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Effect of toremifene on antipyrine elimination in the isolated perfused rat liver

Lorraine K. Webster, Andrew G. Ellis, and James F. Bishop

Experimental Chemotherapy and Pharmacology Unit, Peter MacCallum Cancer Institute, Melbourne, Victoria, Australia

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Summary. Toremifene is a triphenylethylene antioestrogen with significant antitumour activity. It is structurally very similar to tamoxifen. Both drugs undergo extensive hepatic metabolism, and tamoxifen is known to inhibit hepatic mixed-function oxidases (MFO). Using the isolated perfused rat-liver model, we investigated the effect of toremifene on the elimination of antipyrine, a standard marker of MFO activity. Perfusate consisted of 20% red cells in a modified Krebs-Henseleit buffer, and 80 ml was recirculated at 14 ml/min for 3 h. High but clinically relevant steady-state toremifene levels of 3 and 10 μg/ml were achieved using bolus plus constant infusion into the reservoir. Elimination of 2.5 mg antipyrine was not inhibited by steady-state toremifene, but methanol (maximal perfusate concentration, 1.29%), the vehicle used for toremifene administration, caused a statistically significant increase in the antipyrine elimination half-life (mean, 1.4 ± 0.2 h for controls vs 2.2 ± 0.3 h for methanol; $P < 0.05$, $n = 4$). Whereas the methanol had no apparent effect on liver viability as assessed by bile flow and perfusate back-pressure, toremifene at a steady-state concentration of 10 μg/ml caused a statistically significant decrease in bile flow (value at 180 min, 0.22 ± 0.05 ml/h as compared with 0.52 ± 0.06 ml/h in the methanol control; $P < 0.05$) and a statistically significant increase in perfusate back-pressure (value at 180 min, 17.5 ± 1.8 cm vs 11.0 ± 2.6 cm in the methanol control; $P < 0.05$). Therefore, toremifene used at high doses can impair liver function in the isolated perfused rat liver, but it does not have any effect on antipyrine elimination.

Introduction

Toremifene is a triphenylethylene antioestrogen with significant antitumour activity in humans [20]. Structurally, toremifene differs from tamoxifen only in the substitution of a chlorine atom for a hydrogen atom on the ethylene alkyl side chain. In addition to their antioestrogen antitumour activity, tamoxifen and toremifene may be active at high doses in hormone-independent cancer [6, 19]. These substances are also being investigated as potential multidrug resistance modifiers, which also requires high-dose therapy [10].

Both drugs undergo extensive demethylation and hydroxylation to active and inactive metabolites via hepatic mixed-function oxidases (MFO), although none of the metabolites are identical since the chlorine on toremifene is not removed [14, 17]. Examples of toremifene metabolites include *N*-desmethyltoremifene (TOR-I), 4-hydroxytoremifene (TOR-II), deaminohydroxytoremifene (TOR-III) and didemethyltoremifene (TOR-X). The majority of a dose is excreted as metabolites in faeces, and there are quantitative but not qualitative differences between human and rat toremifene-metabolite profiles [1, 17].

Tamoxifen is known to inhibit hepatic MFO activity in vitro [7, 12, 14], and clinical interactions suggestive of inhibition of warfarin metabolism have been reported [13, 18]. No report has been published on the ability of toremifene to influence drug-metabolising enzymes similarly. Because of the structural similarity of tamoxifen and toremifene and since toremifene may be given at high doses and with other drugs, the possibility of an interaction with drug-metabolising enzymes becomes more clinically important. We therefore used the isolated perfused rat liver model to investigate the effect of toremifene on the elimination of antipyrine, an index of MFO activity.

Materials and methods

Liver perfusion. Non-fasting male Sprague-Dawley rats (250–350 g) were anaesthetised with sodium pentobarbitone (60 mg/kg; i.p.), and their livers were surgically isolated by standard techniques [2] and perfused as previously described [22]. Briefly, the portal vein, thoracic inferior vena cava and bile duct were cannulated, and the liver was connected to a recirculating perfusion circuit in a constant-temperature (37° C) cabinet. The perfusate flow rate was constant at 1.1 ml min^{-1} g

Correspondence to: L. K. Webster, Experimental Chemotherapy Unit, Peter MacCallum Cancer Institute, 481 Little Lonsdale St., Melbourne, Victoria 3000, Australia

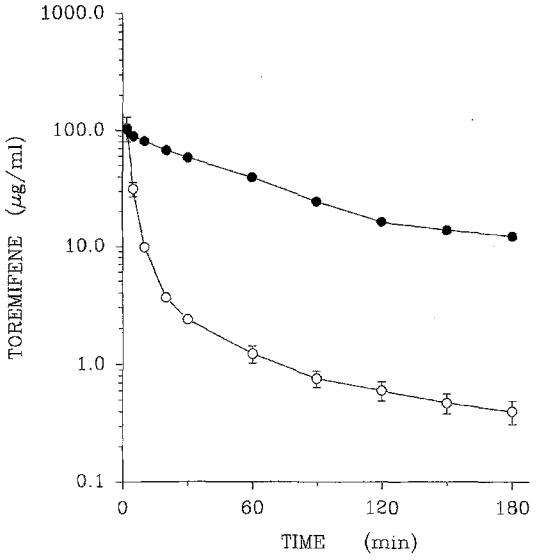

Fig. 1. Semilogarithmic plot of perfusate toremifene concentration versus time following a bolus dose of 8.0 mg toremifene at time zero in the presence (○, $n = 3$) and absence (●, $n = 2$) of a liver in the perfusion circuit. Data represent mean values ± SE

liver^{-1} (14.4 ± 1.8 ml/min). The perfusate (80 ml) consisted of 20% (v/v) washed human red blood cells, 1% (w/v) bovine serum albumin (Fraction V; Commonwealth Serum Laboratories, Melbourne, Australia) and 0.1% (w/v) D-glucose in a modified Krebs-Henseleit buffer (pH 7.4). Sodium taurocholate (Sigma) was infused into the perfusate reservoir at 30 μmol/h to maintain the bile flow. Liver viability was confirmed by normal values for bile flow (0.5–1.0 ml/h), perfusion back-pressure (less than 10 cm H_2O), oxygen consumption (2.0–4.0 μmol g liver^{-1} min^{-1}) and homogeneous appearance. Except where noted, livers remained viable for the duration of the 3-h experiments. All drugs were delivered to the perfusate reservoir to simulate systemic administration. Samples for drug estimation were taken from the reservoir and were replaced with equal volumes of fresh perfusate. Drug amounts lost through sampling were less than 2%.

Drug administration and sampling. In preliminary studies, toremifene (Farmos, Turku, Finland) was added to the perfusate reservoir as a bolus dose of 8 mg ($n = 3$), and the resulting toremifene clearance value was used to calculate doses for steady-state studies.

Elimination of antipyrine (Sigma Chemicals; 2.5 mg dissolved in 1 ml perfusate) was studied in the presence of toremifene at nominal steady-state concentrations of 3.0 ($n = 4$) or 10.0 μg/ml ($n = 4$) for 3 h; investigations using toremifene at 30 μg/ml (steady-state) were also attempted ($n = 3$). These steady-state concentrations were achieved using a bolus dose of toremifene [3 mg in 384 μl 50:50 (v/v) methanol: 0.9% saline) at the beginning of a 3-h constant 36.4-, 130.0- or 390.0-μg/min infusion of toremifene in 50:50 (v/v) methanol: saline (equivalent to 280, 280 and 870 μl methanol/h, respectively). The toremifene bolus dose was found to cause an initial overshoot in the required steady-state concentration of 3.0 μg/ml, and it thus was not increased in the higher-dose experiments. Antipyrine elimination was also examined in the absence of drug or methanol (control, $n = 4$) and after a 384-μl bolus of 50:50 (v/v) methanol: saline plus a continuous 280-μl/h infusion of methanol in saline (methanol control, $n = 4$). This was equivalent to the 3- and 10-μg/ml toremifene infusion schedules.

Perfusate (1.0 ml) was sampled for drug estimations at 2, 5, 10, 20, 30, 60, 90, 120, 150 and 180 min, and the red cells were removed by centrifugation. Bile was collected on ice in pre-weighed vials at 30-min intervals. All samples were stored at –20° C and assayed within 14 days. After the last sample collection the perfusate was flushed from the liver, which was homogenised in 3 vol. 0.067 M Sorenson's phosphate buffer (pH 7.4) for later assay.

Table 1. Toremifene pharmacokinetics in the isolated perfused rat-liver system

	n	Clearance (ml/h)	Elimination half-life (h)	Volume of distribution (ml)
Non-liver control	2	64 ± 1	1.2 ± 0.1	110 ± 10
Toremifene:				
Bolus 8.0 mg	3	690 ± 174	1.25 ± 0.13	1230 ± 260
Steady-state 3 μg/ml	4	690 ± 187	NC	NC
Steady-state 10 μg/ml	4	796 ± 88	NC	NC

Data represent mean values ± SD. NC, Not calculated

Drug assays. Levels of toremifene (TOR), didemethyltoremifene (TOR-X), *N*-desmethyltoremifene (TOR-I) and deaminohydroxytoremifene (TOR-III) were determined using a reverse-phase high-performance liquid chromatographic (HPLC) assay [24]. Briefly, 200-μl samples were prepared by protein precipitation with acetonitrile containing the internal standard (Fc-1226 a; Farmos, Turku, Finland). A 100-μl aliquot of supernatant was injected onto a Waters NovaPak C_{18} Rad-Pak column housed in a Z-module (Waters). The mobile phase consisted of acetonitrile: 100 mM ammonium acetate: triethylamine (65:35:0.05, by vol.) at pH 6.35, and the flow rate was 2.0 ml/min. Ultraviolet detection was carried out at 277 nm. The quantitation limit for toremifene and its metabolites was approximately 200 ng/ml.

Antipyrine concentrations in perfusate plasma were determined in separate 200-μl aliquots using modifications of previously described HPLC assays [9, 15]. Conditions for sample preparation and chromatography were similar to those used in the toremifene assay. The mobile phase consisted of 45% methanol, 1% triethylamine, and 54% water at pH 3.0. Detection was carried out at 244 nm and the limit of antipyrine quantitation was approximately 200 ng/ml.

Data analyses. All results were expressed as mean values ± SD. Pharmacokinetic parameters were calculated using standard model-independent pharmacokinetic formulae. Statistical analyses included one-way analysis of variance followed by specific between-group comparisons using a Bonferroni *t*-test [21].

Results

Preliminary studies of toremifene disposition showed that a bolus of 8.0 mg ($n = 3$) underwent biphasic elimination from the perfusate (Fig. 1). The distribution half-life was approximately 9 min; other pharmacokinetic parameters are listed in Table 1. The clearances calculated during the steady-state experiments were consistent with the results of the bolus-dose studies. Mass-balance calculations (Table 2) showed that less than 1.0% of the delivered dose was present in the perfusate as toremifene or as its assayed metabolites after 180 min perfusate recirculation. Approximately 1.0% of the dose was excreted in bile over 3 h, and only 0.6–2.0% was removed from the perfusate in samples for analyses. At least 21% of the dose was retained by the liver. Similar values were obtained in the steady-state studies. Therefore, a major proportion of the dose was not accounted for as toremifene or assayed metabolites in any biological sample.

In non-liver control experiments, perfusate concentrations of an 8-mg bolus of toremifene decreased monoexponentially to approximately 10% of the initial concentra-

Table 2. Mass-balance results and relative percentages present as toremifene and metabolites in the isolated perfused rat liver. The relative proportions may be influenced by an unknown degree of metabolite binding to the circuit components

	% of total dose accounted for at 180 min	Proportion (%) present as			
		Toremifene	TOR-1	Tor-III	TOR-X
Bolus 8 mg:					
Samples[a, b]	2.0				
Perfusate	0.6	64	36	–	–
Bile[b]	1.0	93	6	0.3	0.7
Liver	21.0	59	39	1	1
Steady-state 3 μg/ml:					
Samples[a, b]	0.9				
Perfusate	3.5	89	11	–	–
Bile[b]	0.5	90.5	6	0.5	3
Liver	32.0	76	23	0.5	0.5
Steady-state 10 μg/ml:					
Samples[a, b]	0.6				
Perfusate	4.2	93	7	–	–
Bile[b]	0.5	91	5	0.4	3.6
Liver	32.0	80	19	0.5	0.5

[a] Drug removed in samples taken for assays
[b] Cumulative amounts over 3 h

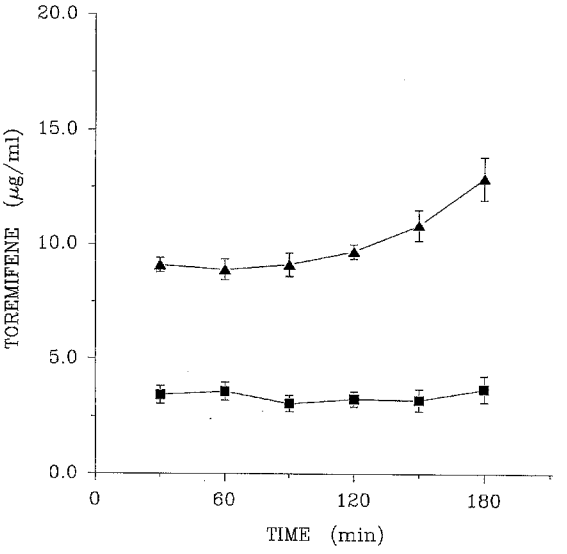

Fig. 2. Steady-state perfusate toremifene concentrations achieved using a bolus dose of 3.0 mg followed by either a 36.4- (■) or a 130.0 μg/min toremifene infusion (▲). Data represent mean values ± SE ($n = 4$). Livers were exposed to these concentrations of toremifene in the antipyrine-elimination experiments

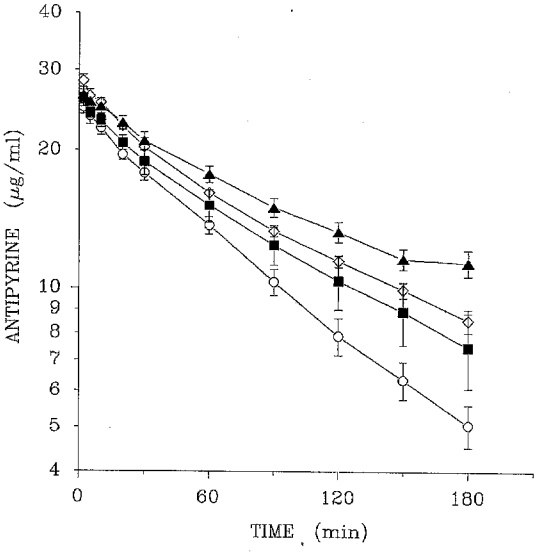

Fig. 3. Semilogarithmic plot of perfusate antipyrine elimination in isolated perfused rat livers in the absence (○) or presence of 3.0- (■) and 10.0-μg/ml (▲) steady-state concentrations of toremifene or in the presence of methanol vehicle (◇). Data represent mean values ± SE ($n = 4$)

tion after 180 min (Fig. 1). The conversion of toremifene to metabolites in the absence of a liver was minimal. Clearance by the perfusion circuit alone was 10% of that observed in the presence of a liver, and the volume of distribution was similar to the circuit volume of 80 ml (Table 1). This clearance of toremifene in the absence of a liver occurred even when glassware was silicone-coated or when a 10 times lower dose of toremifene was used. It also occurred after the removal of either the red blood cells or the bovine serum albumin from the perfusate. Red cell uptake was shown to be minimal. When a non-liver experiment was followed by rinses of the perfusion circuit with 0.9% saline, air and then methanol, more than 20% of the delivered bolus dose was recovered in the methanol, probably eluting from the perfusion-circuit tubing.

Although not extensive, there was some metabolism of toremifene by the isolated perfused rat liver. TOR-I was the major metabolite detected, with the concentrations in perfusate after a bolus dose of toremifene ranging from 15% to 55% of toremifene concentrations at 30 and 180 min, respectively. Of the 21% of the toremifene dose that remained in the liver at 180 min after bolus administration of toremifene, 39% occurred as TOR-I (Table 2). TOR-III and TOR-X were found only in the bile and the liver. The proportions of metabolites produced in the steady-state experiments were similar to those detected in the bolus-dose experiments.

Despite the elimination of toremifene by the perfusion circuit, moderate (3.4 ± 0.8 μg/ml) and high (10.1 ± 1.0 μg/ml) steady-state concentrations of toremifene were achieved for the antipyrine-elimination studies (Fig. 2). The perfusate elimination profiles of antipyrine are shown in Fig. 3. No statistically significant difference in antipyrine clearance or elimination half-life was found between the 3- and 10-μg/ml toremifene experiments and the methanol-control values (Table 3), although antipyrine concentrations were elevated in the presence of 10 μg/ml toremifene (Fig. 3). In contrast, a comparison of the control and the methanol-control experiments indicated that methanol did cause a statistically significant increase in the elimination half-life of antipyrine (Table 3).

There was some evidence that liver viability was compromised in the higher-dose experiments. In the 10-μg/ml toremifene experiments, perfusate toremifene concentra-

Table 3. Antipyrine pharmacokinetics during exposure to steady-state toremifene concentrations in the isolated perfused rat liver

Treatment	Clearance (ml/h)	Half-life (h)	Volume of distribution (ml)
Control	56±10	1.4±0.2	109±4
Methanol control	35± 4**	2.2±0.3*	112±3
Toremifene:			
Steady-state 3 μg/ml	43±16	2.1±0.7	113±8
Steady-state 10 μg/ml	29± 4***	2.6±0.3***	107±8

Data represent mean values ± SD ($n = 4$)
* $P < 0.05$ relative to control values; ** $P < 0.054$ relative to control values; *** $P < 0.05$ relative to control values but not relative to methanol control values

tions increased during the 3rd h (Fig. 2). Examination of the liver-viability parameters (Table 4) revealed a statistically significant decrease in bile flow and a statistically significant increase in back-pressure at 180 min in the 10-μg/ml toremifene studies as compared with the methanol control. The methanol control had no statistically significant effect on these parameters in comparison with the control. A steady-state toremifene concentration of 30.0 μg/ml could not be maintained; perfusate toremifene concentrations continually increased, and the bile flow ceased completely after 90 min. However, the hourly methanol infusion rate required to deliver the toremifene at this concentration was 3 times higher than those required for the two lower toremifene doses or in the methanol control. Experiments using methanol infusion at this higher rate resulted in a decrease in bile flow at 180 min (0.173±0.45 ml/h, $n = 2$).

Discussion

The isolated perfused rat-liver model is well suited to studies of hepatic metabolism and biliary excretion [2, 22, 23]. Many experimental parameters can be controlled, and sampling and drug administration are simplified. Using this model, we have previously examined the hepatic metabolism of trimetrexate and have shown that cimetidine can inhibit its elimination [23]. Antipyrine elimination is a valuable model for investigating hepatic drug metabolism [11], and its use in the isolated perfused rat liver has been established [8, 9]. Patients receiving high-dose toremifene therapy (up to 300 mg/m^2 daily) show plasma toremifene concentrations ranging from 1.5 to 4.0 μg/ml [3], and the doses used in the present study are therefore clinically relevant.

The hepatic mixed-function oxidases, or cytochromes P450, form a large family of enzymes with overlapping substrate specificity. They are involved in many oxidation reactions such as demethylation and hydroxylation. Antipyrine is a known substrate for this system, and its disposition has been used for many years as an index of hepatic drug-metabolising capacity, although the specific isoenzymes involved in its metabolism have not yet been identified. In the present study in the isolated perfused rat liver, toremifene did not significantly inhibit the elimination of antipyrine. The individual P450 isoenzyme that is responsible for converting tamoxifen to its major metabolite, *N*-desmethyltamoxifen, has recently been identified [5]. In view of the similarity in structure of tamoxifen and toremifene, it is likely that the same isoenzyme is involved, and we are currently investigating this possibility. There will likely be many other drugs sharing this isoenzyme, and the possibility of interactions and competition exists.

The decrease in bile flow and the increase in back-pressure observed in the higher-dose toremifene studies may be evidence of impaired liver function. An inhibition of drug metabolism resulting from the decrease in liver function might partly explain the increase in perfusate toremifene concentrations in the higher-dose steady-state toremifene studies (Fig. 2). However, it is also possible that toremifene or one of its metabolites may have inhibited its own metabolism. There has been no report of hepatic toxicity in clinical trials of toremifene, and although we did not detect any changes indicative of liver damage in 19 patients in our high-dose phase I trial [3], the results of this pre-clinical study would suggest that patients on high-dose toremifene should be monitored for signs of hepatotoxicity. Liver damage due to tamoxifen is rare but has been reported [4].

In this closed experimental system, it was possible to do a mass-balance calculation at the end of the 180-min perfusion. Of the total dose, less than 5% was recovered as toremifene or measured metabolites in perfusate and bile samples. It is unlikely that a significant amount would have remained undetected as other metabolites; 4-hydroxytoremifene (TOR-II), which was not measured in the present study, is a major metabolite in rats but is present at only double the level of TOR-I [17]. At least 20% of the dose was recovered in the liver homogenate. These findings indicate that the high clearance and volume of distribution of toremifene in this experimental model primarily

Table 4. Liver viability in the toremifene and control experiments

	Bile flow (ml/h)		Back-pressure (cm perfusate)	
	Pre-drug	180 min	Pre-drug	180 min
Control	0.67±0.12	0.49±0.08	5.8±0.6	6.8±0.2
Methanol control	0.84±0.13	0.52±0.06	5.3±0.8	11.0±2.6
Toremifene 3 μg/ml	0.78±0.11	0.39±0.17	6.6±1.0	11.1±2.4
Toremifene 10 μg/ml	0.69±0.05	0.22±0.05*	5.9±0.5	17.5±1.8*

Data represent mean values ± SD (n=4)
* $P < 0.05$ relative to methanol control values

reflect the uptake of toremifene by the rat liver. That approximately 70% of the dose was not accounted for in biological samples suggests that other mechanisms also contributed to toremifene clearance. Control experiments confirmed that toremifene disappeared from the perfusate in the absence of a liver. Since toremifene is lipophilic and highly protein-bound (99.7% bound to serum proteins [16]), it may have bound to some of the circuit components. The toremifene elimination half-life obtained in the present study, which is identical to the half-life observed in the non-liver control, would therefore be an artifact representing the half-life of adsorption to the circuit components. These results serve to illustrate the importance of doing non-liver control experiments when this investigational model is used.

In humans, TOR-I is the major metabolite and is present at twice the concentration of toremifene. Previous studies in rats have shown that toremifene undergoes slow but extensive metabolism to many metabolites, most of which can be recovered in faeces over several days [17]. Although no spontaneous conversion of toremifene to metabolites occurred in the absence of a liver, the metabolite concentrations observed in this study may not represent the true metabolite profiles. This is because the metabolites may have bound to the circuit components in a manner similar to that of the parent compound, and there is no way to predict their relative affinities for such binding. Regardless of this unknown factor, the data remain useful for general comparisons. In the bolus-dose studies, TOR-I was present in perfusate at approximately 60% of toremifene concentrations after a 3-h perfusion. Similarly, a high percentage of the toremifene dose remaining in the liver occurred as TOR-I. In contrast, over 90% of the dose excreted in bile over 180 min was recovered in the form of parent toremifene. Therefore, if only a small percentage is eventually excreted unmetabolised, extensive reabsorption of toremifene due to enterohepatic recycling must be occurring. Enterohepatic recirculation has been reported in both rats and humans [17, 25].

Toremifene is metabolised and excreted by the isolated perfused rat liver, but a significant amount binds to the perfusion circuit, preventing the determination of pharmacokinetics. Although clinically relevant doses of toremifene do not inhibit the elimination of antipyrine, high concentrations may cause liver damage, and monitoring of the liver function of patients receiving high-dose toremifene may be warranted.

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