Interspecies Comparison of *In Vitro* Endoxifen Metabolism

Miguel Muzzio[1], Zhihua Huang[1], Elizabeth R. Glaze[2], and David L. McCormick[1]

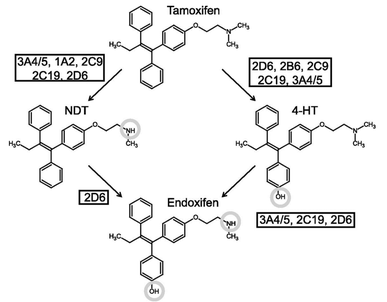
[1]IIT Research Institute, Chicago, IL 60616; [2]National Cancer Institute, Bethesda, MD 20852

ABSTRACT

Endoxifen (N-desmethyl-4-hydroxytamoxifen) is an active metabolite of tamoxifen being developed for use in breast cancer prevention and therapy. To optimize model selection for preclinical toxicology and pharmacology studies, the *in vitro* metabolism of endoxifen was compared using commercially available pools of hepatocytes from humans, rats, dogs, rabbits, minipigs, and non-human primates. Endoxifen (10 µM) was incubated with hepatocytes for 0, 2, or 4 hr. Cells were harvested, extracted, and analyzed by LC-MS/MS to quantitate levels of parent compound and major metabolites. Although substantial differences in patterns of endoxifen metabolism were seen in different species, endoxifen glucuronide and endoxifen sulfate were the primary metabolites identified in all species. Endoxifen was relatively stable when incubated with human hepatocytes: after 2 and 4 hr of incubation, endoxifen levels were > 20-fold and > 5-fold greater than levels of endoxifen glucuronide and endoxifen sulfate. Levels of endoxifen glucuronide and endoxifen sulfate in human hepatocytes were similar at both time points. By contrast, endoxifen was extensively glucuronidated (but not sulfated) in both rabbit and minipig hepatocytes: after 4 hr of incubation, levels of endoxifen glucuronide in rabbit and minipig hepatocytes were > 5 times the level of parent drug. Levels of endoxifen sulfate were very low in both rabbits and minipigs. Rats and dogs demonstrated patterns of endoxifen metabolism that were intermediate to those of humans, rabbits, and minipigs. In rat hepatocytes, levels of parent drug were slightly greater than levels of endoxifen glucuronide at 4 hr; little endoxifen sulfate was generated in rat hepatocytes. In dog hepatocytes, levels of endoxifen and endoxifen glucuronide were comparable at 4 hr; sulfation of endoxifen was greater in dogs than in other species, as levels of endoxifen sulfate at 4 hr were approximately 2/3 of the levels of parent drug and endoxifen glucuronide. Although endoxifen was somewhat less stable in hepatocytes isolated from non-human primates than in human hepatocytes, endoxifen metabolism in hepatocytes from non-human primates demonstrated the greatest similarity to metabolism in human hepatocyte pools: at 4 hr, levels of parent drug in monkey hepatocytes were approximately 4 times the levels of endoxifen glucuronide and 8 times the levels of endoxifen sulfate. These data demonstrate that endoxifen is quite stable when incubated with hepatocytes from humans and non-human primates. By contrast, endoxifen is rapidly and extensively glucuronidated in hepatocytes from rabbits and minipigs. Rats and dogs, the two species used most commonly in preclinical safety studies, both demonstrate substantially more rapid endoxifen metabolism than do humans.

BACKGROUND AND INTRODUCTION

Endoxifen (4-hydroxy-N-desmethyltamoxifen; 4-[(1*E*/*Z*)-1-[4-[2-(methylamino)ethoxy]-phenyl]-2-phenyl-1-buten-1-yl]-phenol) is a metabolite of tamoxifen that is produced by the sequential action of cytochromes P4503A4/5 (CYP3A4/5) and CYP2D6. Two stereoisomers, (*Z*)-endoxifen and (*E*)-endoxifen, are produced during P450-mediated metabolism of tamoxifen; (*Z*)-endoxifen is the more potent of the two isomers.



Endoxifen binds with high affinity to estrogen receptor α (ERα), and demonstrates *in vivo* and *in vitro* antiestrogenic activities that are comparable to that of the well-studied tamoxifen metabolite, 4-hydroxytamoxifen.

Tamoxifen and Endoxifen in Breast Cancer Prevention and Therapy

Tamoxifen is a standard first-line therapy for women with ER+ breast cancer, and is also effective in breast cancer prevention. Tamoxifen is a prodrug that requires CYP-mediated biotransformation; patient genotype (most notably, the presence of CYP2D6 polymorphisms) is a key determinant of tamoxifen efficacy. Data from clinical studies demonstrate that tamoxifen is significantly less active in women harboring CYP2D6 polymorphisms that reduce their ability to metabolize tamoxifen. Exposure to inhibitors of CYP2D6 (e.g., serotonin reuptake inhibitors such as paroxetine) may also reduce tamoxifen activity.

After oral administration of tamoxifen, plasma levels of endoxifen in patients with functional CYP2D6 are as much as 6 -fold greater than are plasma levels of 4-hydroxy-tamoxifen. Considering (a) the comparable antiestrogenic potencies of endoxifen and 4-hydroxytamoxifen, and (b) relatively higher plasma levels of endoxifen seen after oral administration of tamoxifen, endoxifen appears to be responsible for much of the pharmacologic activity of tamoxifen.

To obviate reductions in tamoxifen efficacy that may result from reduced metabolism in women with CYP2D6 polymorphisms and/or exposure to CYP2D6 inhibitors, endoxifen is being developed as a potentially more active antiestrogen for breast cancer prevention and therapy.

ACKNOWLEDGEMENT

This research was supported by contract HHSN2612015000241 from the Chemopreventive Agent Development Research Group, Division of Cancer Prevention, National Cancer Institute.

RATIONALE

This study is a component of a larger preclinical program to characterize the toxicity, pharmacokinetics (PK), and metabolism of endoxifen for breast cancer prevention. The primary focus of the program is the preclinical development of a gel formulation of endoxifen that is designed for direct topical application to the skin of the breast. It is proposed that local delivery of endoxifen to the breast will (a) maximize the quantity of endoxifen that reaches the breast epithelium, thus optimizing antitumor activity; and (b) reduce or eliminate systemic toxicity that may result from oral drug administration.

In addition to genetic toxicology studies (bacterial mutagenesis and mammalian cell structural chromosome aberration assays), dermal irritation/sensitization studies, and *in vitro* phototoxicity studies, we have completed subchronic oral toxicity studies of neat endoxifen in rats, dogs, and minipigs, and subchronic topical toxicity studies of endoxifen gel in minipigs.

The minipig is the standard species used for preclinical toxicology studies of agents being developed for topical application. However, the results of our studies in minipigs demonstrated that endoxifen undergoes very rapid Phase 2 metabolism to endoxifen glucuronide. The present studies were performed to compare patterns of endoxifen metabolism in hepatocytes from several species, in order to identify species whose metabolism of endoxifen is most similar to humans and are therefore most appropriate for use in chronic preclinical safety evaluations.

MATERIALS AND METHODS

Study Design: Commercially available pools of hepatocytes from human, minipig, rat, rabbit, dog, and monkey were used to compare *in vitro* patterns of endoxifen metabolism. Hepatocytes were incubated with endoxifen (10 µM) for 0, 2, or 4 hr, followed by analysis of endoxifen and endoxifen metabolites by LC-MS/MS.

Hepatocyte Pools: Cryopreserved hepatocytes from female donors (Table 1) were purchased from XenoTech, Lenexa, KS. Hepatocytes were freshly isolated, pooled and cryopreserved by the vendor, and were shipped and stored at ≤ -80 °C prior to use.

Table 1: Species and Identifiers for Hepatocytes

Species/Strain	Gender	ID/Lot Number	Pool Number
Human, CryostaX	Female	HPCH.Custom/1710060	10
Minipig, Gottingen	Female	Z6500.H15/PR15006	1
Rat, Sprague-Dawley	Female	R1500.H15/1510028	12
Rabbit, New Zealand	Female	L1500.H15/1610310	3
Dog, Beagle	Female	D1500.H15/1210438	3
Monkey, Cynomolgus	Female	P2500.H15A/1310011	3

Immediately prior to use, cryopreserved hepatocytes were rapidly thawed in a 37°C water bath and dispensed in pre-warmed OptiThaw Hepatocyte Kit Media Component (Xenotech). Cells were then centrifuged at 100 × g at RT for 5 min and the resulting pellet was separated from the supernatant and resuspended in OptiIncubate Hepatocyte Media (Xenotech). Cell counts and viability were determined by trypan blue exclusion. Cell suspensions were then diluted to a density of approximately 2 x 10^6 viable cells per mL with additional medium for use in metabolism studies. Verification of hepatocyte metabolizing capacity was demonstrated using testosterone as a positive control.

***In Vitro* Metabolism Studies:** Solutions of endoxifen, vehicle (DMSO), or positive control (testosterone) were diluted to appropriate target concentrations and transferred to uncoated 24-well plates. The same volume of hepatocyte suspension (containing 4 x 10^5 viable hepatocytes) was added to each well. Final concentration of endoxifen was 10 µM in an incubation volume of 400 µL; final concentration of testosterone was 250 µM. All incubations were run in triplicate.

Plates were gently mixed and placed in a 37°C/5% CO_2 humidified incubator for the length of the experiment (2 or 4 hr). Reaction wells were sampled at 0 hr (initial mixing), 2 hr, and 4 hr. At each sampling time, 100 µL aliquots were collected and mixed with 200 µL ice cold acetonitrile (ACN) to stop the reactions; samples were stored at -70°C until analyzed. For analysis, samples were thawed, vortex-mixed and centrifuged at 4°C and 7000 RPM for 10 minutes. Supernatant was collected, transferred to autosampler vials, and diluted with either 200 µL water (endoxifen samples) or 0.9 mL 20% (v/v) ACN in water (testosterone samples for analysis).

Analysis of Endoxifen and Metabolites: Levels of endoxifen and metabolites were quantitated using an AB SCIEX Model 4000 QTrap LC-MS/MS equipped with an Agilent Model 1200 HPLC.

Table 2: HPLC Conditions used to Quantitate Endoxifen and Metabolites

HPLC Column	Phenyl-Hexyl 2.6 µm 50×2.1 mm (Phenomenex)		
Column Temperature	25°C		
Injection Volume	5 µL		
Flow Rate	0.3 mL/min		
Mobile Phase A	0.1% formic acid in water		
Mobile Phase B	0.1% formic acid in acetonitrile		
Program	**Time (min)**	**Mobile Phase A (%)**	**Mobile Phase B (%)**
	0.00	65	35
	0.30	65	35
Retention Time	Endoxifen – approximately 1.7 minutes		
Run Time	5 minutes		

Table 3: MS/MS Conditions used to Quantitate Endoxifen and Metabolites

Scan Type	MRM		
Ion Source	Turbo Spray ESI		
Ion Spray Voltage	5500 Volts		
Polarity	Positive		
Ion Source Temperature	500 °C		
Collision Energy	45 Volts		
Ions Monitored (Q1®Q3):	**Biotransformation**	**(M+H)$^+$**	**Fragmentation Ions**
	Endoxifen (parent)	374.2	58.1
	N-demethylation	359.2	58.1
	Mono-hydroxylation	390.2	58.1
	Di-hydroxylation	406.2	58.1
	N-oxidation	390.2	74.1
	O-glucuronidation	550.2	58.1
	N-glucuronidation	550.2	234.2
	O-sulfation	454.2	58.1
	N-sulfation	454.2	138.2
	Glutathione Conjugation	679.3	58.1
Resolution	Unit		
Data System	Analyst® 1.6.3 (Sciex)		

RESULTS

Endoxifen-O-glucuronide and endoxifen-O-sulfate were the only metabolites that were consistently identified. Other Phase 1 and Phase 2 metabolites listed in Table 1 were below the limit of detection in all or most of the samples analyzed. For this reason, this data presentation is limited to relative levels of parent compound and the O-glucuronide and O-sulfate metabolites.

Endoxifen was quite stable when incubated with human hepatocytes (Figures 1 and 2). After 2 or 4 hr of incubation with human hepatocytes, endoxifen levels were > 20-fold greater than levels of endoxifen glucuronide and > 5 fold greater than levels of endoxifen sulfate. Production of endoxifen glucuronide and endoxifen sulfate by human hepatocytes was similar at both time points.

Figure 1: Levels of Endoxifen and Major Metabolites After 2 Hour Incubation with Hepatocytes from Different Species

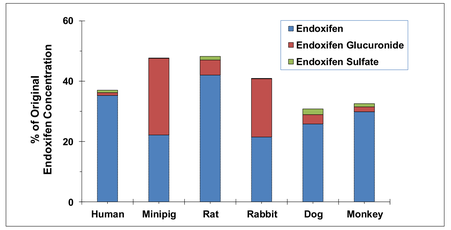
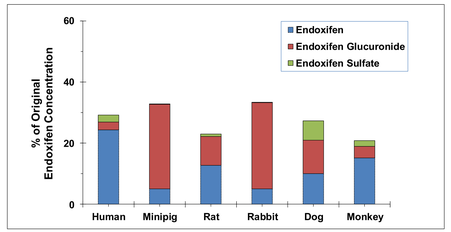


Figure 2: Levels of Endoxifen and Major Metabolites After 4 Hour Incubation with Hepatocytes from Different Species



At 2 hr, endoxifen was also metabolically stable when incubated with hepatocytes from rats, dogs, and monkeys (Figure 1); levels of parent compound measured after these incubations were substantially greater than levels of any metabolite. Each of these species demonstrated slightly greater metabolism to the O-glucuronide than to the O-sulfate.

After a 2 hr incubation, minipigs and rabbits both demonstrated much greater metabolism of endoxifen than did the other species studied. Endoxifen metabolism in both species was almost entirely to O-glucuronide; little metabolism to endoxifen-O- sulfate was seen after incubation for two hours with hepatocytes from either minipigs or rabbits.

Levels of metabolites were also much lower than levels of endoxifen after 4 hr of incubation with human or monkey hepatocytes (Figure 2). As seen after 2 hr, levels of endoxifen-O-glucuronide generated by human and monkey hepatocytes slightly exceeded levels of endoxifen-O-sulfate, At 4 hr, most endoxifen had been glucuronidated by minipig and rabbit hepatocytes (Figure 2); relatively little parent compound was measured. Rats and dogs demonstrated an intermediate level of endoxifen metabolism; both species generated greater levels of endoxifen-O-glucuronide than endoxifen-O-sulfate.

The relative production of Phase 2 metabolites by hepatocytes from each species is tabulated in Table 4. As described in Figure 1, total production of Phase 2 metabolites after a 2 hr incubation was less than 2% of the administered endoxifen dose in human hepatocytes, and less than 3% of the administered endoxifen dose in monkey hepatocytes. By contrast, production of Phase 2 metabolites (primarily endoxifen-O-glucuronide) at 2 hours by minipig hepatocytes was more than 25% of the administered dose of endoxifen, and was nearly 20% of the administered dose in at 2 hours in rabbit hepatocytes.

Table 4: Interspecies Comparison of *In Vitro* Phase 2 Metabolite Generation

Species	Metabolite Concentration (% of Administered Endoxifen Dose)	
	2 Hr Incubation	4 Hr Incubation
Endoxifen-O-Glucuronide		
Human	0.84	2.5
Minipig	25	28
Rat	5.0	9.4
Rabbit	19	28
Dog	3.1	11
Monkey	1.6	3.8
Endoxifen-O-Sulfate		
Human	0.89	2.3
Minipig	0.14	0.09
Rat	1.2	0.79
Rabbit	0.18	0.21
Dog	1.9	6.4
Monkey	1.0	1.9

CONCLUSIONS

1. Endoxifen-O-glucuronide and endoxifen-O-sulfate were the only metabolites consistently generated by hepatocytes from the species studied. Other Phase 1 and Phase 2 metabolites were below the limit of detection in most or all samples analyzed.
2. Endoxifen was relatively stable when incubated with human hepatocytes: after 2 and 4 hr of incubation, endoxifen levels were > 20-fold and > 5-fold greater than levels of endoxifen-O-glucuronide and endoxifen-O-sulfate, respectively. Levels of endoxifen-O-glucuronide and endoxifen-O-sulfate generated by human hepatocytes were similar at both incubation times.
3. Although endoxifen was slightly less stable when incubated with monkey hepatocytes than when incubated with human hepatocytes, endoxifen metabolism by monkey hepatocytes was most similar to metabolism by human hepatocytes: at 4 hr, levels of parent drug remaining in incubations with monkey hepatocytes were approximately 4 times the levels of endoxifen-O-glucuronide and 8 times the levels of endoxifen-O-sulfate.
4. By contrast, endoxifen was extensively glucuronidated (but not sulfated) by both rabbit and minipig hepatocytes; after 4 hr incubation, levels of endoxifen glucuronide in incubations with rabbit and minipig hepatocytes were > 5 times the levels of parent drug. Levels of endoxifen sulfate generated by both rabbit and minipig hepatocytes were very low. These data are in agreement with our recent report (Society of Toxicology, 2018) that parent drug is almost undetectable in minipigs following oral or topical administration of endoxifen to minipigs.
5. Rats and dogs demonstrated patterns of endoxifen metabolism that were intermediate to those of humans, rabbits, and minipigs.
   - In incubations with rat hepatocytes, endoxifen levels at 4 hr were slightly greater than were levels of endoxifen-O -glucuronide. Very little endoxifen-O-sulfate was produced by rat hepatocytes.
   - In incubations with dog hepatocytes, levels of endoxifen and endoxifen-O-glucuronide were comparable at 4 hr. Sulfation of endoxifen by dog hepatocytes was substantially greater than in other species: levels of endoxifen-O-sulfate after a 4 hr incubation were 2/3 of levels of parent drug and endoxifen-O-glucuronide.
6. From the perspective of comparative metabolism, the monkey is far superior to the other four animal species evaluated for studies of endoxifen pharmacokinetics and pharmacodynamics. In comparison to the rat, dog, rabbit, and minipig, the monkey best approximates both the qualitative and quantitative profile of endoxifen metabolism in humans.