Effects of Troglitazone on HepG2 Viability and Mitochondrial Function

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Troglitazone (TRO), a member of the thiazolidinedione class of drugs, has been associated with hepatotoxicity in patients. The following in vitro study was conducted to investigate the effects of TRO on mitochondrial function and viability in a human hepatoma cell line, HepG2. TRO induced a concentration- and time-dependent increase in cell death, as measured by lactate dehydrogenase release. Exposure to 50 or 100 µM TRO produced total loss of cell viability within 5 h. Preincubation of HepG2 cells with P450 inhibitors did not significantly protect against TRO-induced cell death suggesting that P450 metabolism was not required to induce cell death. Preincubation with the mitochondrial permeability transition inhibitor, cyclosporin A, provided complete protection against TRO-induced cell death. Our results also indicated that TRO produced concentration-dependent decreases in cellular ATP levels and mitochondrial membrane potential (MMP). Ultrastructural analysis demonstrated that TRO induced mitochondrial changes at concentrations of ≥10 µM after 2 h. Decreased MMP and altered mitochondrial morphology occurred at time points that preceded cell death and at sublethal concentrations of TRO. These observations in HepG2 cells suggest that TRO disrupts mitochondrial function, leading to mitochondrial permeability transition and cell death.

Key Words: troglitazone; HepG2; hepatotoxicity; mitochondrial permeability transition; cyclosporin A; thiazolidinedione; ATP depletion.

Troglitazone (Rezulin; TRO) is a member of the thiazolidinedione class of insulin-sensitizing drugs used for treatment of type-2 diabetes mellitus. This class of drugs, including TRO, is known to bind and activate the peroxisome proliferator-activated receptor-γ (PPARγ). The antidiabetic activity of thiazolidinedione drugs has been linked with activation of PPARγ (Fujiwara and Horikoshi, 2000). Over a 2-year period, approximately 40 cases of TRO-induced acute liver failure, characterized by hepatic necrosis and fibrosis, were reported in the U.S. (Tolman, 2000).

The mechanism responsible for the idiosyncratic TRO-induced hepatotoxicity in humans is not known. Structurally, TRO contains a chromanol moiety similar to α-tocopherol and has been reported to inhibit lipid peroxidation of low-density lipoprotein (LDL) in vitro (Noguchi et al., 1996) TRO is extensively metabolized and can be converted to a quinone-type metabolite (TRO-quinone) by human liver microsomes (Yamazaki et al., 1999). Yamazaki et al. (1999) identified CYP2C8 and CYP3A4 as major forms of cytochrome P-450 catalyzing TRO-quinone formation in human liver. In recent studies using rat and human liver microsomes (He et al., 2001; Kassahun et al., 2001) and human hepatocytes (He et al., 2001), CYP3A was identified as the major P450 isoform responsible for TRO-quinone formation. Liver toxicity was not observed in preclinical animal testing, which included monkeys that had a similar metabolite profile to humans (Yamazaki et al., 1999). Although TRO was not hepatotoxic in vivo studies, in vitro evidence suggested that TRO induced cell death. Toyoda et al. (2001) reported that 15 µM TRO, when incubated for 20 h with rat hepatocytes, produced cell death in about 80% of the cells in culture. Kostrubsky et al. (2000) demonstrated that human hepatocyte cultures exposed to 50 µM TRO for 24 h in serum-free media had a 50% decrease in cell viability. In addition, Kostrubsky reported that TRO inhibited protein synthesis in human hepatocyte cultures exposed to 25 µM TRO (sublethal concentration). These researchers concluded that TRO itself rather than a quinone metabolite was cytotoxic in vitro. To date, most of the in vitro toxicity studies with TRO have been conducted in rat or human cultured hepatocytes. Human hepatocytes contain P450 activity, but these activity levels may vary considerably, reflecting inherent interindividual differences in P450 levels in humans (Schuetz et al., 1993). Hepatocyte P450 activity is also influenced by culture conditions. Rat hepatocytes, to a greater extent than human hepatocytes, are known to rapidly lose P450 activity, including CYP3A, during culture (Schuetz, et al., 1993). The amounts of P450 activity in the human hepatoma cell line, HepG2, are low but well characterized. Schuetz et al. (1993) concluded that CYP3A7 was exclusively expressed in HepG2 cells. As such, HepG2 cells provide an excellent model system to examine whether TRO itself or a P450-activated metabolite is responsible for cell death in vitro.
Previous research in our group has shown that TRO induces alterations in mitochondrial function in cultured cells (Narayanan et al., 2001). We hypothesized that TRO-induced hepatocellular death was a consequence of mitochondrial dysfunction—an established mechanism of hepatocellular toxicity (Pessayre et al., 1999). We conducted the following study in order to examine the effects of TRO on mitochondrial function and viability in HepG2 cells and to specifically address if activation by P450 was required for TRO-induced toxicity.

MATERIALS AND METHODS

Chemicals and materials. Troglitazone (purity >96%) was obtained from SmithKline Beecham. Stock solutions of TRO were prepared in dimethylsulfoxide (DMSO), aliquoted into small volumes, and stored at 4°C. Mitotracker® Red CMXRos, calcein AM, and 5,5′,6,6′-tetrachloro-1′,3′,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were purchased from Molecular Probes (Eugene, OR). The ATP Bioluminescent Somatic Cell Assay Kit was obtained from Sigma (St. Louis, MO). P450 inhibitors, cyclosporin A, carbonyl cyanide-m-chlorophenylhydrazone (CCCP), and all other chemicals used in this study were obtained from Sigma.

HepG2 culture and incubation conditions. HepG2 cells were obtained from American Type Culture Collection (Manassas, VA) and used prior to passage 16. HepG2 cells were cultured in T75 flasks in Minimum Essential Medium Eagle Alpha modification with 10% heat inactivated fetal bovine serum and 100 I.U. penicillin/100 µg streptomycin at 37°C in a 5% CO2 atmosphere. One day prior to the experiment, confluent HepG2 cells were trypsinized and plated in 12-well plates at a density of 10^6 cells/well for viability experiments; in poly-L-lysine-coated 96-well plates at a density of 20,000, dissolved in sterile water, to wells for 10 min, then by adding 100 µl of ATP releasing reagent and 100 µl of water. Aliquots of 100 µl were transferred to white 96-well assay plates. Luminescence was monitored on a Gemini XS SPECTRAmax dual scanning microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) in the luminescence mode following addition of 100 µl luciferin and luciferase.

Mitochondrial potential measurements. Cells plated in 96-well plates were preloaded with 10 µg/ml JC-1 dissolved in HBSS for 30 min at 37°C. The JC-1 containing HBSS solution was removed and cells were washed twice with HBSS. JC-1-loaded cells were incubated with TRO, cyclosporin A/TRO, or CCCP at 37°C for a 2-h period. JC-1 exists as a monomer (em 527 nm) at low mitochondrial potentials but forms J-aggregates (em 590 nm) at high mitochondrial potentials, which can be assessed with JC-1 by monitoring fluorescence emission ratios at 590:527 nm. Fluorescence values (488 nm ex, 527/590 nm em with a 527 nm cutoff filter) were monitored on plates at zero time, and every 30 min, with a Gemini XS SPECTRAmax dual-scanning microplate spectrophotometer. Values are expressed as a percent of 0 time readings.

Confocal laser scanning microscopy. Cells were rinsed twice with HBSS. Troglitazone® Red CMXRos (100 nM) and calcein AM (1 µM) dissolved in DMSO were added to each well to give a final concentration of 0.2% DMSO (v/v) in HBSS, incubated for 15 min, and rinsed with HBSS. Selected wells were preincubated with 5 µM cyclosporin A for 30 min, followed by addition of either DMSO or 25 µM TRO dissolved in DMSO (0.1% DMSO final concentration). Cells were incubated for 1 h, then rinsed with HBSS. Images of cells were collected using a Zeiss LSM-510 confocal laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a 250 mW argon/krypton laser ( Omnichrome, Inc., Chino, CA). Fluorescence images were collected using appropriate band-pass filters at excitation wavelengths of 488 and 568 nm and emission wavelengths of 520 and 590 nm.

Transmission electron microscopy. Following TRO incubations, cells were fixed with 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2 for 2 h. Cells were harvested, pelleted, washed with sodium phosphate buffer, and post-fixed with 1% osmium tetroxide for 1 h. The pellets were dehydrated in increasing concentrations of ethanol and embedded in epoxy resin. Thin sections (~80 nm) were cut with a Reichert Ultracut S ultramicrotome (Leica Microsystems, Bannockburn, IL) and stained with uranyl acetate and lead citrate. Sections were examined with a JEOL 1200 EX transmission electron microscope (Peabody, MA), and images were collected using a GATAN BioScan 792 digital camera (Pleasanton, CA).

Statistics. All values are expressed as means ± SEM. All data, except as indicated, were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test. Cytochrome P450 data were analyzed by ANOVA, followed by the Tukey multiple-comparison test. ANOVA was performed with Sigma Stat v.2.0 statistical package (Jandel Corporation, San Rafael, CA).

ATP statistical analysis was performed in SAS v. 8.1 (SAS Institute, 1999). Because observations from the same experiment are correlated, ATP data from the 5 experiments were analyzed using mixed-effects models in SAS PROC MIXED (separate analysis was done for 1- and 2-h ATP data). The models contained the fixed-effect TREATMENT and the random-effects EXPERIMENT × TREATMENT interaction. Restricted maximum likelihood estimation was used to estimate the model parameters. The models were checked using residual diagnostic plots. Two families of significance tests were considered: Family 1 included the TRO + Cyclosporin A vs. TRO one-sided comparisons testing for an increase in ATP levels in TRO + Cyclo groups). Family 2 contained one-sided comparisons of all the 10 treatment levels against the control (testing for a decrease of ATP levels in the treatment). Family 1 and Family 2 p values were adjusted for multiplicity, using the Bonferroni and Dunnett-Hsu method (Hsu 1992), respectively.
RESULTS

The concentration- and time-dependent effects of TRO on HepG2 cell viability are summarized in Figure 1. Both 50- and 100-μM concentrations of TRO reduced cell viability to nearly 0% after 5 h of exposure. No significant reduction in cell viability was observed during the first 1 h at concentrations up to 100 μM TRO, or for 2 h at 50 μM TRO. Concentrations of 25 μM TRO did not significantly increase HepG2 cell death during 5 h of exposure.

We investigated whether cytochrome P450 inhibitors protected HepG2 cells against TRO-induced cell death. At the concentrations used in this study, SKF-525A (non-selective cytochrome P450 inhibitor; Echizen et al., 2000) and ketoconazole (CYP3A inhibitor; Clarke, 1998) provided no protection against TRO-induced (50 μM) HepG2 cytotoxicity (Fig. 2). Incubations of HepG2 cells with SKF-525A or ketoconazole, in the absence of TRO, produced less than 5% cell death after 5 h (data not shown).

The mitochondrial permeability transition inhibitor, cyclosporin A, provided complete protection against 50 μM TRO-induced cytotoxicity during the 5-h incubation period (Fig. 3). Cyclosporin A alone did not alter HepG2 cell viability during the 5-h exposure.

TRO concentrations ≥25 μM decreased mitochondrial membrane potentials (Fig. 4) as measured by the fluorescent probe JC-1. The mitochondrial uncoupler CCCP was used as a positive control in these experiments. TRO produced significant effects on mitochondrial potentials after only 30 min, and continued to reduce mitochondrial membrane potentials throughout the 2-h incubations. In control cells, the 590:527 nm ratio increased slightly throughout the experiment, while in 25–50 μM TRO-exposed cells, there was a continual decrease in the 590:527 nm ratio. In 50 μM TRO-exposed cells, the 590:527 nm ratio was reduced to less than 25% of 0 time values after 2 h. The addition of cyclosporin A did not significantly protect against 50 μM TRO-induced loss of mitochondrial potential at any of the time points analyzed in this study (data not shown).

Mitochondria preloaded with the mitochondrial-potential dye, MitoTracker® Red, also exhibited TRO-induced changes in dye distribution (Fig. 5). MitoTracker® Red is a positively charged cell-permeant fluorochrome that is preferentially sequestered in actively respiring mitochondria. Red fluorescence, in control cells preloaded with MitoTracker® Red, was localized to mitochondria, which appeared brightly fluorescent, elongate and branching, and was evenly distributed in the cell cytoplasm. Exposure to 25 μM TRO for 1 h caused a large decrease in fluorescence with many fewer mitochondria being
detected. The mitochondria observed were round and swollen. TRO also induced leakage of dye from mitochondria, resulting in diffuse weak cytoplasmic fluorescence. In addition to MitoTracker® Red, cells were also preloaded with the viability stain, calcein AM. Calcein AM is cell permeant-stain, which after cell entry, is cleaved by endogenous esterase activity to yield fluorescent calcein. Calcein fluorescence was not altered during the 1-h exposure period, suggesting that mitochondrial changes occurred prior to cell death (data not shown). Cyclosporin A pretreatment protected to some extent against TRO-induced leakage of MitoTracker® Red, although mitochondria often appeared round or ovoid and lacked the branching seen in controls. Cyclosporin A alone had no effect on mitochondrial morphology or MitoTracker® Red staining (data not shown).

TRO induced a concentration- and time-dependent decrease in cellular ATP concentrations (Fig. 6). CCCP was used as a positive control. Concentrations ≥ 25 μM TRO decreased ATP concentrations at both 1- and 2-h time points, but 10 μM TRO had no effect. After 2 h, cellular ATP concentrations were reduced to 50% of controls with 50 μM TRO. Cyclosporin A significantly protected against TRO-induced loss of ATP at both 1 and 2 h. Decreased ATP concentrations are unlikely a result of lost cell viability, since ATP decreased at time points that preceded cell death and at concentrations of TRO (25 μM) that were not cytotoxic.

Electron microscopic examination revealed extensive alterations in mitochondrial morphology (Fig. 7). At 10 μM for 2 h,
TRO caused swelling with reduced matrical density and abnormal cristae in a majority of mitochondria. Many mitochondria were cup-shaped with cytoplasmic invaginations. Small numbers of mitochondria retained normal appearance. At 25 μM, TRO produced strikingly abnormal mitochondria with cytoplasm, filling most of the matrical space, duplicated double membranes, and reticular cristae-like structures usually occupying one pole of a mitochondrion. Normal mitochondria were not observed in cells exposed to 25 μM TRO.

**DISCUSSION**

Our observations indicated that TRO, at concentrations ≥50 μM, induced significant cytotoxicity in HepG2 cells. Schuetz et al. (1993) demonstrated that HepG2 cells only express CYP3A7 activity. This limited P450 profile makes HepG2 cells a suitable cell system for addressing the involvement of P450 activation in TRO toxicity. In our studies, preincubations with the P450 inhibitors ketoconazole (CYP3A) and SKF-525A (nonselective) failed to prevent TRO-induced toxicity. Previous studies have suggested that TRO is metabolized by CYP2C (Yamazaki et al., 1999), and CYP3A (He et al., 2001; Kassahun et al., 2001; Yamazaki et al., 1999) to TRO-quinone. This suggests that the P450-formed TRO-quinone metabolite is not the cytotoxic component in HepG2 cells. Our results agree with those obtained by Kostrubsky et al. (2000), who examined TRO toxicity and metabolism in human and porcine cultured hepatocytes. These researchers exposed hepatocytes to TRO and monitored TRO, TRO-quinone, TRO-sulfate, and TRO-glucuronide concentrations by liquid chromatography-tandem mass spectrometry. The development of toxicity in both pig and human hepatocytes was associated with the parent drug and not TRO-quinone. Kostrubsky et al. (2000) found that 50 μM TRO produced cell death and inhibited protein synthesis in human hepatocytes during a 24-h incubation and concluded that TRO itself or possibly an unidentified metabolite was responsible for the toxicity observed in cultured hepatocytes. In studies conducted by Tettey et al. (2001), 25 μM TRO was toxic to HepG2 cells and reduced cell viability by about 70% after 24 h. In comparison, TRO-quinone was much less toxic to HepG2 cells than TRO. TRO-quinone only produced significant toxicity at concentrations in excess of 100 μM after 24 h. Finally, in agreement with our data, Yamamoto et al. (2001) recently demonstrated that TRO induced cytotoxicity in HepG2 cells and that the addition of the P450 inhibitor, ketoconazole, did not protect against cytotoxicity. TRO quinone, an electrophile, forms a biliary glutathione conjugate in rats (Kassahun et al., 2001; Tettey et al., 2001), but there is no evidence directly linking TRO-quinone formation to hepatotoxicity.

In our studies, TRO rapidly (0.5–2 h) produced both structural and functional changes in HepG2 mitochondria. These mitochondrial effects were observed at concentrations of TRO not associated with cell death (25 μM). TRO significantly decreased mitochondrial membrane potentials after 30 min and continued to reduce mitochondrial membrane potentials during the entire 2-h incubation period. This rapid onset of mitochondrial effects suggests that TRO directly influenced mitochondrial homeostasis. TRO also decreased cellular ATP concentrations after 1- and 2-h incubations. In general, there was an excellent agreement between the extent of mitochondrial membrane potential loss, as measured by JC-1, and decreases in cellular ATP concentrations induced by TRO. These concentrations of TRO that significantly decreased mitochondrial membrane potential also significantly reduced cellular ATP levels. Results from our confocal microscopic studies, using MitoTracker® Red and calcein AM, were in concordance with JC-1 results and suggested that TRO-induced mitochondrial

![FIG. 7. Ultrastructural changes in mitochondria of HepG2 cells exposed to TRO for 2 h. (A) Control, (B) 10 μM TRO, and (C) 25 μM TRO. Note: Invaginated mitochondria (B), duplicated double membranes, matrical areas filled with cytoplasm, and reticulated cristae (C). M, mitochondria, and N, nucleus. (A) and (C), bar = 0.5 μm; (B), bar = 1 μm.](image-url)
membrane potential changes occurred prior to cell death. Ultrastructural examination indicated concentration-dependent structural abnormalities in mitochondrial membranes, matrix, and cristae in TRO-exposed HepG2 cells. Caldwell et al. (2001) also reported misshapen mitochondria in hepatocytes from liver biopsies of patients with nonalcoholic steatohepatitis treated with TRO.

Both microscopic and biochemical analysis confirm that TRO disrupts mitochondria. Preininger et al. (1999) demonstrated in perfused rat liver that 0.61 μM TRO rapidly increased lactate release. Furnsinn et al. (2000) found similar effects in isolated rat skeletal muscle. Exposure of muscle specimens to 5 μM TRO for 25 h resulted in significant inhibition of insulin-stimulated mitochondrial fuel oxidation. A shift toward glycolysis was detected in muscle exposed to TRO in the absence of insulin after only 90 min (Furnsinn et al., 2000); these effects could be due to TRO disrupting mitochondrial function.

Further support for mitochondrial dysfunction in TRO-induced toxicity in HepG2 cells comes from our studies with the mitochondrial permeability transition inhibitor, cyclosporin A. Studies have indicated that mitochondrial permeability transition is a common event following toxicant exposures leading to cellular necrosis or apoptosis (Lemasters et al., 1998). The mitochondrial permeability transition is triggered by the opening of pores in mitochondrial membranes and is caused by high intracellular concentrations of calcium, membrane depolarization, and oxidation of vicinal thiols in the pore complex (Lemasters et al., 1998). In our studies, pretreatment of cells with cyclosporin A provided complete protection against 50 μM TRO-induced cell death. Cyclosporin also significantly protected against TRO-induced ATP loss but did not protect against mitochondrial membrane depolarization as measured by JC-1. Compounds such as mitochondrial electron transport inhibitors (Fontaine et al., 1998) and uncouplers (Fontaine et al., 1998; Minamikawa et al., 1999) are known to produce cyclosporin A-insensitive mitochondrial depolarization. We propose that TRO-induced mitochondrial depolarization may subsequently trigger mitochondrial permeability transition, severe mitochondrial damage, and cell death. In our studies, cyclosporin A protected against TRO-induced ATP loss but not mitochondrial depolarization. The mitochondrial permeability transition has been linked to the large-scale depletion of cellular ATP (Duchen, 2000; Qian et al., 1999). It has been postulated that the mitochondrial permeability transition uncouples mitochondria and causes consumption of cellular ATP by mitochondrial ATPases.

Our confocal microscopic studies indicate that cyclosporin A protected mitochondria against TRO-induced morphological changes and also protected, to some extent, against TRO-induced loss of mitochondrial membrane potential as measured by cellular distribution of MitoTracker® Red. Our MitoTracker® Red mitochondrial potential results differ from those obtained with JC-1 and may relate to different properties of MitoTracker® Red versus JC-1. The fluorescence intensity of MitoTracker® Red is known to be sensitive to mitochondrial membrane potential changes. However, Minamikawa et al. (1999) observed that even after the mitochondrial membrane potential has collapsed, mitochondria still retain some MitoTracker® Red fluorescence. Minamikawa et al. (1999) hypothesized that this potential insensitive pool of MitoTracker® Red was due to MitoTracker® Red mitochondrial protein binding (Molecular Probes MitoTracker® Red Information Sheet). The almost complete loss of MitoTracker® Red we observed following TRO exposures suggest that both free and protein-bound dye are lost from the mitochondria. Loss of protein-bound MitoTracker® Red from mitochondria would be expected to occur in mitochondria that have undergone severe membrane damage. By protecting against the mitochondrial permeability transition, cyclosporin A may prevent severe mitochondrial damage and the subsequent loss of MitoTracker® Red bound to mitochondrial protein, but not prevent the loss of the potential sensitive pool of free MitoTracker® Red. This hypothesis may explain the incremental decrease in mitochondrial MitoTracker® Red fluorescence seen in cyclosporin A/TRO-treated cells.

The relevance of our findings to idiosyncratic hepatotoxicity in TRO-treated patients is uncertain. Maximal plasma concentrations of TRO in humans taking therapeutic doses of 600 mg TRO per day was 6.4 μM (Spencer and Markham, 1997). Concentrations of TRO used in our study were higher than plasma concentrations measured in patients receiving TRO, but it is difficult at best to equate in vitro concentrations to patient plasma concentrations. Low concentrations of TRO may produce mitochondrial effects if the duration of TRO exposures is increased. There is also evidence that TRO is concentrated in the liver of animals following oral administration (Kawai et al., 1997). Kawai et al. (2000) concluded that orally administered TRO is extracted by the liver and subsequently undergoes enterohepatic circulation in rats. If similar effects occur for TRO in humans, hepatocytes may be exposed to higher concentrations of TRO than those measured in plasma.

Patients receiving TRO may be predisposed to TRO toxicity because of mitochondrial dysfunction related to age, mutations, comedication, or preexisting disease. St. Peter et al. (2001) conducted a retrospective study of 291 patients with type 2 diabetes mellitus receiving TRO to investigate risk factors associated with TRO-induced increases in serum liver enzyme values. They concluded that age and concurrent therapy with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) were factors that significantly increased risk of liver-enzyme elevation in TRO-treated patients. It should be noted that only a small percentage of patients receiving TRO developed hepatotoxicity. It has been clearly established that age leads to an impairment of mitochondrial function (Cortopassi and Wong, 1999). Also, there is evidence suggesting that statins can affect mitochondrial function (De Pinieux et al.,
TRO DISRUPTS MITOCHONDRIAL FUNCTION

1996). Finally, diabetes itself has been associated with increased levels of mitochondrial DNA deletions and DNA damage (Suzuki et al., 1999). Patients with impaired mitochondrial function may be more susceptible to the mitochondrial effects of TRO, and as such may exhibit mitochondrial dysfunction at lower concentrations of TRO.

Mitochondrial dysfunction has been established as a mechanism of drug-induced hepatotoxicity (Pessayre et al., 1999). Cytolytic hepatitis can occur by inhibition of mitochondrial respiration, uncoupling of oxidative phosphorylation, or drug-induced mitochondrial permeability transitions. Tacrine, valproic acid, and salicylic acid are among the drugs which have been proposed to induce hepatotoxicity through the disruption of mitochondrial function (Pessayre et al., 1999). Similar effects may occur in patients with impaired mitochondrial function receiving TRO.

In conclusion, our results indicate that TRO rapidly disrupts mitochondria in HepG2 cells, and this disruption precedes cell death. TRO affects mitochondrial membrane potential, cellular ATP levels, and mitochondrial structure in HepG2 cells. Also, our evidence suggests that TRO does not require metabolic activation to TRO-quinone to produce these effects.

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REFERENCES


