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RESEARCH PAPER

Propranolol is a mechanism-based inhibitor of CYP2D and CYP2D6 in humanized CYP2D6-transgenic mice: Effects on activity and drug responses

Edgor Cole Tolledo[1] | Sharon Miksys[1] | Frank J. Gonzalez[2] | Rachel F. Tyndale[1]

[1]Campbell Family Mental Health Research Institute, Centre for Addiction and Mental Health (CAMH), Department of Pharmacology & Toxicology, Department of Psychiatry, University of Toronto, Toronto, Ontario, Canada

[2]Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Correspondence
Rachel F. Tyndale, Campbell Family Mental Health Research Institute, Centre for Addiction and Mental Health (CAMH), Department of Pharmacology & Toxicology, Department of Psychiatry, University of Toronto, Toronto, Ontario, Canada.
Email: r.tyndale@utoronto.ca

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Background and Purpose: Genetics and drug interactions contribute to large inter-individual variation in human CYP2D6 activity. Here, we have characterized propranolol inhibition of human and mouse CYP2D using transgenic (TG) mice, which express both mouse CYP2D and human CYP2D6, and wild-type (WT) mice. Our purpose was to develop a method for in vivo manipulation of CYP2D6 enzyme activity which could be used to investigate the role of CYP2D6 in drug-induced behaviours.

Experimental Approach: Dextromethorphan metabolism to dextrorphan was used to measure CYP2D activity and to characterize propranolol inhibition in vitro and in vivo. Effects of propranolol pretreatment (24 hr) on serum levels of the CYP2D6 substrate haloperidol and haloperidol-induced catalepsy were also studied.

Key Results: Dextrorphan formation velocity in vitro was threefold higher in liver microsomes of TG compared to WT mice. Propranolol acted as a mechanism-based inhibitor (MBI), inactivating CYP2D in liver microsomes from TG and WT mice, and humans. Pretreatment (24 hr) of TG and WT mice with 20 $mg \cdot kg^{-1}$ intraperitoneal propranolol reduced dextrorphan formation in vivo and by liver microsomes in vitro. Serum haloperidol levels and catalepsy were increased.

Conclusions and Implications: Propranolol was a potent MBI of dextrorphan formation in liver microsomes from TG and WT mice, and humans. The inhibition parameters in TG overlapped with those in WT mice and in humans. Inhibition of CYP2D with propranolol in vivo in TG and WT mice altered drug responses, allowing further investigation of variations in CYP2D6 on drug interactions and drug responses.

1 | INTRODUCTION

Cytochrome P450 2D6 (CYP2D6) is an important drug metabolizing enzyme estimated to be involved in the oxidation of 20–30% of clinically used drugs (Yu, Idle, & Gonzalez, 2004; Zanger & Schwab, 2013). The *CYP2D6* gene is highly genetically polymorphic with numerous *CYP2D6* allelic variants; the resulting CYP2D6 activity is used to describe poor metabolizers (PM), intermediate metabolizers, extensive metabolizers (EM), and ultra-rapid metabolizers (UM; Gaedigk, 2013). *CYP2D6* PM have little to no CYP2D6 enzyme activity (Eichelbaum, Spannbrucker, Steincke, & Dengler, 1979; Mahgoub, Dring, Idle, Lancaster, & Smith, 1977), and their *CYP2D6* genetic status has been associated with greater side effects when taking antineoplastic drugs (Jung & Lim, 2014; Zeng et al., 2013), antidepressants (de Leon et al.,

Abbreviations: CL_{int}, intrinsic clearance; CYP2D, cytochrome P450 2D isoforms from rodent species; CYP2D6, cytochrome P450 2D isoform from humans; EM, extensive metabolizers; MBI, mechanism-based inhibitor; PM, poor metabolizers; TG, transgenic; UM, ultra-rapid metabolizers; WT, wild-type.

2005; Llerena, Berecz, Dorado, & de la Rubia, 2004), and antipsychotics (Brockmoller et al., 2002; Schillevoort et al., 2002). PM also experience little analgesia after taking opioids that require metabolic activation by CYP2D6, such as codeine (Zahari & Ismail, 2014).

Phenoconversion is the process by which a genotype-predicted enzymatic activity is converted to a different phenotype, typically due to enzymatic inhibition or environmental factors such as illness (Shah & Smith, 2015; Zanger & Schwab, 2013). A mechanism-based inhibitor (MBI), also referred to as an irreversible or suicide inhibitor, is a substrate that is metabolized to a reactive intermediate that then covalently binds to the enzyme, rendering it irreversibly inactivated (Silverman, 1995). Inhibition by MBIs require the synthesis of new enzyme to restore enzymatic activity and thus the inhibition can be long lasting (Liston et al., 2002). Drugs such as methylenedioxymethamphetamine (Heydari et al., 2004), **paroxetine** (Bertelsen, Venkatakrishnan, Moltke, Obach, & Greenblatt, 2003), or cimetidine (Madeira, Levine, Chang, Mirfazaelian, & Bellward, 2004) are known to be MBIs of CYP2D6 and, when taken, lead to irreversible inhibition of CYP2D6 and the phenoconversion of UM and EM to intermediate metabolizers/PM (Juřica & Žourková, 2013; O'Mathúna et al., 2008).

Propranolol is a β-adrenoceptor antagonist metabolized by CYP2D6 (Lennard et al., 1984) that requires enzyme-catalysed metabolism for irreversible inhibition to occur in human liver microsomes (Shaw, Lennard, Tucker, Bax, & Woods, 1987) which suggests that propranolol is an MBI of human CYP2D6. Rowland et al. (1994) found evidence that 4-hydroxypropranolol, a propranolol metabolite, may be an MBI of CYP2D6 in vitro in human liver microsomes; however, they did not test propranolol itself as an MBI of CYP2D6 in vitro. While propranolol is also metabolized by **CYP3A4** and **CYP1A2**, it does not appear to be an MBI for these enzymes (Herman, Nakamura, Wilkinson, & Wood, 1983; Walle, Walle, Cowart, Conradi, & Gaffney, 1987). Unlike propranolol, we could find no evidence that other β-adrenoceptor antagonists are suspected to be MBIs of CYP2D6 or other enzymes. Propranolol is an MBI of rat CYP2D (we use CYP2D here to denote the isoform(s) from rodent species) and propranolol pretreatment in rats results in the inhibition of further propranolol metabolism both in vitro and in vivo (Schneck & Pritchard, 1981). In vitro, propranolol pretreatment in rats reduces CYP2D-specific metabolism of imipramine and debrisoquine by rat liver microsomes (Masubuchi et al., 1991).

A transgenic (TG) *CYP2D6* mouse line, which included the complete human *CYP2D6* gene and its regulatory sequence, has been created (Corchero et al., 2001). Recently, another TG mouse line was created with a wider CYP2D6 tissue distribution and with tissue-specific regulation of the human *CYP2D6* transgene (Cheng et al., 2013). These TG mice can model overexpression of CYP2D since they have both the mouse *Cyp2ds* and the human *CYP2D6* genes. Propranolol inhibition in TG mice could be a useful tool to investigate CYP2D6 in vitro and in vivo. However, the inhibition and inactivation characteristics of propranolol have not yet been described in mice or humans. These TG mice allow CYP2D6 activity to be manipulated (increased in TG and decreased via propranolol) in order to investigate more effectively the role of CYP2D6 in drug interactions, drug responses, toxicity, therapeutic effect, drug reward, and/or risk of abuse.

What is already known

- The humanized CYP2D6-transgenic mouse can be used to characterize CYP2D6 enzyme activity.
- Propranolol is a CYP2D mechanism-based inhibitor in rats.

What this study adds

- Characterization of propranolol as a mechanism-based inhibitor of mouse and human CYP2D in vitro.
- Manipulation of CYP2D in vivo with propranolol alters drug levels and response.

What is the clinical significance

- Long-lasting in vivo inhibition of CYP2D by propranolol suggests potential for drug–drug interactions.
- Propranolol pretreatment in transgenic mice can be used to investigate human CYP2D6 variation in vivo.

The aims of this study were to use TG (Cheng et al., 2013) and wild-type (WT) mice (a) to determine the relative velocity of hepatic CYP2D metabolism, (b) to assess propranolol as an as an MBI of CYP2D in vitro in liver microsomes from TG and WT mice and humans, and to derive inhibition and inactivation parameters, and (c) to investigate the in vivo metabolism of **dextromethorphan** and **haloperidol** (both CYP2D6 substrates; Schmid, Bircher, Preisig, & Küpfer, 1985; Shin, Kane, & Flockhart, 2001). Haloperidol induces catalepsy, an acute behavioural response, which was studied to assess the possible in vivo pharmacodynamic effects of propranolol pretreatment on metabolism catalyzed by CYP2D.

2 | METHODS

2.1 | Animals

All animal care and experimental procedures complied with the NIH guidelines for the care and use of laboratory animals and were approved by the University of Toronto Animal Care Committee. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010; McGrath & Lilley, 2015) and with the recommendations made by the *British Journal of Pharmacology*. Adult (8–12 weeks old) male TG (Cheng et al., 2013) and WT C57BL/6 mice (Charles River, St-Constant, Canada) were housed in groups of three to four under a 12 hr light/dark cycle with water and chow supplied ad libitum. All TG mice used for the

study were homozygous; all TG pups were genotyped for the presence of the human CYP2D6 transgene prior to use according to the published methods (Cheng et al., 2013). The homozygosity of founders was confirmed via back-breeding and genotyping for the transgene. All procedures were conducted in the light phase. Equal animal numbers from TG and WT mice were randomly assigned a pretreatment for each experiment; drug levels, enzymatic activity, and drug behavioural responses were assessed blinded to pretreatment group. In the rare case of technical failure, additional animals were assessed for final n = 10 per group, as suggested by Curtis et al. (2018). Based on pilot data for each approach, we determined that n = 10 per mouse line and pretreatment would be sufficient to determine differences.

2.2 | In vitro dextrorphan formation

Liver microsomes were prepared as previously described (Siu, Wildenauer, & Tyndale, 2006) using pooled (n = 7) untreated mice from each mouse line for baseline and inhibition assessments. Pooled human liver microsomes (Xenotech, Lenexa, USA) were used to compare in vitro CYP2D metabolism between TG mice and humans. The test substrate dextromethorphan undergoes CYP2D-specific O-demethylation to **dextrorphan** (Schmid et al., 1985). The assay conditions were adapted from Felmlee, Lon, Gonzalez, and Yu (2008), with time and protein concentration optimized for linear dextrorphan formation. Final incubation concentrations contained 100-mM potassium phosphate buffer (pH 7.4) and 1-mM **NADPH**. After preincubation with 50-μg microsomal protein (final concentration of 0.1 $\mu g \cdot \mu l^{-1}$) from TG, WT, or human liver for 2 min at 37°C, reactions were initiated by adding 50 μl of dextromethorphan (final concentration of 0.3–100 μM) for a total volume of 500 μl. Reactions were stopped with 500 μl of hexane–butanol (95:5 v/v) after 10 min.

2.3 | Propranolol inhibition of in vitro dextrorphan formation by liver microsomes

A schematic time course of the preincubation schedule for inhibitory studies can be found in Figure 1. To generate Dixon plots, reaction mixtures containing 50-μg microsomal protein (final concentration of 0.1 $\mu g \cdot \mu l^{-1}$) from TG or WT liver and propranolol (final concentration of 0–50 nM) in 100-mM potassium phosphate buffer were prewarmed at 37°C for 2 min before adding NADPH (final concentration of 1 mM). After a 5 min preincubation, reactions were initiated by adding dextromethorphan (final concentration of 2.5, 5, or 10 μM) for a total volume of 500 μl.

To generate IC_{50} plots, reaction mixtures containing 50-μg microsomal protein (final concentration of 0.1 $\mu g \cdot \mu l^{-1}$) from TG, WT, or human liver and propranolol (final concentration of 0–100 μM) were prewarmed at 37°C for 2 min, before adding NADPH after either 0 or 5 min during a subsequent 5 min preincubation (Figure 1). Reactions were initiated by adding dextromethorphan (final concentration of 5 μM, approximate K_m) for a total volume of 500 μl.

To generate inactivation curves, reaction mixtures containing 50-μg microsomal protein (final concentration of 0.1 $\mu g \cdot \mu l^{-1}$) from TG, WT, or human liver and propranolol (final concentration of 0–10 μM) were prewarmed at 37°C for 2 min, before adding NADPH after 0, 2.5, 4, or 5 min during a subsequent 5 min preincubation (Figure 1). Reactions were initiated by adding dextromethorphan (final concentration of 5 μM) for a total volume of 500 μl.

2.4 | In vivo dextrorphan formation

Mice were pretreated i.p. 24 hr before experimental testing with either propranolol hydrochloride (20 $mg \cdot kg^{-1}$ in saline) or saline alone (n = 10 per group). After 24 hr, dextromethorphan hydrobromide (30 $mg \cdot kg^{-1}$ in saline i.p.) was injected and saphenous vein blood was

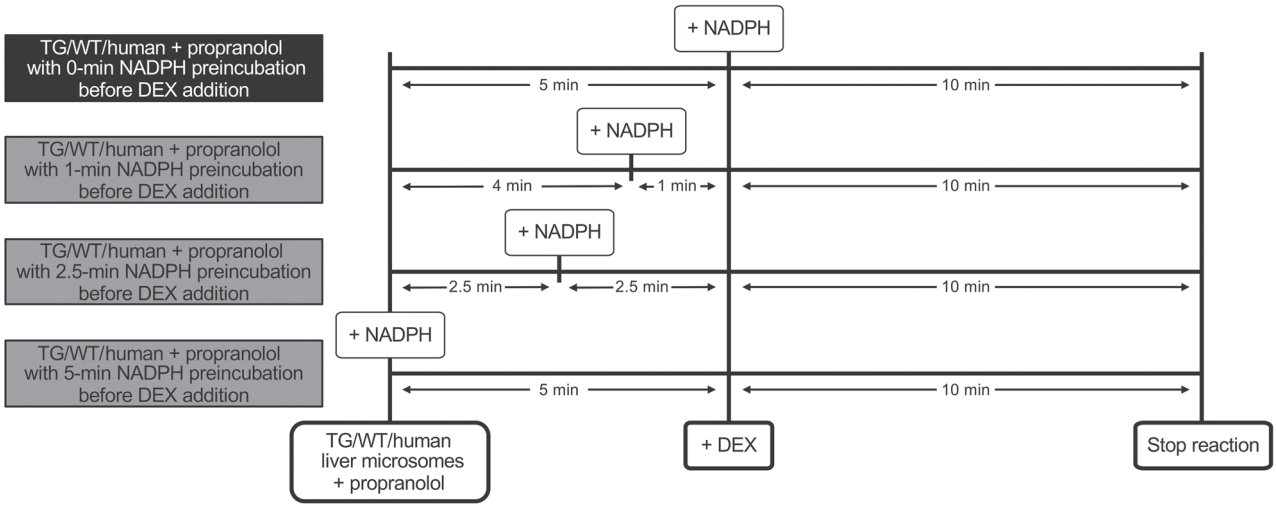


FIGURE 1 Schematic times courses of the conditions used for propranolol incubations with different preincubation times. Pooled liver microsomes of TG, WT, and humans were co-incubated with propranolol, and NADPH was added at different times listed above, before **dextromethorphan** (DEX) was added to each sample, and the reaction was carried out for another 10 min

collected 30 min later. Blood was collected after dextromethorphan T_{max} (estimated as 15 min in mice; Sakai et al., 2014) so that we are at the descending limb of the dextromethorphan curve. Mice were immediately killed, and tissues were collected and stored at −80°C. Dextromethorphan and dextrorphan levels in serum and from in vitro incubates were quantified using HPLC, with standard curves for dextromethorphan and dextrorphan (5–500 ng·ml^{-1}) as previously described (Miksys et al., 2017). The ratio of serum dextrorphan / **dextromethorphan** was used as an index of in vivo hepatic CYP2D activity. Given that the half-life of propranolol in mice is ~45 min (Levy, Ngai, Finck, Kawashima, & Spector, 1976), the 24 hr pretreatment represents approximately 32 half-lives and thus we did not attempt to measure propranolol concentrations.

2.5 | Haloperidol-induced catalepsy

Catalepsy was assessed as the latency to removal of a mouse's forepaws from a surface raised 9 cm above the cage floor, with a 420-s maximum cut-off time (Miksys et al., 2017). Twenty-four hours after i.p. propranolol or saline pretreatment (n = 10 per group), mice received haloperidol (0.1 mg·kg^{-1} s.c., injectable formulation, 5 mg·ml^{-1} base in water adjusted to pH 3–3.8 with lactic acid), and catalepsy was measured 120 min later. Timing and dose were selected from dose–response curves (testing from 0 to 180 min against 0.05, 0.1, and 0.2 mg·kg^{-1} s.c. injections of haloperidol) produced from TG and WT mice to enable detection of increases or decreases in catalepsy. Saphenous vein blood samples were taken at 60 and 180 min after haloperidol injection to assess the effects of pretreatments on serum haloperidol levels. Blood was collected at equal times before and after the time when catalepsy was assessed to minimize the stress from blood collection on catalepsy testing while ensuring that the earliest blood draw was after haloperidol T_{max} in mice (Zetler & Baumann, 1985). The effects of vehicle and propranolol pretreatments were tested within animals, with a 2-week washout period between tests. Serum haloperidol levels were quantified using LC-MS as previously described (Miksys et al., 2017); no haloperidol metabolites were able to be measured. The internal standard, 0.5-ng haloperidol-d4 dissolved in 30:70 of 5-mM ammonium formate/acetonitrile, was added to each sample and to haloperidol calibration standards (1–500 ng·ml^{-1}).

2.6 | Data and statistical analyses

The data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). The data were analysed using GraphPad Prism (RRID: SCR_002798; version 6.0c; La Jolla, California, USA). Michaelis–Menten parameters, K_m and V_{max}, as well as the corresponding standard error for both parameters were estimated using non-linear regression. Michaelis–Menten parameters were also confirmed using the Eadie–Hofstee method. Intrinsic clearance (CL_{int}) was calculated as the ratio of V_{max} to K_m. The inhibition constant K_i was determined according to Dixon (1953). IC_{50} was estimated using non-linear regression and calculated as the concentration of propranolol that was halfway between the top and bottom plateaus of the curve for a one-site enzyme (Neubig, Spedding, Kenakin, & Christopoulos, 2003). For two-enzyme systems, $IC_{50,High}$ and $IC_{50,Low}$ are defined as IC_{50} for the high affinity sites and the low affinity sites, respectively. To estimate inactivation constants, the initial rate constant of inactivation of dextrorphan formation by each propranolol concentration (K_{obs}) was assessed by linear regression analysis of the natural logarithm of the percentage of activity remaining against preincubation time data (Kitz & Wilson, 1962). Thereafter, the K_{obs} values were used to determine the inhibitor concentration needed to cause a half-maximal rate of enzyme inactivation (K_I) and the maximal rate of inactivation ($k_{inactivation}$). The K_I and $k_{inactivation}$ were estimated by non-linear regression using the following equation (Jones et al., 1999): $K_{obs} = \frac{k_{inactivation} * [I]}{K_I + [I]}$. Serum dextrorphan / **dextromethorphan** ratios and hepatic dextrorphan formation velocities were analysed by two-tailed, unpaired sample t tests, while catalepsy scores and serum haloperidol levels were analysed by two-tailed, paired sample t tests. Statistical analyses were only performed with measures that had at least five animals per group; n = 10 individual animals per group (treatment and mouse line) were the basis of the independent values, using within or between statistical analyses as indicated. All values are expressed as mean ± *SEM* unless otherwise stated. A P value of <.05 was considered statistically significant. Data were normalized to mean saline pretreatment controls (fold mean of the controls) within each mouse line to illustrate the effect of propranolol pretreatment; however, all statistical analyses were run with raw, non-normalized data. Outliers were included in data analysis and figures.

2.7 | Materials

We obtained propranolol hydrochloride and dextromethorphan hydrobromide from Sigma-Aldrich Canada (Oakville, Canada); haloperidol from Omega (Montreal, Canada) and haloperidol-d4 from Toronto Research Chemicals (Toronto, Canada).

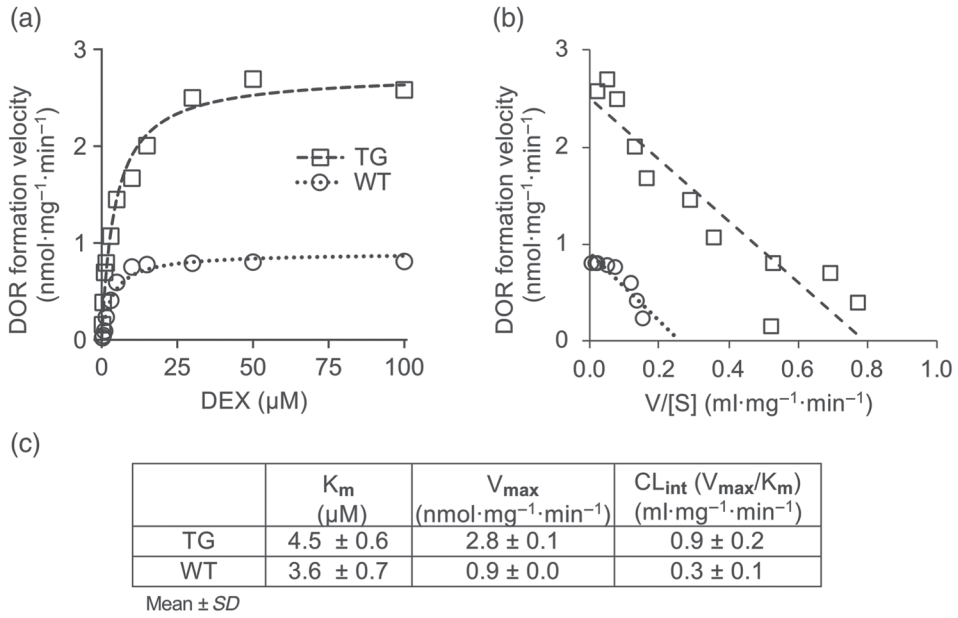
2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

3 | RESULTS

3.1 | TG mice exhibit faster in vitro dextrorphan formation compared to WT mice

Using liver microsomes, V_{max} values of 2.8 nmol·mg^{-1}·min^{-1} for TG and of 0.9 nmol·mg^{-1}·min^{-1} for WT mice and a K_m of 4.5 μM for TG



(c)

	K_m (μM)	V_{max} (nmol·mg^{-1}·min^{-1})	CL_{int} (V_{max}/K_m) (ml·mg^{-1}·min^{-1})
TG	4.5 ± 0.6	2.8 ± 0.1	0.9 ± 0.2
WT	3.6 ± 0.7	0.9 ± 0.0	0.3 ± 0.1

Mean ± *SD*

FIGURE 2 TG mice show faster dextrorphan (DOR) formation by liver microsomes compared to WT mice. Michaelis–Menten (a) and Eadie–Hofstee (b) plots of liver microsomal formation of dextrorphan from **dextromethorphan** (DEX). Enzyme kinetic values were obtained from these plots (c)

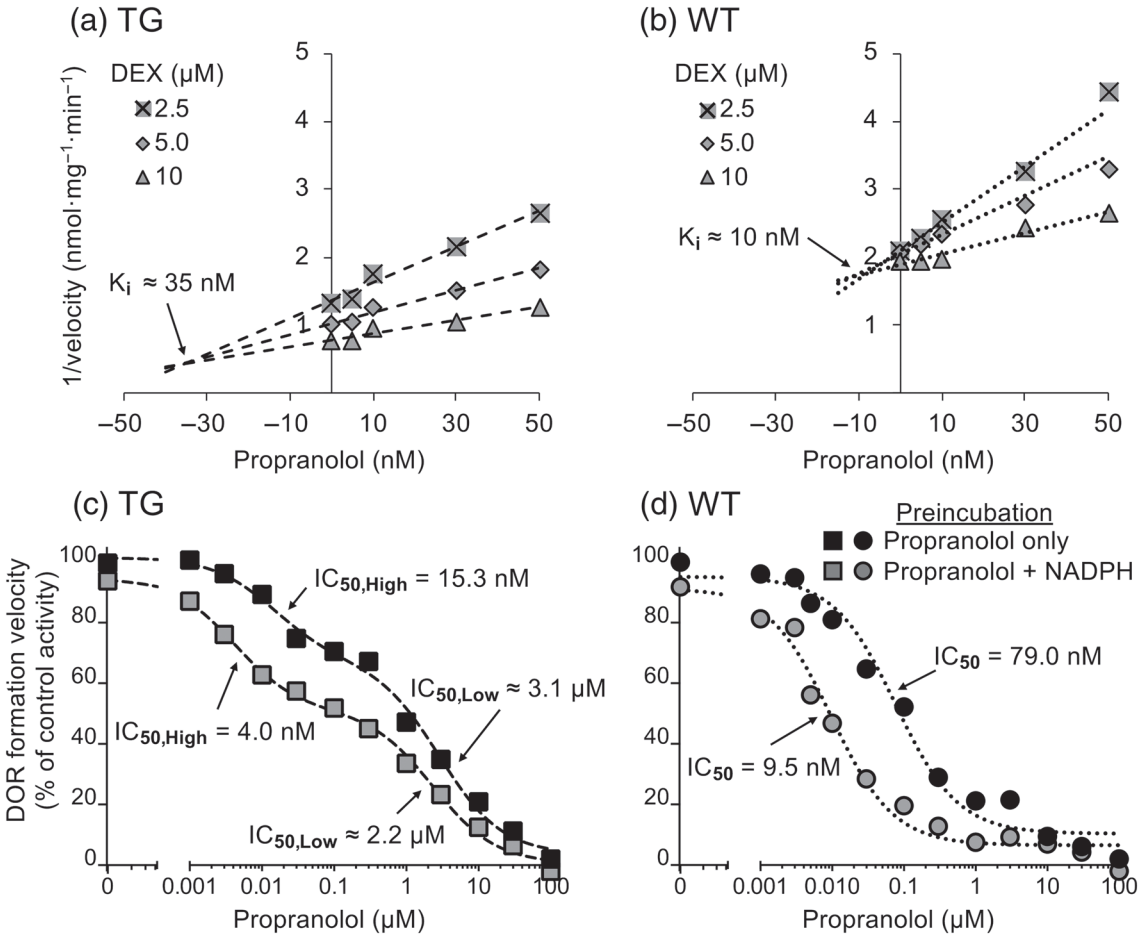


FIGURE 3 Propranolol acts as an MBI of dextrorphan (DOR) formation in TG and WT mouse liver microsomes in vitro. Dixon (a and b) and IC_{50} plots (c and d) of dextrorphan formation by pooled TG (a and c) and WT (b and d) mouse liver microsomes. In (a) and (b), pooled TG or WT mouse liver microsomes (0.1 mg·ml^{-1}) were incubated with propranolol (0–50 nM) for 5 min before 2.5, 5, or 10 μM of dextromethorphan (DEX) was added. In (c) and (d), pooled TG or WT mouse liver microsomes (0.1 mg·ml^{-1}) were incubated with propranolol (0–100 μM), with or without NADPH, for 5 min before dextromethorphan (5 μM) was added. The difference in dextrorphan formation between propranolol preincubation, with and without NADPH, before the dextromethorphan was added, suggests that propranolol acts as an MBI of CYP2D in liver microsomes from TG (c) and WT mice (d)

and of 3.6 μM for WT mice were derived from Michaelis–Menten and Eadie–Hofstee plots (Figure 2a,b). The CL_{int} was estimated to be threefold faster in TG compared to WT mice. The kinetic parameters for each mouse line are summarized in Figure 2c.

3.2 | Propranolol irreversibly inhibits in vitro dextrorphan formation by liver microsomes

A K_i of 35 nM for TG and of 10 nM for WT mouse liver microsomes was observed for propranolol, with **dextromethorphan** as substrate (Figure 3a,b). The IC_{50} plots demonstrated that the inhibitory effect of propranolol on dextrorphan formation velocity in TG and WT mouse liver microsomes was increased by preincubation with NADPH. The data suggested propranolol inhibition of a one-site system for WT and of a two-site system for TG mouse liver microsomes. Propranolol preincubation with NADPH decreased the $IC_{50,High}$ (3.8-fold) and $IC_{50,Low}$ (1.4-fold) in TG mouse liver microsomes and decreased the IC_{50} (8.3-fold) in WT mouse liver microsomes (Figure 3c,d). Propranolol inhibited in vitro dextrorphan formation velocity in a concentration- and time-dependent manner for TG and WT mouse liver microsomes (Figure 4a,b). The $k_{inactivation}$ and K_I for CYP2D were 0.11 min^{-1} and 84 nM for TG and 0.24 min^{-1} and 11 nM for WT mouse liver microsomes, respectively (Figure 4c,d).

Propranolol preincubation with NADPH decreased the IC_{50} in human liver microsomes (1.8-fold) compared to preincubation with propranolol alone (Figure 5a). Propranolol also inhibited in vitro dextrorphan formation velocity in a concentration- and time-dependent manner for human liver microsomes (Figure 5b), and the $k_{inactivation}$ and K_I for CYP2D6 were 0.05 min^{-1} and 420 nM, respectively (Figure 5c).

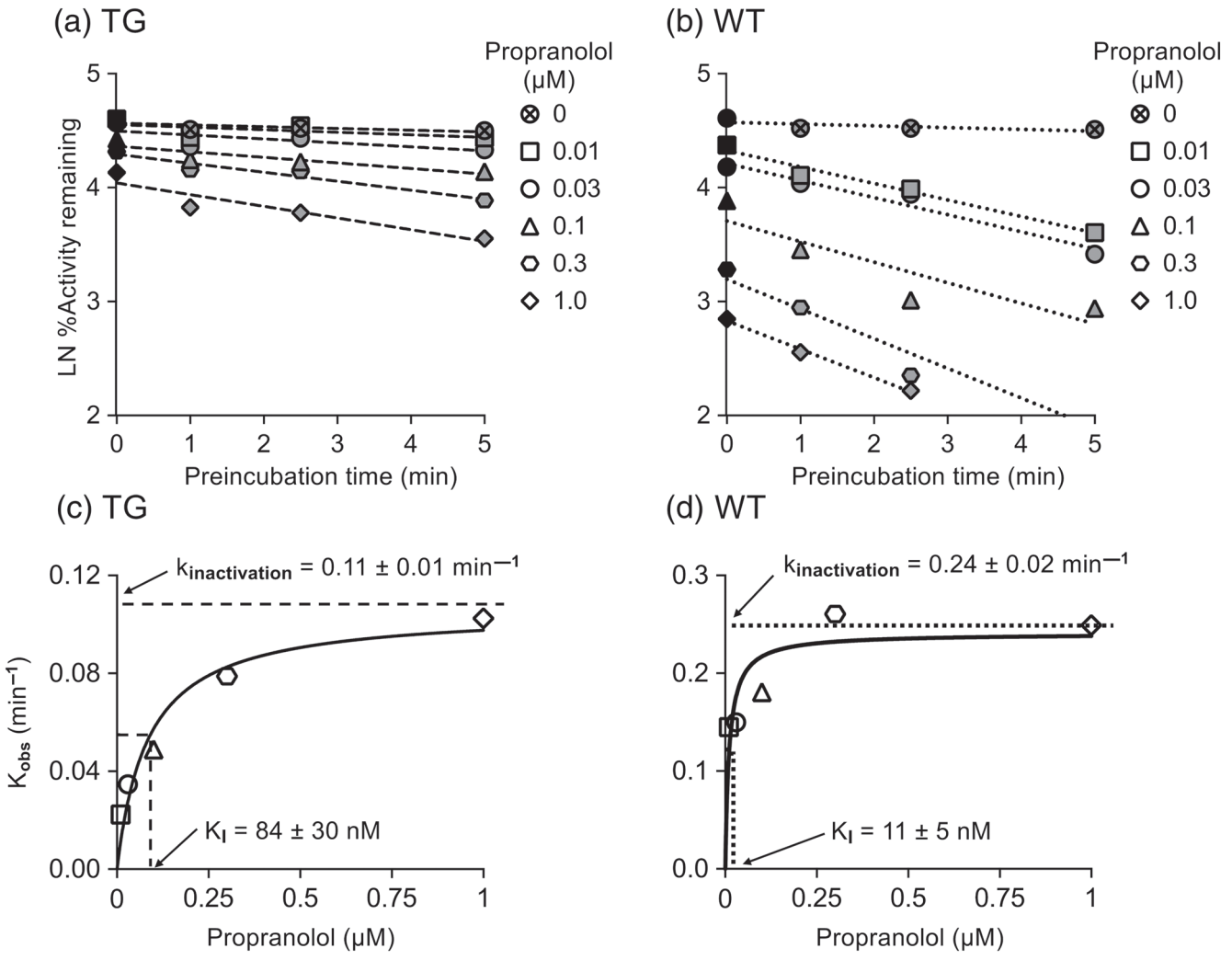


FIGURE 4 Propranolol inhibits dextrorphan formation velocity by liver microsomes from TG and WT mice in a time- and concentration-dependent manner. In (a) and (b), propranolol (0-1 μM) inhibition of dextrorphan formation velocity by pooled TG and WT mouse liver microsomes (0.1 $mg \cdot ml^{-1}$) was preincubation time and dose-dependent. In (c) and (d), the rate of inactivation of CYP2D (K_{obs}) by each propranolol concentration was plotted to determine $k_{inactivation}$ and K_I

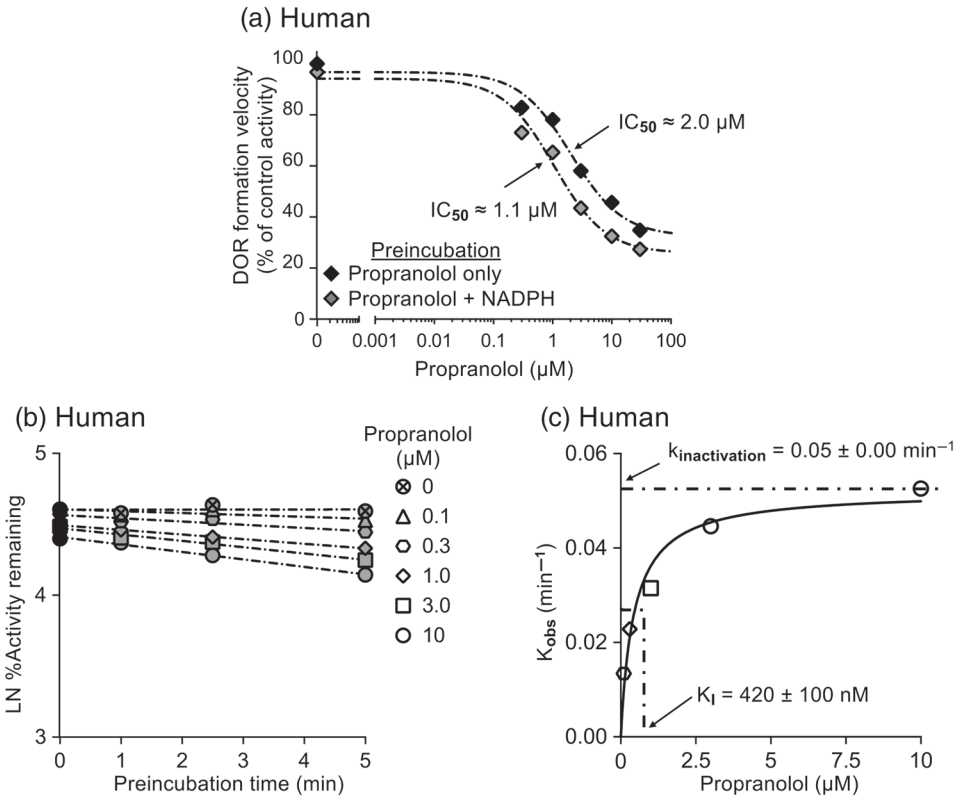


FIGURE 5 Propranolol acts as an MBI of CYP2D6 in human liver microsomes in vitro. In (a), pooled human liver microsomes (0.1 mg·ml^{-1}) were preincubated with propranolol (0–30 μM) for 5 min before dextromethorphan (5 μM) was added. The difference in dextrorphan (DOR) formation between propranolol preincubation with and without NADPH, before dextromethorphan was added, suggests that propranolol acts as an MBI of CYP2D6 in liver microsomes from humans. In (b), propranolol (0–10 μM) inhibition of dextrorphan formation velocity by pooled human liver microsomes (0.1 mg*ml^-1) was preincubation time and dose-dependent. In (c), the rate of inactivation of CYP2D6 (K_{obs}) by each propranolol concentration was plotted to determine $k_{inactivation}$ and K_I

3.3 | Propranolol pretreatment irreversibly inhibits dextrorphan formation in TG and WT mice in vivo and in vitro

The serum dextrorphan / dextromethorphan ratio was significantly reduced in both TG (by 26%, Figure 6a) and WT mice (by 33%, Figure 6b) by 24 hr i.p. pretreatment with propranolol, compared to saline. Using the liver microsomes from these pretreated mice, dextrorphan formation velocity in vitro was significantly reduced in both TG (by 22%, Figure 6c) and WT mice (by 25%, Figure 6d). Our results - the long-lasting (greater than 24 hr) inhibition by propranolol of subsequent in vivo and in vitro dextrorphan formation - suggest that propranolol pretreatment irreversibly inhibits CYP2D in TG and WT mice in vivo, consistent with the inhibition previously observed in vitro (Figure 4a,b).

3.4 | Propranolol pretreatment increases serum haloperidol levels and haloperidol-induced catalepsy

Serum haloperidol levels were significantly increased by 24 hr i.p. pretreatment with propranolol, compared to saline pretreatment within animals, in both TG (by 19% and 28%, at 60 and 180 min, respectively, Figure 7a; AUC_{60-180} by 22%, Figure 7c) and WT mice (by 30% and 37%, at 60 and 180 min, respectively, Figure 7b; AUC_{60-180} by 32%, Figure 7d). Similarly, the mean catalepsy scores at 120 min after the haloperidol injection were significantly increased by 24 hr i.p. pretreatment with propranolol, compared to saline pretreatment within animals, in both TG (by 31%, Figure 7e) and WT mice (by 35%, Figure 7f).

4 | DISCUSSION

We have shown that TG have faster hepatic dextrorphan formation in vitro compared to WT mice, and that propranolol irreversibly inhibits dextrorphan formation in liver microsomes of TG and WT mice and humans in vitro. Pretreatment of the mice with propranolol (24 hr, i.p.) resulted in a significant reduction of dextrorphan formation in vivo in both TG and WT mice, and a significant reduction of dextrorphan formation velocity was observed in liver microsomes prepared from these pretreated animals. After showing that propranolol acts as an MBI of CYP2D-mediated dextromethorphan metabolism to dextrorphan, haloperidol was used to confirm the effects of propranolol mechanism-based inhibition of CYP2D by measuring serum haloperidol levels and response to halperidol. Propranolol pretreatment resulted in a significant increase in serum haloperidol levels and haloperidol-induced catalepsy. These data indicate that propranolol exerts mechanism-based inhibition of hepatic CYP2D in vitro and in vivo in TG and WT mice, and that this inhibition is sufficient to alter drug-induced behaviour (catalepsy).

The CYP2D6 MBI paroxetine is known to have long-lasting effects in vivo in humans, as a short-term (6 weeks) paroxetine treatment required a 4-week washout period before CYP2D6 disinhibition (Juřica & Žourková, 2013). Longer term (~18 weeks) paroxetine use was reported to need an even longer 6-week washout period, and

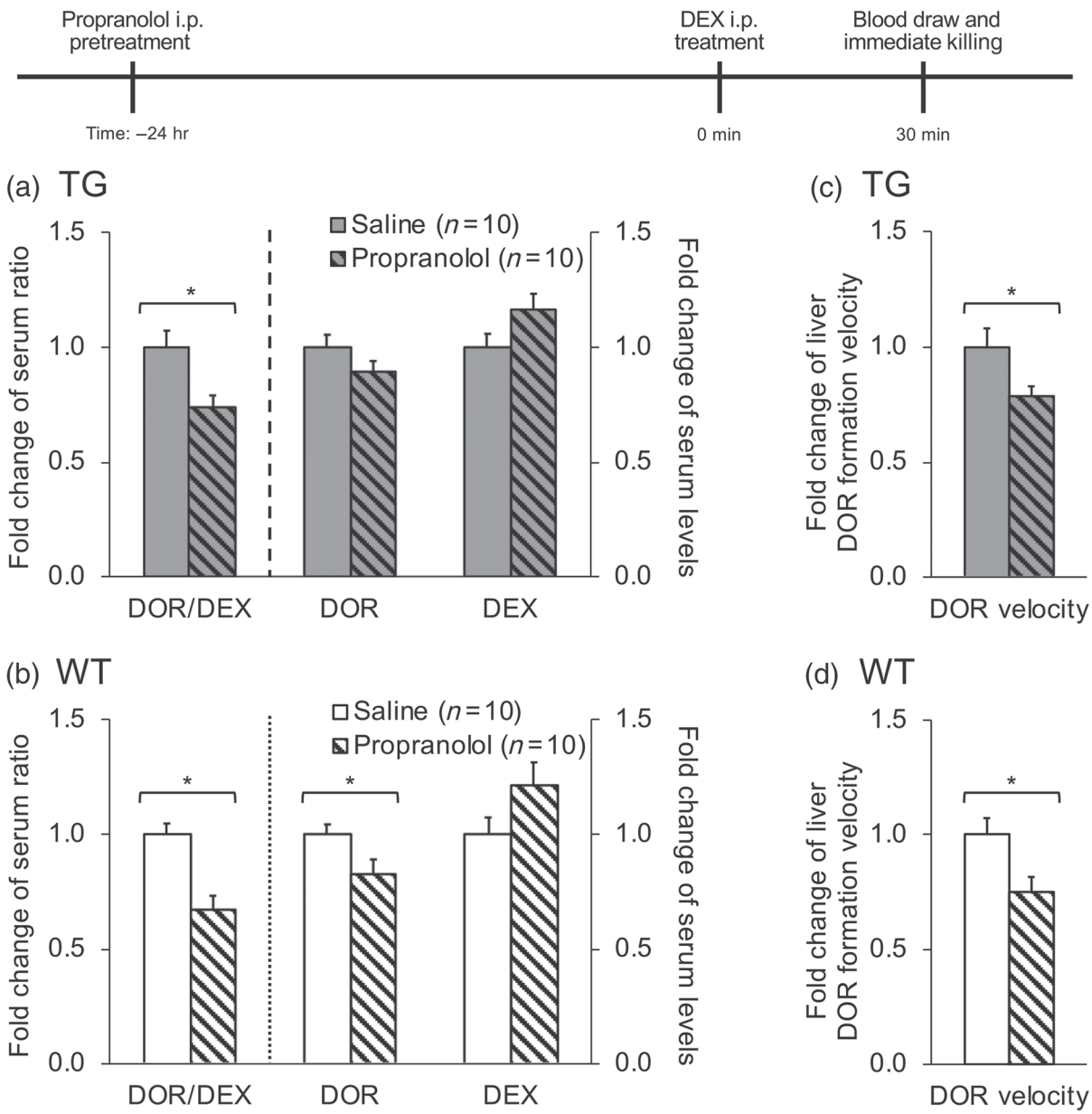


FIGURE 6 Pretreatment (24 hr) with 20 mg·kg^{-1} i.p. propranolol inhibited dextrorphan (DOR) formation in vivo and in vitro in liver microsomes from TG and WT mice. After 24 hr i.p. pretreatment with either saline or propranolol, all mice were given an i.p. injection of 30 mg·kg^{-1} of dextromethorphan (DEX) and blood was collected via saphenous vein 30 min after dextromethorphan injection. In (a) and (b), the in vivo serum dextrorphan/dextromethorphan ratio, serum dextrorphan and serum dextromethorphan levels in TG and WT mice were assessed. In (c) and (d), the dextrorphan formation by liver microsomes (0.1 mg·ml^{-1}) prepared from pretreated mice was assessed after incubation with dextromethorphan (5 μM) for 10 min. Data presented are expressed as mean (with SEM) values of individual animals normalized to the saline pretreated group, within the same mouse line. *P < .05, significantly different from saline pretreated group; two-tailed, unpaired t test on the raw data

CYP2D6 inhibition was still present in three of eight patients after 6-weeks (Juřica & Žourková, 2013). Co-medication with propranolol increased drug concentrations of other CYP2D6 substrates (Greendyke & Kanter, 1987; Jones et al., 1999; Kiss et al., 2019) and increased adverse events (Drake & Gordon, 1994; Zhou, Anthony, Roden, & Wood, 1990). These results taken together suggest that patients taking propranolol with other CYP2D6 substrates, especially when those substrates are CYP2D6 MBIs themselves (e.g., methylenedioxymethamphetamine, paroxetine, and cimetidine), are at greater risk for adverse events. Here, we have derived inactivation parameters of CYP2D6 mechanism-based inhibition by propranolol in human liver microsomes. These data can be useful in improving our understanding of the clinical effects of CYP2D6 inhibition by propranolol. Careful consideration of treatment with CYP2D6 substrates may be required in patients taking propranolol, even if propranolol has been discontinued prior to the administration of the CYP2D6 substrate.

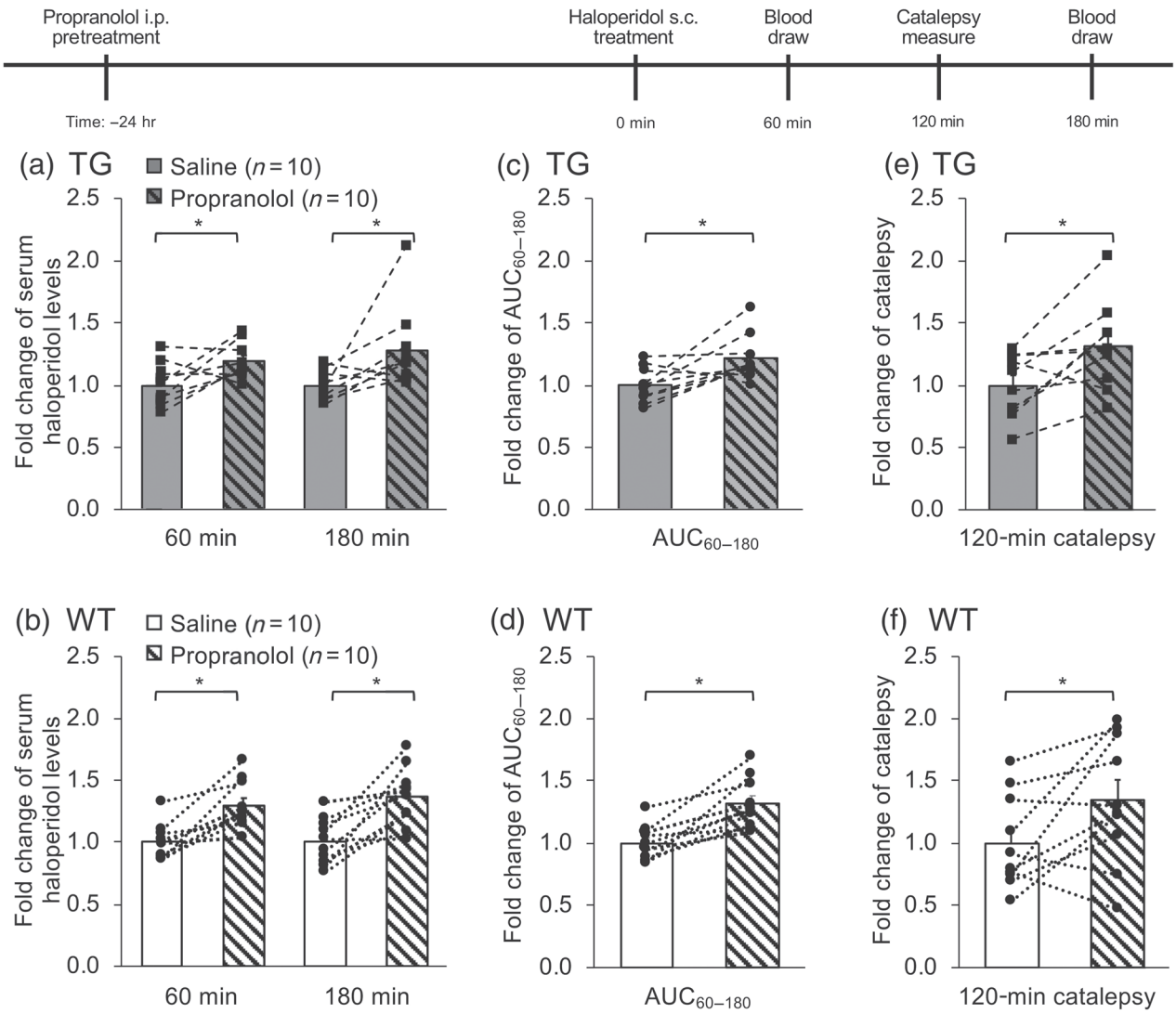


FIGURE 7 Serum haloperidol, serum haloperidol AUC_{60-180}, and acute haloperidol-induced catalepsy are increased (within animals) in TG and WT mice after 24 hr i.p. propranolol pretreatment. After 24 hr i.p. pretreatment with either saline or propranolol, all mice were given a s.c. injection of 0.1 mg·kg^{-1} of haloperidol, and serum haloperidol levels were assessed at 60 and 180 min after the haloperidol injection (a and b), and serum haloperidol AUC from 60 to 180 min was derived (c and d). Catalepsy was tested at 120 min after haloperidol injection (e and f). Data are expressed as mean (with SEM) values of each individual animal normalized to the saline pretreated group, within the same mouse line. Pretreatments were given 2 weeks apart. *$P < .05$, significantly different from saline pretreated group; two-tailed, paired t test on the raw data

Propranolol acts as an MBI against mouse CYP2D in TG and WT, both in vitro and in vivo. We did not investigate whether other CYPs were also inhibited by propranolol pretreatment as our conditions for in vitro and in vivo testing were optimized to specifically investigate CYP2D activity (as measured by dextrorphan formation and the dextrorphan / **dextromethorphan** ratio). The propranolol IC_{50} plots with WT mouse liver microsomes suggest one-site inhibition, while the propranolol IC_{50} plots with TG mouse liver microsomes suggest two-site inhibition. These two sites in TG mouse liver microsomes may correspond to mouse CYP2D and human CYP2D6, but it is difficult to distinguish between the two as the dextrorphan formation K_m of mouse CYP2D (Felmlee et al., 2008) and human CYP2D6 (Kerry, Somogyi, Bochner, & Mikus, 1994) overlap. The higher affinity site in TG has a similar IC_{50} value to WT mice, suggesting that this higher affinity site represents the mouse CYP2D. The lower affinity site in TG mouse liver microsomes has a similar IC_{50} value to human liver microsomes, and similar to the IC_{50} value previously observed (6.6 μM; Obach et al., 2006), suggesting that the lower affinity site represents human CYP2D6. The mouse *Cyp2d* cluster is composed of nine genes (*Cyp2d9*, *d10*, *2d11*, *2d12*, *2d13*, *2d22*, *2d26*, *2d34*, and *2d40*; Nelson et al., 2004) and the mRNA of six are transcribed to different extents in the liver (*Cyp2d9*, *2d10*, *2d11*, *2d22*, *2d26*, and *2d40*; Renaud, Cui, Khan, & Klaassen, 2011). Other organs in WT mice also express CYP2D isozymes (Miksys, Cheung, Gonzalez, & Tyndale, 2005), and this variation in organ-specific expression is even greater in TG mice with the addition of the differential expression of human CYP2D6 (Cheng et al., 2013). Inhibition of CYP2D by propranolol may be useful in studying the

role of CYP2D and CYP2D6 metabolism within other organs, such as intestine, heart, and brain.

In this study, we used a two-step method without dilution at lower microsome and inhibitor concentrations to determine if propranolol was an MBI. This two-step method has the advantage of using a lower microsome concentration to reduce inhibitor binding to microsomes, as well as to reduce reactive-intermediate formation in the primary incubation that can affect the secondary incubation (Parkinson et al., 2011). The disadvantage of this method is that without a dilution step, there is an increased chance of inactivation and reversible inhibition occurring in the secondary incubation with the test substrate, thereby decreasing sensitivity (Grimm et al., 2009). Another approach to determine mechanism-based inhibition is to start with high microsome and inhibitor concentrations and then to dilute them 10-fold after a primary preincubation step, prior to the addition of the test substrate to assay the CYP activity in a secondary incubation (Grimm et al., 2009). As described earlier, the dilution step reduces the degree of inactivation and reversible inhibition, and should thus result in increased sensitivity. However, concerns have been raised about this dilution step, given that higher initial microsome concentrations can lead to higher amounts of reactive intermediates formed in the primary incubation which can potentially inflate the extent of protein inactivation that can occur in the secondary incubation (Parkinson et al., 2011). Thus, in this study, we used the former approach.

Our data showing that TG have threefold higher V_{max} and turnover than WT mice suggest that the TG mouse could be used to represent CYP2D6 UM, while the WT mouse could model CYP2D6 EM, as previously suggested (Cheng et al., 2013; Corchero et al., 2001). A similar fold difference in activity was observed by Kiss et al. (2018) in human liver microsomes where the rate of dextrorphan formation from dextromethorphan in UM was ~2.5-fold higher compared to EM. For a number of drugs, high drug levels in vivo in CYP2D6 PM, as a result of genetic status (Brockmoller et al., 2002; Llerena et al., 2004), or from CYP2D6 MBIs (Juřica & Žourková, 2013; O'Mathúna et al., 2008), could increase the occurrence of adverse events. Using the long-lasting, mechanism-based inhibition by propranolol of mouse CYP2D and human CYP2D6 in TG, and of mouse CYP2D in WT, we can model the reduction of CYP2D6 UM and EM metabolism from an acute exposure to a CYP2D6 MBI. Propranolol pretreatment was sufficient to alter in vivo dextromethorphan metabolism; the decrease in serum dextrorphan / dextromethorphan ratio of saline pretreated TG mice (0.43), compared to propranolol pretreated TG mice (0.31) resulted in ratios that were similar to saline pretreated WT mice (0.33). Propranolol pretreatment was also sufficient to affect in vivo haloperidol metabolism (as seen in the modest increase in serum haloperidol AUC) and behaviour (as seen with the significant increase in haloperidol-induced catalepsy), and was sufficient to show clear proof of MBI inactivation (as seen within the in vitro inhibition of liver microsomes from these mice). This was observed despite this propranolol pretreatment being given 24 hr prior to testing. This 24 hr propranolol pretreatment did not affect baseline catalepsy response, suggesting that haloperidol-induced catalepsy was increased due to irreversible inhibition of CYP2D-mediated haloperidol metabolism and not through the effects of propranolol itself. If even under these circumstances we see a modest shift in in vivo metabolism and response in propranolol pretreated mice, then chronic or concurrent propranolol treatments in patients may have a substantial effect on their overall CYP2D6 metabolism. For example, propranolol inhibited the metabolism of another CYP2D6 substrate (debrisoquine) in vivo in humans taking propranolo for a week (Rowland et al., 1994). This approach could also be useful for investigating novel CYP2D substrates—using both the relative rate of metabolism between the WT and TG mice, and the effects of propranolol's CYP2D mechanism-based inhibition of in vivo drug metabolism (e.g., changes in serum drug levels) and drug response, including side effects, as we have done here using haloperidol-induced catalepsy. This approach of giving an MBI such as propranolol 24 hr prior to testing is also useful for avoiding the possible confounding pharmacodynamic effects that competitive inhibitors have in vivo which can hinder their usefulness in modelling decreases in activity. The combined use of propranolol and TG mice could also be used to investigate drug interactions, therapeutic effects, and risk for drug dependence and abuse.

In conclusion, we provide evidence for mouse lines that can be used to model extensive and ultra-rapid CYP2D6 metabolism, and the effect of acute propranolol (mechanism-based inhibition) in vitro and in vivo on resulting drug levels and response. Our data also provides evidence for long-lasting inhibition of CYP2D by propranolol, suggesting caution when prescribing propranolol with other CYP2D6 substrates.

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AUTHOR CONTRIBUTIONS

E.C.T. performed the experiments and analysed the data. E.C.T., S.M., and R.F.T. contributed to the study design. E.C.T., S.M., F.G., and R.F.T. contributed to the manuscript writing. All authors approved the final version of this paper.

CONFLICT OF INTEREST

R.F.T. has consulted in the past for Quinn Emanuel and Ethismos on unrelated topics. E.C.T., S.M., and F.G. declare no conflict of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and

Animal Experimentation, and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

ORCID

Edgor Cole Tolledo https://orcid.org/0000-0003-2655-1815
Sharon Miksys https://orcid.org/0000-0001-7642-3745
Frank J. Gonzalez https://orcid.org/0000-0002-7990-2140
Rachel F. Tyndale https://orcid.org/0000-0003-1297-2053

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