolcapone required to affect mitochondria.

However, the concentrations of tolcapone found to be active in these experiments, cannot be directly compared with those measured in the plasma of patients treated with tolcapone, since tolcapone binds strongly to serum albumin (8). Thus, the great majority (99.9%) of tolcapone measured in the plasma is bound to serum albumin, and only a small percentage (0.1%) is free (not bound to albumin), and as such is pharmacologically active. Therefore, the experiments mentioned above were also conducted in a medium more similar to the plasma i.e., in a buffer that contained either bovine serum albumin (BSA 2% w/v, equivalent to 2 g/dl) or diluted (1/5) human serum as a source of human albumin. In the presence of albumin tolcapone did not affect mitochondrial respiration (Fig. 1) or membrane potential (Fig. 2, see over) even at the highest concentration tested (300 µM, equivalent to 82 µg/ml). These results demonstrate that it is the fraction of tolcapone not bound to albumin that is responsible for the uncoupling activity of tolcapone. Therefore, comparisons between the concentrations measured in the plasma and those found to be active in in vitro experiments carried out in a protein-free medium can only be performed considering unbound concentrations.

The unbound concentration of tolcapone
has been measured in the plasma of elderly healthy volunteers and in patients with moderate liver disease (8). After a single administration of 200 mg of tolcapone, the unbound concentration of tolcapone (Cmax) in the plasma of elderly volunteers was 6.3 ng/ml (range 4.4-8 ng/ml) and 13.3 ng/ml (range 5.3-23 ng/ml) in patients with moderate liver disease (Table I). In healthy subjects, the average unbound Cmax of tolcapone is approximately 43-fold lower than the lowest concentration of tolcapone shown to affect mitochondrial respiration in vitro (1 μM equivalent to 270 ng/ml; Table I). Even in patients with liver disease impairment, in whom the metabolic capacity is presumably reduced, the average concentration of unbound tolcapone is still 21-fold lower than the lowest concentration of tolcapone shown to affect mitochondria (Table I).

These data show that there is large a difference between the plasma concentrations of tolcapone that produce a therapeutic effect and those that affect mitochondrial respiration in vitro. This conclusion is further supported by the results of experiments carried out in various animal species:

1) In the rat, DNP causes hyperthermia whereas tolcapone does not increase core body temperature even when administered at doses much higher (> 20-fold) than those required to produce virtually complete inhibition of liver COMT (manuscript in preparation).

2) In the dog and rat, long-term toxicological studies showed that tolcapone was well tolerated and no hepatotoxicity was observed even at doses that produced plasma Cmax 20-30-fold higher than those measured in patients treated at a therapeutic dose.

**DOES TOLCAPONE LEAD TO THE FORMATION OF REACTIVE PRODUCTS AND OXYGEN RADICALS?**

Tolcapone exhibits structural features that might generate reactive species. The catechol- and the nitro-residues of tolcapone (see structure in Fig. 3) may undergo redox cycling accompa-
nied by the generation of reactive oxygen species (ROS). Therefore, it could be hypothesised that tolcapone itself or its metabolites might generate reactive products capable of reacting with cellular components and potentially impairing cellular function. The capacity of the liver to counteract such processes is very high, due to its high content of glutathione (GSH) and other scavengers and to the presence of powerful antioxidant defence systems. However, under abnormal conditions (e.g., pathological depletion of non-protein thiols, alcohol consumption, fasting) it cannot be excluded that reactions, triggered by a postulated reactive product, may become toxicologically relevant.

In a first set of experiments, we investigated whether tolcapone induced the formation of ROS in rat hepatocytes kept in culture. Formation of ROS was measured by monitoring the reduction

Table I - Comparison of the concentrations of unbound tolcapone measured in the plasma after administration of 200 mg of tolcapone and the lowest concentration affecting mitochondrial respiration *in vitro*.

<table>
<thead>
<tr>
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<th>Unbound plasma conc. of therapeutic tolcapone</th>
<th>Lowest conc. affecting mitochondria</th>
<th>Safety margin</th>
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<tbody>
<tr>
<td>Elderly volunteers</td>
<td>6.3 ng/ml (4.4-8)</td>
<td>270 ng/ml</td>
<td>43 (62-34)</td>
</tr>
<tr>
<td>Cirrhotic liver patients</td>
<td>13.3 ng/ml (5.3-23)</td>
<td>270 ng/ml</td>
<td>21 (52-12)</td>
</tr>
</tbody>
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The unbound plasma concentrations of tolcapone measured in humans are taken from Jorga et al. 1998 (8). The lowest concentration of tolcapone affecting mitochondria (1 μM, equivalent to 270 ng/ml) was taken from the experiments shown in Figs. 1 and 2.

![Metabolic pathways of tolcapone](image)

**Fig. 3 - Metabolic pathways of tolcapone.** Tolcapone is extensively metabolised *in vivo*. Only 0.5% of the administered dose is eliminated unchanged. The major metabolic elimination route in humans is by glucuronidation. In the rat the major elimination pathways are oxidation and glucuronidation.
of nitroblue tetrazolium (NBT) to formazan (9). Incubation of rat hepatocytes with tolcapone, at concentrations of 10 and 30 µM for 30 minutes, had no effect on the rate of formation of formazan. These results indicate that no significant amount of ROS was generated by tolcapone. To verify the sensitivity of this method of detecting ROS formation, two compounds known to generate ROS were tested in these experiments. In agreement with previous reports (10), nitrofurantoin and menadione increased the formation of formazan, indicating the generation of substantial amounts of ROS in rat hepatocytes.

To further investigate whether tolcapone and its metabolites can generate reactive products we measured the formation of tolcapone-GSH adducts in a culture of rat hepatocytes (11). In these experiments, rat hepatocytes were incubated for 6 or 24 hours either with tolcapone or with selected metabolites (see Fig. 3 for a schematic representation of the metabolic pathways of tolcapone). At the end of the incubation, the culture medium of the hepatocytes was collected and the presence of GSH-adducts was assessed by mass spectrometry analysis. The results obtained are summarised in Table II. Incubation of rat hepatocytes with tolcapone, at the concentrations of 9.5 and 95 µM, resulted in the production of trace amounts of GSH adducts. In addition, two glutathione ester conjugates of the carboxylic metabolites of tolcapone were detected. They represent two (innocuous) products of condensation reactions not originating from radical precursors. Neither the methylated product of tolcapone nor the amine metabolite formed detectable amounts of GSH adducts in rat hepatocyte culture (Table II).

A pre-requisite for the formation of reactive products from catechols is the formation of the corresponding quinones. Extensive stability tests indicated that tolcapone does not spontaneously form quinones. Furthermore, this oxidised form has never been detected in vivo in humans and animals. However, since it cannot be completely excluded that this quinone may be formed in vivo, we synthesised the quinone derivative of tolcapone and tested its ability to form GSH adducts. No GSH adducts were detected in cultures of rat hepatocytes incubated with this derivative of tolcapone.

Two compounds known to form GSH-adducts, acetaminophen and diclofenac (11), were also included in these experiments as active controls. GSH-adducts of both acetaminophen and diclofenac were detected in the incubation medium of treated hepatocytes, demonstrating the suitability and sensitivity of this approach. Minor amounts of GSH adducts were also detected when hepatocytes were incubated with the structurally related COMT-inhibitor entacapone.

<table>
<thead>
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<th>Table II - Formation of GSH-adducts in rat hepatocytes.</th>
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<tr>
<td>Compound</td>
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<td>----------------</td>
</tr>
<tr>
<td>Diclofenac</td>
</tr>
<tr>
<td>Acetaminophen</td>
</tr>
<tr>
<td>Entacapone</td>
</tr>
<tr>
<td>Tolcapone</td>
</tr>
<tr>
<td>Methyl-tolcapone</td>
</tr>
<tr>
<td>Amine-tolcapone</td>
</tr>
<tr>
<td>Quinone-tolcapone</td>
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<tr>
<td>Levodopa</td>
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whereas no adducts were observed with levodopa.

The concentrations of tolcapone used for the experiments carried out with rat hepatocytes ranged from 9.5 to 95 μM (equivalent to approximately 2,600 and 26,000 ng/ml, respectively). Since no protein fraction (foetal calf serum or bovine serum albumin) was added to the culture medium of the hepatocytes, all the tolcapone added was available to the hepatocytes. Thus, these concentrations are much higher (400 and 4,000-fold higher, respectively) than the unbound concentrations of tolcapone measured in patients treated at therapeutic doses (6.3 ng/ml; Table I).

In conclusion, the results obtained with rat hepatocytes suggest that tolcapone does not induce formation of substantial amounts of ROS. The toxicological relevance of the extremely low amount of GSH adducts measured in vitro, is probably negligible in normal situations. Hypothetical reactive products originating from tolcapone will have a biological significance only in conditions in which they cannot be detoxified by the protective systems of the organism. The lack of toxicity observed in long-term toxicological studies supports this conclusion.

IS HEPATOTOXICITY DUE TO THE FORMATION OF TOXIC METABOLITES?

Tolcapone is extensively metabolised in vivo, only a minor part of the administered dose (0.5%) is excreted unchanged. Various metabolising enzymes participate in the metabolism of tolcapone (Fig. 3). Rat hepatocytes are able to generate the same metabolic products observed in humans. The difference between man and the rat is a quantitative one and not a qualitative one. Thus, oxidative products are more pronounced in the rat than in man, due to the contribution of an additional cytochrome P450, CYP 2B, not expressed in substantial amounts in human liver. When compared to rats, humans extensively glucuronidate tolcapone by UGT1A isoenzymes. Reduction of the nitro group is a further metabolic pathway in both species, while sulfatation and methylation represent minor pathways.

Given the variety of metabolic pathways that can metabolise tolcapone, it is theoretically possible that some of the metabolites generated may exert toxic effects either directly or through the formation of reactive species. To explore whether a specific metabolic pathway is associated with toxicity, we first incubated rat hepatocytes with increasing concentrations of tolcapone in order to determine the range of concentrations in which tolcapone has cytotoxic effects. These experiments were then repeated in the presence of inhibitors of a specific metabolic pathway. If a given metabolic pathway generates toxic products leading to cell damage, its inhibition would be expected to result in a decrease of cytotoxicity. Thus, these sets of experiments allow the identification of metabolic pathway(s) involved in the generation of putative reactive products and/or toxic metabolites.

Rat hepatocytes kept in a serum-free medium were incubated for 24 hours with increasing concentrations of tolcapone in the presence and in the absence of either 10 μM SKF 525-A (to inhibit cytochrome P-450-dependent metabolism) and 1 mM borneol (to inhibit glucuronidation of tolcapone) or 30 μM p-chloro-mercurybenzoate (to inhibit the reduction of the nitro residue of tolcapone). Cytotoxicity was quantified by monitoring the leakage of lactate dehydrogenase (LDH) into the culture medium. LDH is an intracellular enzyme that is released extracellularly when the integrity of the hepatocytes is compromised and its presence in the culture medium can be taken as an indicator of cellular damage and cell death.

Incubation of rat hepatocytes with high concentrations of tolcapone led to a concentration-dependent increase in cytotoxicity (Fig. 4, see over). Leakage of LDH was first observed at a concentration of 30 μM and was maximal at 100 μM. It
should be noted that these experiments were carried out in a serum-free medium. Thus, the effects reported here refer to unbound concentrations of tolcapone much higher than those measured in the plasma of patients on therapeutic doses (see mitochondrial section). The lowest concentration of tolcapone that showed a minimal effect on LDH release (30 $\mu$M, equivalent to approximately 8,200 ng/ml) is more than 1,000-fold higher than the unbound concentration of tolcapone measured at therapeutic doses (6.3 ng/ml; Table I).

The cytotoxic effects of tolcapone were completely abolished when the experiments were carried out in a culture medium supplemented with 1% BSA (Fig. 4). No leakage of LDH was observed even at the highest tolcapone concentration tested (300 $\mu$M). These results demonstrate that it is the unbound fraction of tolcapone that is responsible for the observed cytotoxicity.

Inhibition of cytochrome P-450 mediated metabolism and glucuronidation by SKF 525-A and borneol did not protect the cells from the cytotoxicity induced by tolcapone, indicating that these metabolic processes are not associated with cytotoxicity. On the contrary, the cytotoxic effect of tolcapone was increased by SKF 525-A and borneol (Fig. 4) and maximal release of LDH was observed with 30 $\mu$M tolcapone (experiment carried out in serum-free medium). Inhibition of the reduction of the nitro group of tolcapone did not have any effect on the cytotoxic effect of tolcapone (results not shown). In the absence of tolcapone, SKF 525-A, borneol and p-chloro-mercury-benzoate had no effect on the release of LDH.

These results indicate that tolcapone, at extremely high concentrations, can have a toxic effect on rat hepatocytes in vitro. This toxicity does
not seem to be caused by the formation of toxic metabolites but is instead due to tolcapone itself. Glucuronidation and cytochrome P-450-mediated metabolism reduce the intracellular concentration of tolcapone and act as detoxifying mechanisms.

The concentrations of tolcapone that induced cytotoxicity were comparable to those shown to uncouple oxidative phosphorylation in isolated rat liver mitochondria (Fig.s 1 and 2). Thus, the acute toxicity observed in these experiments is most likely to be due to disruption of mitochondrial function.

CONCLUDING REMARKS

The results presented here do not identify a major clinical cause of toxicity. Tolcapone displayed toxic effects only at concentrations much higher than those measured in the plasma of patients treated at therapeutic doses. As regards all potential causes of toxicity considered in this review, large safety margins have been demonstrated.

The results of the experiments focusing on the hypothesis of mitochondrial uncoupling indicate that tolcapone does not uncouple oxidative phosphorylation at therapeutic concentrations and provide evidence in support of the existence of a large safety margin.

Toxic effects on hepatocytes were observed only at very high concentrations unlikely to be reached in vivo. The cytotoxicity observed in cultured rat hepatocytes is very likely to be related to tolcapone itself and not due to the generation of reactive or toxic metabolites.

The most significant finding of this study is that the toxicity of tolcapone was mostly manifest under in vitro conditions of reduced metabolism and elimination. This suggests that variations in metabolic activity, due to genetic factors, co-medications and co-morbidities that impair metabolism of tolcapone, could be associated with an increased risk of toxicity.

REFERENCES

9. Ogata I, Mochida S, Fujiwara K. Formazan formation in hepatic macrophages