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A Natural Product Ligand of the Oxysterol Receptor, LXR

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Cellular and Molecular

List of non-standard abbreviations:

TIF2: Transcription intermediary factor 2, SRC-1: Steroid receptor coactivators-1, ER: Estrogen receptor, FXR: farnesoid X receptor, PPAR: peroxisome proliferator-activated receptor, bDNA: branched DNA, OHC: hydroxycholesterol, LXR: Liver X receptor, NHR: nuclear hormone receptor, PXR: Pregnane X receptor, RXR, retinoid X receptor, LBD: Ligand binding domain, ABC: ATP binding cassette transporter, SREBP: Sterol regulatory element binding protein

Abstract

Natural products have been identified as ligands for a number of members of the nuclear hormone receptor (NHR) superfamily. Often these natural products are used as dietary supplements to treat a myriad of ailments ranging from perimenopausal hot flashes to hypercholesterolemia and reduced cognitive function. Examples of some natural product ligands for NHRs include genestein (estrogen receptors; NR3A1, NR3A2), guggulsterone (farnesoid X receptor; NR1H4) and St. John's wort (pregnane X receptor; NR1I2). In this study, we identified the first non-oxysterol natural product that functions as a ligand for the liver X receptor (LXRα and LXRβ; NR1H3, NR1H2), a NHR that functions as the receptor for oxysterols and plays a key role in regulation of cholesterol metabolism and transport as well as glucose metabolism. We show that paxilline, a fungal metabolite, is an efficacious agonist of both LXRα and LXRβ in biochemical and *in vitro* cell-based assays. Paxilline binds directly to both receptors, and is an activator of LXR-dependent transcription in cell-based reporter assays. We also demonstrate that paxilline binding to the receptors results in efficient activation of transcription of two physiological LXR target genes, ABCA1 and SREBP. The discovery of paxilline, the first reported non-oxysterol natural product ligand of the LXRs, may provide insight into the mechanism of ligand recognition by these receptors and reaffirms the utility of examining natural product libraries for identifying novel NHR ligands.

Natural products are proven sources of biologically active molecules that have played a critical role in pharmacology. Many of these natural products have been demonstrated to be useful for medicinal purposes. A limited number of natural products appear to act as ligands of nuclear hormone receptors. One of the most well characterized natural product nuclear receptor ligands is genestein, a product of soy, which binds directly to estrogen receptors (NR3A1 and NR3A2) and is used as a dietary supplement to alleviate menopausal symptoms in women (Kuiper et al., 1998; Tham et al., 1998). Interestingly, genestein has been recently shown to also function as a low affinity ligand for yet another nuclear receptor, peroxisome proliferator-activated receptor γ (NR1C3) (Dang et al., 2003). A related isoflavone soy product also used in dietary supplements, daidzein, is another estrogen receptor agonist, albeit weaker than genestein (Wiseman, 2000). The active compound within St. John's wort, an herbal remedy for depression, is a potent agonist of the xenobiotic nuclear receptor, pregnane X receptor (PXR; NR1I2) (Moore et al., 2000; Watkins et al., 2003). Although the antidepressent activity of St. John's wort does not appear to be mediated by its PXR activity, the induction of cytochrome P450 (CYP) 3A4 is mediated by activation of this receptor potentially leading to a significant increase in the metabolism of a variety of drugs taken by individuals using this supplement (Moore et al., 2000). The most recent report of a natural product ligand of a nuclear receptor is the discovery that guggulsterone, found in the resin of the guggul tree, is an efficacious antagonist of the farnesoid X receptor (FXR; NR1H4), the bile acid receptor (Urizar et al., 2002; Wu et al., 2002). Guggul tree extract has been suggested to lower low-density lipoprotein levels in animal models, has been successfully used in Ayurveda medicine to treat obesity and lipid disorders, and a modern antihyperlipoproteinemic drug based on the actions of guggulsterone is

marketed in India (Satyavati, 1988; Singh et al., 1990; Dev, 1997). Beyond the use of natural products directly as dietary supplements, identification of novel natural product ligands for a given receptor often provides pharmacological tools and unique insight into drug design.

The liver X receptors (LXRα (NR1H3) and LXRβ (NR1H2)) are additional members of the nuclear receptor superfamily and were originally identified as orphan receptors (Shinar et al., 1994; Song et al., 1994; Seol et al., 1995; Teboul et al., 1995; Willy et al., 1995). Subsequently, oxysterol cholesterol metabolites were demonstrated to be physiological ligands for LXR (Janowski et al., 1996). These two receptors play a key role in regulation of cholesterol metabolism and transport as well as glucose metabolism and inflammation (Repa and Mangelsdorf, 2002; Cao et al., 2003; Joseph et al., 2003). Modulation of the activity of these receptors may be useful in the treatment of a number of pathophysiological states including dyslipidemia, atherosclerosis, and diabetes (Joseph et al., 2002; Tangirala et al., 2002; Cao et al., 2003).

In this study, we describe the discovery of the first non-oxysterol natural product ligand of LXR. The indole alkoid fungal metabolite from *Penicillium paxilli*, paxilline, functions as a ligand for both LXRα and LXRβ. Paxilline binds directly to both receptors leading to recruitment of coactivators and activates the receptors in a cell-based context. In addition, paxilline efficaciously induces the expression of LXR target genes, ABCA1 and SREBP. Our data confirm that natural product libraries are a rich source of ligands for nuclear receptors and may provide pharmacological agents for investigation of the function of these receptors as well as potential drugs.

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Methods

LXR Ligands

22(R) hydroxycholesterol and paxilline were obtained from Sigma Chemical (St. Louis, MO).

Radioligand Binding Assay

The LXR radioligand binding assay was performed using scintillation proximity technology as previously described (Thomas et al., 2003). We utilized 800 ng of baculovirus-expressed, His-tagged LXRα-LBD protein (aa 162-447) or 600 ng of LXRβ-LBD protein (aa 202-461), 25 nM ^{3}H-25 Hydroxycholesterol (Amersham, Piscataway, NJ), 0.05 mg Yttrium Silicate polylysine coated SPA beads (Amersham, Piscataway, NJ), and varying concentrations of competitor per well of a 96-well OptiPlate (Packard, Meriden, CT). Protein, radioligand, and competitor were added to the plate. SPA beads were then added to the assay plate followed by 10 minutes gentle shaking at room temperature protected from light. The plates were incubated in the dark at room temperature for two hours prior to reading in a TopCount plate reader (Packard, Meriden, CT).

Coactivator Interaction Assay

Interaction between LXRα/LXRβ and the coactivators SRC-1 or TIF-2 were assayed using AlphaScreen™ (amplified luminescent proximity homogenous assay) technology (Perkin-Elmer Life Sciences). The assay was performed in white, low volume, 384-well plates utilizing a final volume of 15 µl containing final concentrations of 20 nM of His-tagged baculovirus expressed LXRα-LBD or LXRβ-LBD protein, 5nM of GST-TIF-2 or GST-SRC-1 protein that contained the entire nuclear receptor interacting domain of the coactivator protein fused to GST and 10 µg/ml of both Ni^{2+} chelate donor beads and anti-GST acceptor beads (Perkin-Elmer Life Sciences). The assay buffer contained 25 mM HEPES (pH 7.0), 100 mM NaCl, 0.1 % BSA, and 2 mM DTT. All manipulations involving assay beads were done in ambient light. Assay plates

were covered with a clear seal and incubated in the dark for two hours after which the plates were read for 1sec/well in a Perkin-Elmer Fusion microplate analyzer using the manufacturers standard AlphaScreen™ detection protocol.

Cell Culture and Transfections

HEK293 cells were cultured in 3:1 DMEM:F-12 containing 10% fetal bovine serum and supplemented with 1% penicillin and streptomycin, 1% L-glutamine, and 20mM Hepes. Forty-eight hours before transfection, cells were seeded at 6 x 10^6 cells/T225 flask in 30 ml growth media. Cells were transfected with Fugene transfection reagent (Roche) according to the Fugene protocol with 330 ng pcDNA3-hRXR-α, 33ng pCMV6 LXR-α or –β, 660 ng pGL3B-E1b-3XLXRE luc (25), and 10 μL Fugene per 10^6 cells. Growth media was replaced during transfection with 3:1 DMEM:F-12 containing 10% Charcoal/dextran treated, heat-inactivated fetal bovine serum and supplemented with 1% penicillin and streptomycin, 1% L-glutamine, and 20mM Hepes. After 24 hours, cells were harvested and plated into 96-well white plates at 50,000 cells/well in 90 μL complete transfection media, allowed to attach for 2 hours, then treated with 10uL of 10X compound and DMSO controls. After 24 hours, cells were lysed and assayed for luciferase activity.

ABCA1 mRNA Quantitation

ABCA1 mRNA expression was measured in THP-1 macrophage cells using a bDNA assay (QuantiGene® High Volume Kit (Bayer Diagnostics)). THP-1 cells were grown in suspension at 37ºC 5%/95% CO_2/air incubator in growth media (RPMI 1640 media containing 0.05 mM 2-mercaptoethanol and 10% Fetal Bovine Serum (FBS)) at a density of 250,000 cells per ml and allowed to reach a density of 1 million cells per ml. Growth media was then changed to growth

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media containing 10 nM phorphol 12-Myristate 13 Acetate (PMA) and cells were plated in 96 well dishes at a density of 100,000 cells per well. After an overnight incubation, media was changed to a growth media containing 10% lipoprotein depleted FBS. Cells were treated with various concentrations of compounds serially diluted to obtain a 10-point concentration curve from a final concentration of 20 μM to 0.001 μM. After a 24 hours incubation with compound, cells were lysed using 50 μl/well of bDNA assay kit lysis reagent. The kit reagents as well as ABCA1 specific primer sets were used to process the samples for the bDNA assay as previously described (Zhang et al., 2002). After a 15-minute incubation at 37°C, 100 μl of the lysis buffer from each well were transferred to the corresponding wells of the capture plate. The capture plate was incubated overnight at 53°C. The capture plate was then washed twice with QuantiGene® wash buffer followed by addition of 100 μl/well QuantiGene® amplifier working reagent. The plate was incubated for 60 minutes at 46°C followed by two washes. The mRNA to be measured was then labeled by addition of 100 μl QuantiGene® label probe working buffer followed by a 60 minute incubation at 46°C. The capture plate was then washed twice followed by addition of 100 μl/well QuantiGene® substrate plus QuantiGene® enhancer reagent. The plates were incubated at 37°C for up to 30 minutes and then read on a luminometer to detect the luminescent signal. The induction of ABCA1 mRNA expression was calculated as a ratio of compound-treated luminescent levels compared to untreated control levels.

SRE Assay and SREBP mRNA Quantitation

As previously described (Thomas et al., 2003), HepG2 cells stably transfected with a 3XSRE thymidine kinase luciferase reporter construct were treated for 24 h with an LXR ligand in order to assess SREBP activity. SREBP mRNA was quantitated by Taqman real time PCR as previously described (Thomas et al., 2003).

Data Analysis

Dose responses and displacement curves were analyzed in GraphPad Prism allowing calculation of both EC_{50} and K_i values. Each point of data represents minimal triplicate wells and the results shown are representative of at least three independent experiments.

Results

A screen of a natural product library for compounds with the ability to modulate the activity of LXR yielded the identification of the first non-oxysterol natural product ligand for this receptor, paxilline. Paxilline is an indole alkoid metabolite from the fungus, *Penicillium paxilli*. The structure of paxilline is shown in figure 1 and is compared to several LXR ligands including a natural ligand, 22(R) hydroxycholesterol as well as synthetic ligands GW3965, T0901317, and APD (Repa et al., 2000; Schultz et al., 2000; Collins et al., 2002; Sparrow et al., 2002).

We examined the ability of paxilline to bind directly to both LXRα and LXRβ using a radioligand binding assay. A scintillation proximity assay format was employed using tritiated 25-hydroxycholesterol as the radioligand. As illustrated in figure 2, paxilline displaced 25-hydroxycholesterol from both receptors (LXRα K_i=660 nM; LXRβ K_i=1100 nM). In contrast a natural oxysterol ligand, 22R-hydroxycholesterol (22(R)OHC) displayed higher affinity in this assay (LXRα K_i=250 nM; LXRβ K_i=490 nM) (Figure 2). Paxilline did not bind and/or activate any other nuclear receptor examined (IC_{50} and/or EC_{50} >10 μM for ERα, ERβ, TR, RXR, FXR) (data not shown). Paxilline also induced LXR recruitment of both coactivators SRC-1 or TIF-2 in a cell free AlphaScreen™ assay system (Figure 3). Using purified recombinant LXRα or LXRβ along with purified GST-SRC-1 or GST-TIF2 we demonstrated that increasing amounts of paxilline resulted in dose-dependent recruitment of these coactivators to both LXRs. The EC_{50} for paxilline mediated LXRα recruitment of SRC-1 was 1800 nM (22(R)OHC = 2600 nM) while TIF-2 was 660 nM (22(R) OHC = 1400 nM). The EC_{50} for paxilline mediated LXRβ recruitment of SRC-1 was 930 nM (22(R)OHC = 300 nM) while TIF-2 was 1200 nM (22R OHC = 780 nM). The ability of paxilline to induce coactivator recruitment by LXR suggested that paxilline might function as an agonist. This was confirmed in a transfection experiment in which

HEK293 cells were contransfected with either LXRα or LXRβ along with a reporter containing 3 copies of a DR4 element derived from the ABCA1 promoter (Figure 4). Paxilline activated transcription of both LXRα and LXRβ with equivalent potency and efficacy as 22(R)OHC (EC_{50} ~ 4000 nM for both receptors and ligands). The apparent discrepancy between the affinity of 22(R)OHC for LXR in the binding assay and the potency in a cotransfection assay has been previously described and is apparently a function of the physiochemical properties of this compound (Janowski et al., 1999). Interestingly, paxilline functions as partial agonist in the coactivator interaction assay (60-90% 22(R)OHC) but as a full agonist in the cotransfection assays. This suggests that either additional coactivators are recruited in the cell-based assay that allow for retention of full efficacy or that only a threshold of coactivator recruitment is required to reach full agonism. Consistent with its function as an agonist in both the coactivator recruitment and cotransfection assay, paxilline induced the expression of ABCA1 in THP-1 cells very efficaciously with a maximal induction of approximately 7-fold (EC_{50} = 1300 nM) (Figure 5). In this paradigm, the dose response for 22(R)OHC was limited by toxicity above 10 μM; however, the potency is greater than 5000 nM and the maximal efficacy at 10 μM is approximately 6-fold. Paxilline also increased the expression of a second LXR target gene, SREBP, in HepG2 cells (Figure 6A). We utilized a SREBP responsive luciferease reporter stably transfected into HepG2 cells (Thomas et al., 2003) to investigate the dose-responsiveness of paxilline induction of SREBP expression. As illustrated in figure 6B, paxilline efficiently increased transcription from the SRE reporter with an EC_{50} of 2800 nM. These data demonstrate that paxilline binds directly to both LXRα and LXRβ, functions as an agonist with the ability to mediate recruitment of coactivators to the receptors, and activates transcription of a reporter gene as well as two natural LXR target genes.

Discussion

Since the identification of LXRs as orphan members of the nuclear hormone receptor superfamily in mid-1990s, several ligands both natural and synthetic have been identified. Endogenous oxysterols were the first ligands identified for LXR, which suggested a role for this receptor in regulation of cholesterol homeostasis via action as a "cholesterol sensor" (Janowski et al., 1996). The first target gene identified for LXR, cholesterol 7α-hydroxylase (CYP7A), was consistent with this suggestion illustrating a role for this receptor in regulation of the rate limiting step in the conversion of cholesterol to bile acids (Lehmann et al., 1997), which was later confirmed in the LXR null mouse (Peet et al., 1998). In addition to its function in regulation of CYP7A, which appears to be rodent specific, an array of additional target genes have been identified that establish LXR as a key regulator of cholesterol homeostasis. These genes include sterol transporters such as ABCA1, ABCG1, ABCG5, and ABCG8, as well as other genes demonstrated to be critical in lipid metabolism including apoE, apoCII, LPL, PLTP, and CETP (Luo and Tall, 2000; Repa et al., 2000; Venkateswaran et al., 2000; Laffitte et al., 2001; Zhang et al., 2001; Cao et al., 2002; Mak et al., 2002; Repa et al., 2002). The manner in which LXR regulates these genes suggests that activation of this receptor may be anti-atherogenic, which has been confirmed in mouse models (Joseph et al., 2002; Tangirala et al., 2002). The therapeutic potential of a LXR agonist has recently expanded by the demonstration that activation of this receptor results in both anti-inflammatory and anti-diabetic activity by regulating an array of genes involved in either inflammatory processes or gluconeogenesis, respectively (Cao et al., 2003; Fowler et al., 2003; Joseph et al., 2003).

Development of selective, high affinity ligands for LXR such as T1317 and GW3965 has proven to provide essential tools in characterization of the physiological and pathophysiological roles of

LXR. In this study, we identified and characterized an additional ligand of LXR, which represents the first non-oxysterol natural product ligand for this receptor. Natural products have proven to be an abundant source of agents both for pharmacological characterization of biomolecules as well as for medicinal purposes. Although natural product libraries have not been as profitable for identification of ligands for nuclear hormone receptors as they have been for other fields such as ion channel pharmacology, key ligands have been identified in the past targeting receptors such as ER, FXR, PXR, and PPARγ. We identified paxilline, an indole alkoid fungal metabolite from *Penicillium paxilli*, as an efficacious LXR agonist. Paxilline binds directly to both LXRα and LXRβ resulting in coactivator recruitment and activation of LXR-dependent gene transcription. Paxilline exhibits similar potency and efficacy as the natural ligand, 22(R)OHC, in both cotransfection assays and in terms of induction of expression of a natural target gene, ABCA1. Additional pharmacological activities of paxilline precluded examination of modulation of LXR activity *in vivo* due to toxicity. Paxilline is a tremorgenic mycotoxin that is a well-characterized antagonist of high conductance calcium-activated K channels (BK channel) with potencies in the range of 100 nM, which is clearly greater than the potencies we detected for the LXRs in the low single digit μM range (Knaus et al., 1994).

Given the limitations for evaluation of paxilline *in vivo*, this compound still provides an additional tool for pharmacological characterization of LXR. In addition, the novel chemical structure provides insight into the diversity of chemical structures that can recognize the ligand binding pockets of both LXRα and LXRβ leading to coactivator recruitment and transcriptional activation. Furthermore, identification of an additional natural product ligand for a nuclear hormone receptor indicates that natural product libraries may be a rich source for ligands of additional nuclear hormone receptors including the orphans.

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Legends for Figures:

Figure 1. Chemical structures of LXR ligands. Paxilline is compared to previously described ligands including the natural ligand, 22(R) hydroxycholesterol. The structures of 3 LXR ligands, T0901317 (T1317), GW 3965, and APD, identified by Tularik, GlaxoSmithKline, and Merck are also shown.

Figure 2. Paxilline binds to both LXRα and LXRβ. A scintillation proximity radioligand binding assay was performed utilizing either recombinant LXRα or LXRβ along with a selective radioligand ^{3}H-25 OHC. Paxilline displaced the radiolabeled 25-OHC with a K_i of 660 nM for LXRα and 1100 nM for LXRβ. The natural ligand 22(R)OHC displayed higher affinity for both receptors with a K_i of 250 nM for LXRα and 490 nM for LXRβ.

Figure 3. Binding of paxilline to LXR results in coactivator recruitment. Utilizing Alpha-Screen™ technology, we evaluated whether paxilline would recruit coactivators (SRC-1 or TIF-2) to either LXR subtype in a biochemical model. Recombinant LXRs were attached to the donor bead via His-tag Ni^{2+} ion interaction while GST-fusion coactivators were attached to acceptor beads via a GST-Ab. Paxilline treatment resulted in dose-dependent recruitment of coactivators to either receptor subtype. For paxilline, the EC_{50} for recruitment of SRC-1 and TIF-2 to LXRα was 1800 nM and 660 nM, respectively. The EC_{50} for recruitment of SRC-1 and TIF-2 to LXRβ was 930 nM and 1200 nM, respectively. For the natural LXR ligand, 22(R)OHC, the EC_{50} for recruitment of SRC-1 and TIF-2 to LXRα was 2600 nM and 1400 nM,

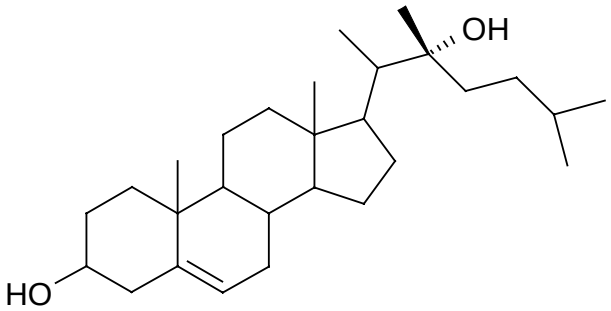
respectively. The EC for recruitment of SRC-1 and TIF-1 to LXRβ was 300 nM and 780 nM, respectively. In all cases, paxilline appears to have partial agonist activity compared to 22(R)OHC with maximal efficacies ranging from 60-90% of 22(R)OHC levels.

Figure 4. Paxilline activates LXR mediated transcription from a reporter gene. Full-length LXRα or LXRβ (along with RXRα) were transfected into HEK 293 cells along with a luciferase reporter containing 3 copies of a LXRE derived from the DR4 element of the ABCA1 promoter. Paxilline and 22(R)OHC had similar potency and efficacy for both LXRα and LXRβ. EC_{50}s were approximately 4000 nM for each ligand for both receptors.

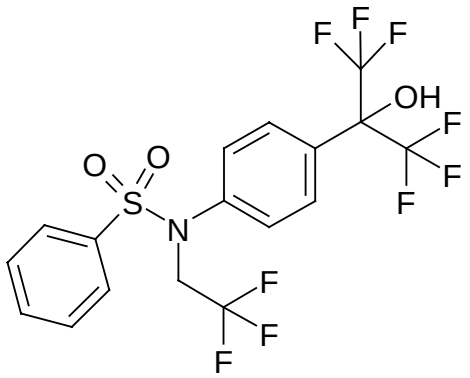
Figure 5. Paxilline induces the expression of a natural target gene, ABCA1, in THP-1 cells. THP-1 cells were differentiated as described in the materials and methods followed by treatment with either paxilline or 22(R)OHC for 24 hours. After this treatment, the cells were harvested and ABCA1 mRNA levels were assessed by bDNA measurement. Paxilline stimulated ABCA1 mRNA expression approximately 7.5-fold with an EC_{50} of 1300 nM. 22(R) OHC was less potent with an EC_{50} of greater than 5000 nM and maximal efficacy was not measured due to limiting toxicity.

Figure 6. Paxilline induces the expression of a natural target gene, SREBP, in HepG2 cells. A) HepG2 cells were treated for 24 hours with either 100 nM T1317 or various concentrations of paxilline for 24 hours before lysis of the cells, preparation of total RNA and quantitation of SREBP mRNA by real time PCR as previously described (Thomas et al., 2003). Paxilline dose-dependently increased SREBP expression. B) HepG2 cells stably transfected with a SRE

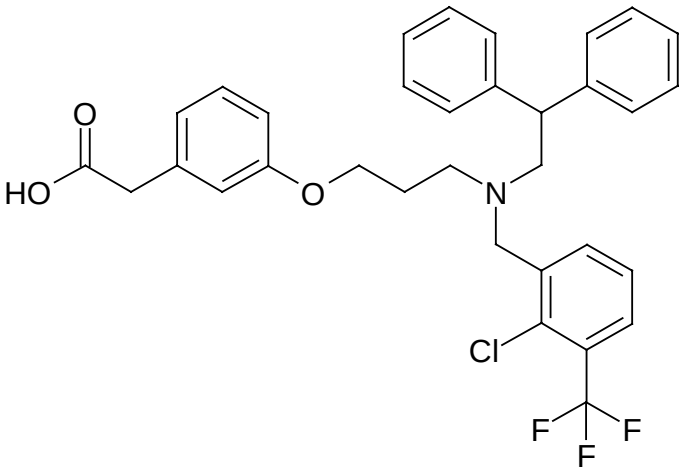
reporter construct were treated with various concentrations of paxilline to assess the ability of this ligand to induce SREBP. Paxilline increased reporter expression dose-dependently displaying an EC_{50} of 2800 nM.



22(R) Hydroxycholesterol



T0901317



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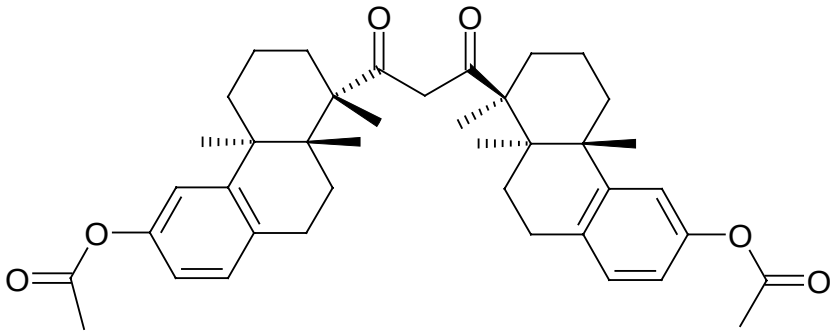
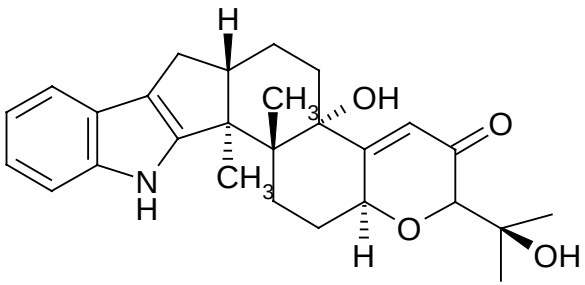


APD



Paxilline

Figure 1

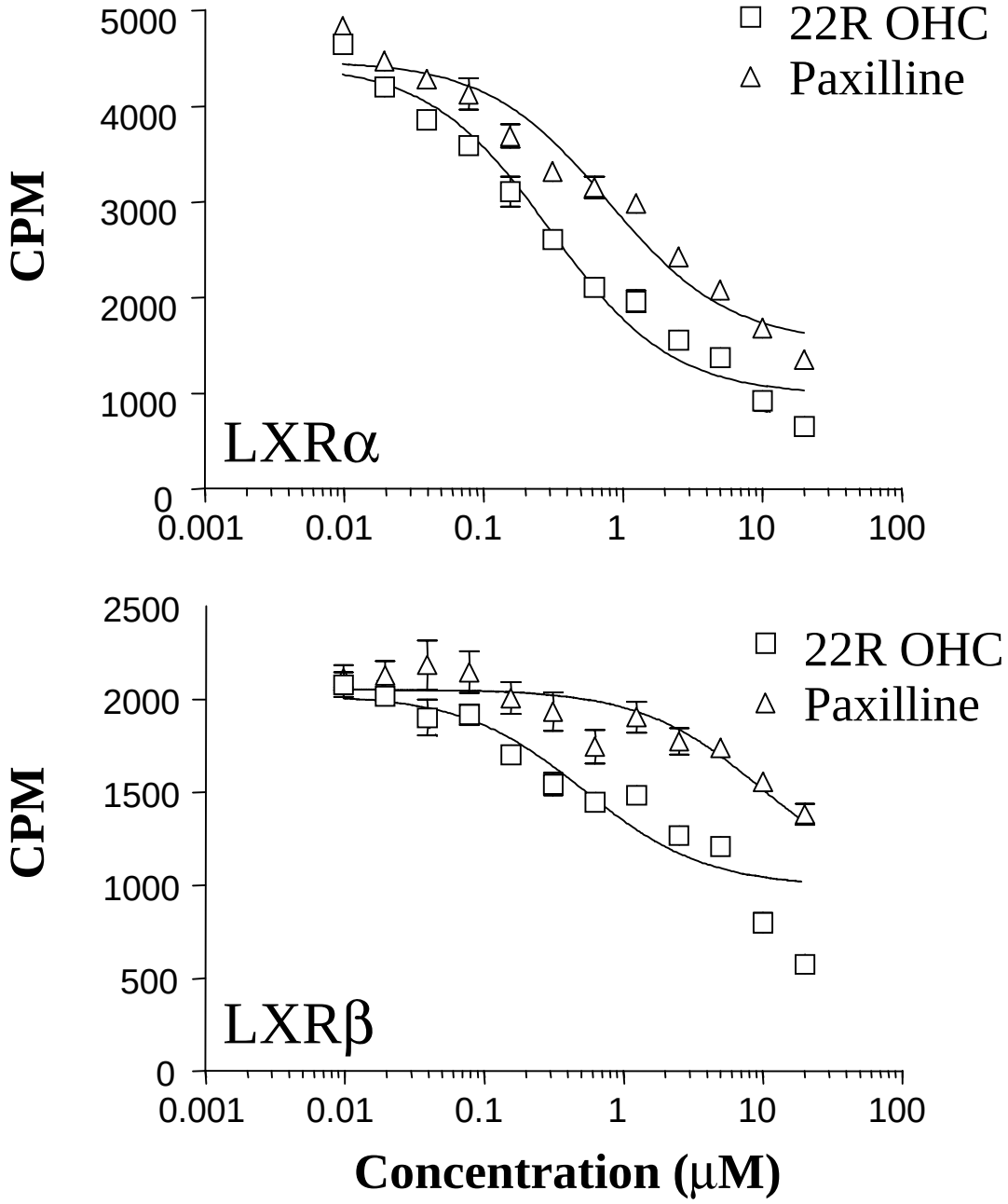


Figure 2

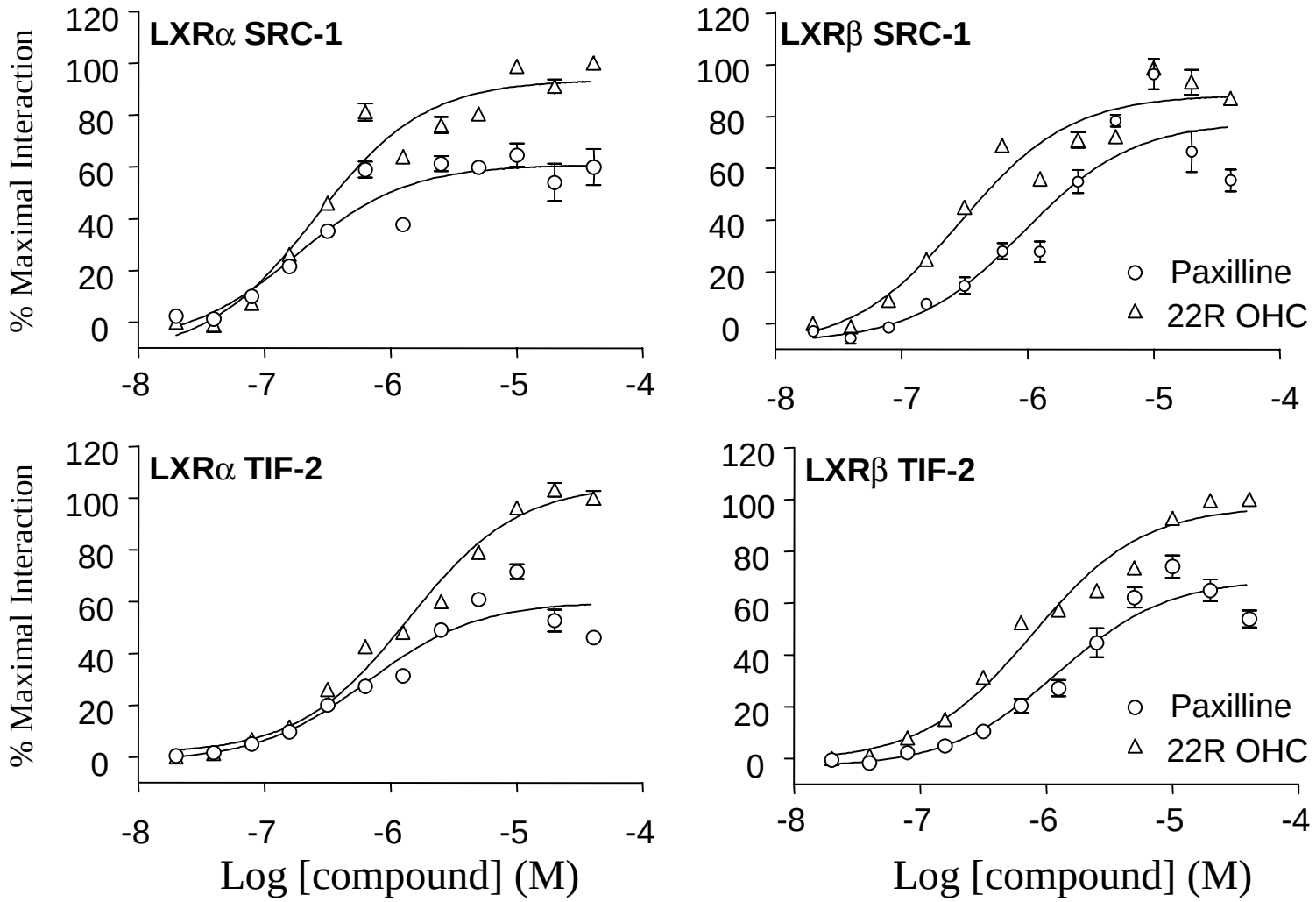


Figure 3

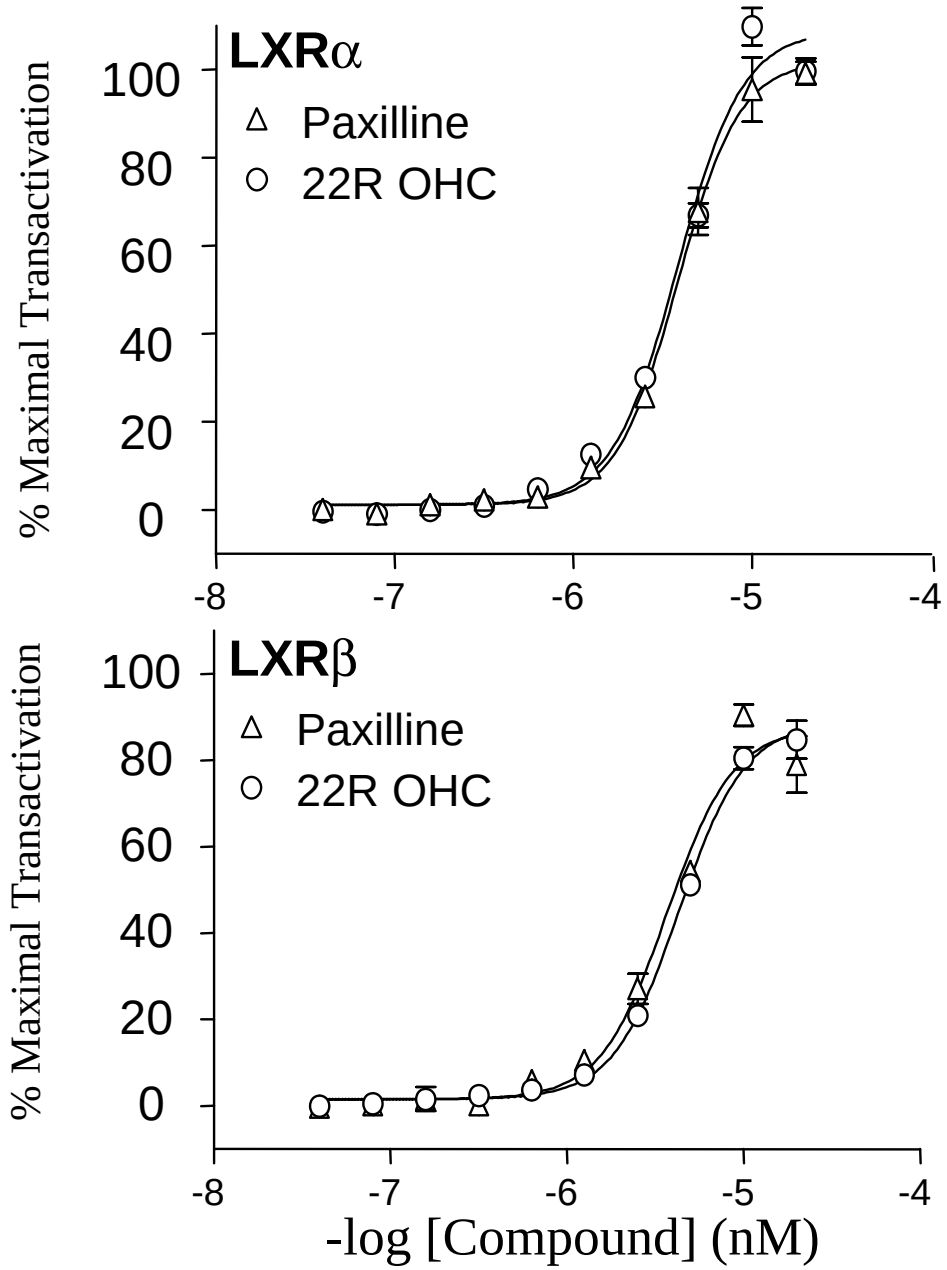


Figure 4

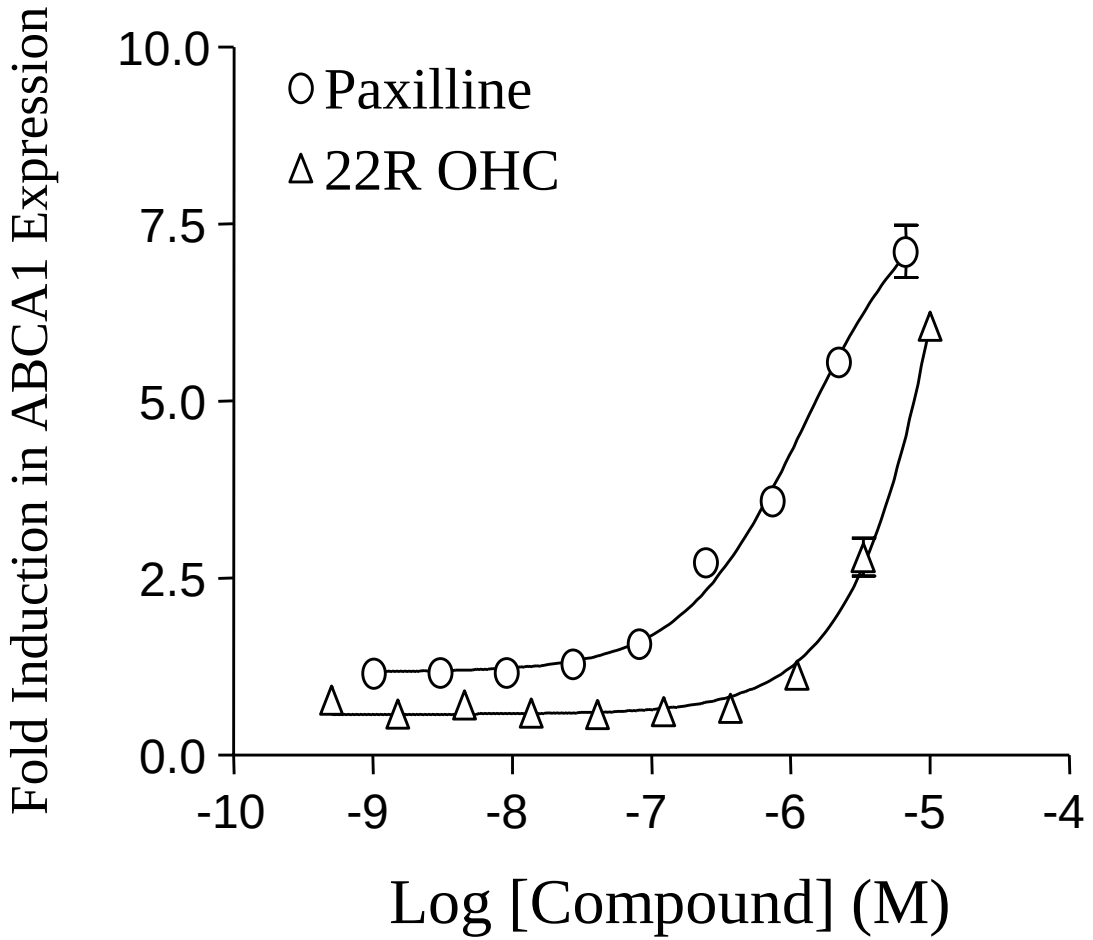


Figure 5

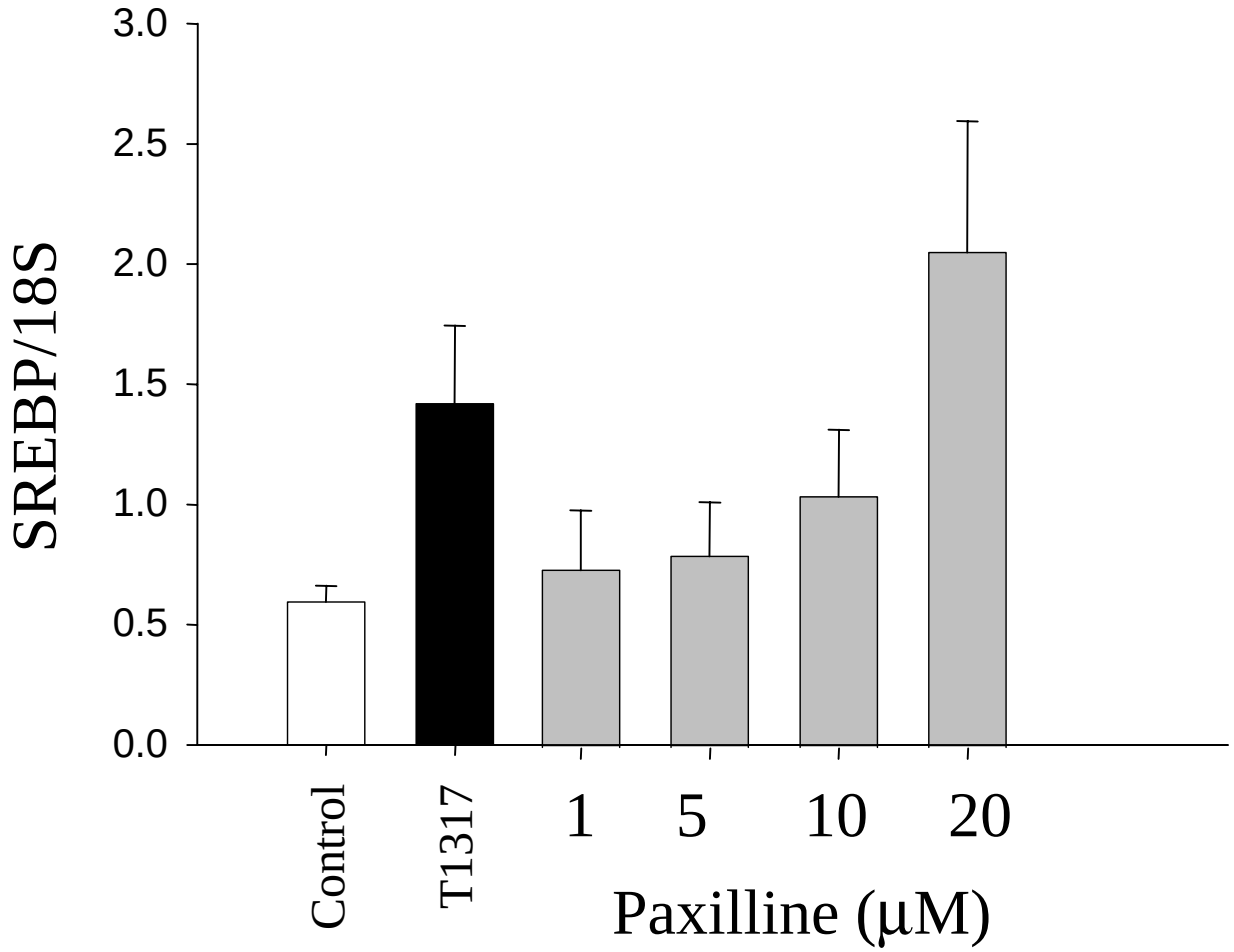


Figure 6A

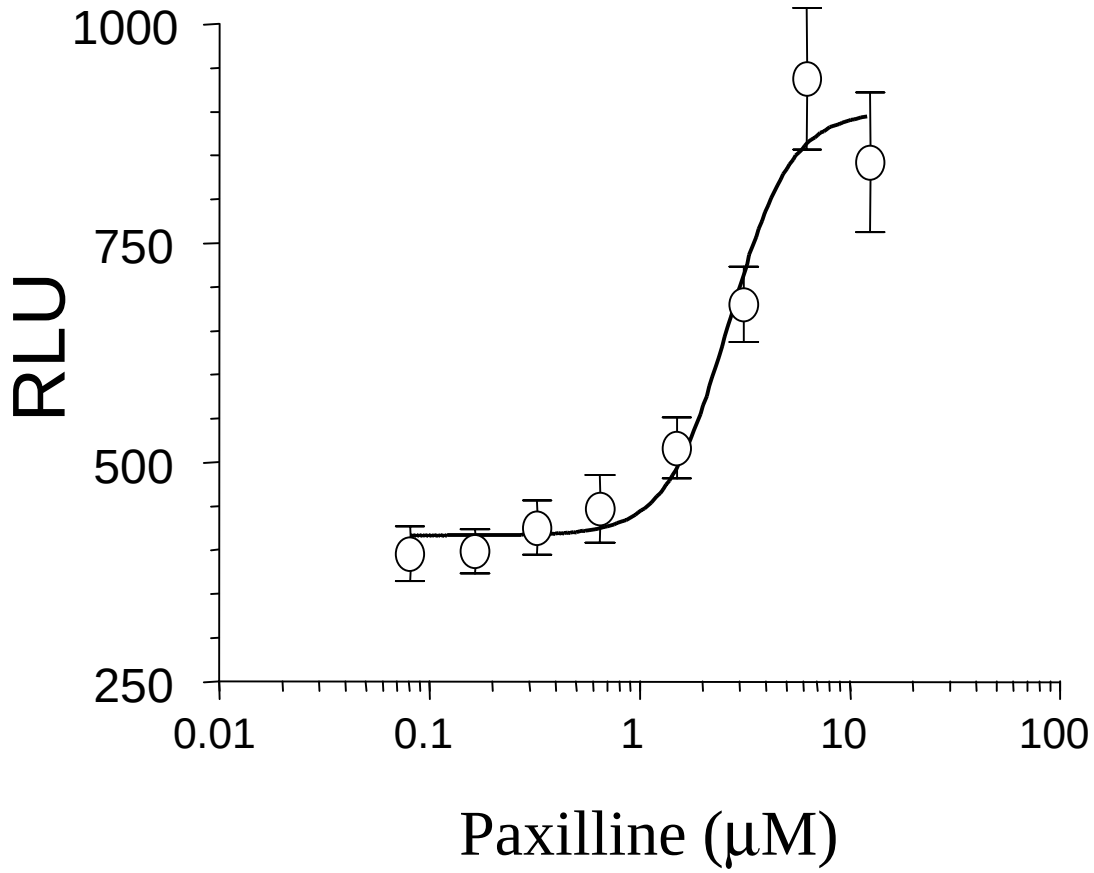


Figure 6B