A link between cholesterol levels and phenobarbital induction of cytochromes P450

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ABSTRACT

Squalestatin1 (SQ1), a potent inhibitor of squalene synthase produced a dose-dependent induction of cytochromes P450 CYP2H1 and CYP3A37 mRNAs in chicken hepatoma cells. The effect of SQ1 was completely reversed by 25-hydroxycholesterol. Bile acids elicited an induction of CYP3A37 and CYP2H1 mRNA. Bile acids also reduced the phenobarbital induction of CYP2H1 but not of CYP3A37 mRNA. The effects of SQ1 and its reversal by 25-hydroxycholesterol and the effects of bile acids were reproduced in reporter gene assays with a phenobarbital-responsive enhancer unit of CYP2H1. These data suggest that an endogenous molecule related to cholesterol homeostasis regulates induction of drug-inducible CYPs.

Keywords: Cytochrome P450, phenobarbital, squalestatin, cholesterol homeostasis, sterols, bile acids, CYP2H1, CYP3A37, drug-induction

INTRODUCTION

Drug-mediated induction of cytochrome P450 hemeproteins (CYPs) has major effects on the biotransformation of endogenous and exogenous molecules including steroid hormones, bile acids, leukotrienes and xenobiotics such as most drugs. Phenobarbital (PB) is the prototype of a large group of small, lipophilic molecules regulating the drug- and steroid-metabolizing CYP2B, CYP2C and CYP3A subfamilies (1, 2). PB induction is observed in all species including plants, fish, birds, insects and mammals, with the notable exception of yeast (3). These observations have led us to speculate that PB induction of CYPs involves an endogenous regulation common to all living organisms (4). Recently, enhancer regions in the 5'-flanking region of PB-inducible CYPs have been isolated in chicken, rat, mouse and humans and DNA-binding proteins associated with these elements have been identified (for reviews, see refs. 1, 5). Members of the nuclear receptor superfamily, in particular the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) in mammals (1) and the chicken xenobiotic receptor (CXR) (6) are transcription factors that mediate the drug-response. These receptors have considerable overlap in their inducer profiles and can interchangeably bind to their respective response elements even across species boundaries (7). Interestingly, CAR, PXR and CXR belong to the family of orphan nuclear receptors comprising also receptors involved in cholesterol homeostasis, namely the oxysterol receptor LXR (liver X receptor) and the bile acid receptor (BAR, alternatively also designated FXR) (8). Moreover, all these receptors form heterodimers with the retinoid X receptor (RXR) for binding to their recognition sequences.

In the absence of uptake of exogenous cholesterol, intracellular cholesterol content is controlled via two interconnected pathways. The mevalonate pathway ensures the *de novo* synthesis of cholesterol from acetyl-coA whereas the bile acid pathway transforms cholesterol into

hydroxylated metabolites leading to the primary bile acids, chenodeoxycholic acid (CCA) and cholic acid (CA) (reviewed in refs. 9-11). The regulation of both pathways occurs via negative feedback control loops activated by their respective end products (cholesterol or oxysterols for the mevalonate pathway, and bile acids for cholesterol catabolism). The pathways are interconnected by the stimulatory action of oxysterols in the catabolic pathway via LXRα (12) and the activation of BAR by bile acids which leads to inhibition of cholesterol metabolism (13, 14).

Previous observation by Kocarek et al. (15) with lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase in rat hepatocyte primary cultures suggested a link between cholesterol homeostasis and regulation of drug-metabolizing enzymes. More recently, squalestatin1 (SQ1), a potent squalene synthase inhibitor was observed to induce the expression of PB-inducible rat CYPB1/B2, *both in vivo* and *in vitro* in rat hepatocytes (16). Moreover, the effect of SQ1 was reversed by oxysterols. However, the mechanism of this interaction remained unexplained. We therefore have further investigated the effects of SQ1, oxysterols and bile acids on CYP induction.

In the experiments described here, we have compared the effect of SQ1, oxysterols and bile acids on both the basal and inducible expression of representatives of two avian PB-inducible CYP subfamilies, CYP2H1 and CYP3A37, in a chicken hepatoma cell line (LMH). These cells retain the unique property of being inducible by PB-type xenobiotics in contrast to mammalian hepatoma cells (7). We show that SQ1 potently induces both CYPs and shares signalling pathways with the PB regulation of these genes. Moreover, we discovered that bile acids also induce CYP2H1 and confirm recent data in mammals that bile acids regulate CYP3A (17, 18).

Our data suggest that an endogenous molecule related to cholesterol homeostasis interacts with the PB-response enhancer sequences (PBRUs) in the 5'-flanking region of PB-inducible CYPs.

MATERIALS AND METHODS

Reagents: Phenobarbital sodium salt was obtained from Fluka, Buchs, Switzerland. Tripotassium squalestatin1 was kindly provided by Glaxo-Welcome, Stevenage, Hertfordshire, UK. 25-hydroxycholesterol, cholic acid and chenodeoxycholic acid were from Sigma Chemicals, Buchs, Switzerland. Cell culture mediums and supplements, Trizol Reagent and fetal calf serum were obtained from Gibco-BRL, Life Technologies, Basel, Switzerland.

Cell culture conditions and transfection: LMH cells were cultured and transfected as described previously (7). For induction experiments, cells were seeded at 80-90% confluence and after attachment, maintained in fetal calf serum deprived medium for 48 hours in order to eliminate any cholesterol exogenous sources. During this period, the medium was renewed at least once. Cells were then treated with the inducers at the indicated concentrations in serum-free medium. After 16 or 24 hours incubation, cells were collected using the Trizol reagent's manufacturer procedure.

RNA isolation and semiquantitative PCR analysis: RNA from LMH cells was isolated with the RNeasy Kit (Qiagen AG, Basel, Switzerland). One μg total RNA was reverse-transcribed with the MMLV reverse transcriptase (Roche Molecular Biochemicals, Rotkreuz, Switzerland). PCR was performed using the Taqman PCR Core Reagent Kit (PE Applied Biosystems, Rotkreuz, Switzerland) and the transcript level quantitated with an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Rotkreuz, Switzerland) according to the manufacturers protocol. Briefly, relative transcript levels in induced cells and non-treated control cells were determined using the relative quantitation method measuring the ΔΔCt. The following primers and fluorescent probes were used in these PCR reactions: CYP2H1: probe 5' TCG CAG

TTG CCT CCA GGT CTC CC 3', forward primer 5' AGG GTG GTG AGG GCA AAT C 3', reverse primer 5' ACA GGC ATT GTG ACC AGC AA 3', CYP3A37: probe 5' TTG GCC CAG GAA TGC CCA GCT 3', forward primer 5' GTC CCA AAG AAA GGC AAT GGT 3', reverse primer 5' GGC CAT TTG GGT TGT TCA AG 3', glyceraldehyde 3-phosphate dehydrogenase (GAPDH): probe 5' TGG CGT GCC CAT TGA TCA CAA GTT T 3', forward primer 5' GGT CAC GCT CCT GGA AGA TAG T 3', reverse primer 5' GGG CAC TGT CAA GGC TGA GA 3'. CYP2H1, CYP3A37 and GAPDH transcript levels were measured in separate tubes and GAPDH was used for normalisation of the CYP2H1 and the CYP3A37 values.

Plasmids: pSRE-luc was a generous gift of Dr. A. Miserez, University Hospital, Basel. This vector contains the promoter of the human HMG CoA synthase gene including a canonical sterol responsive element (SRE). The 264bp-pBLCAT5 vector has already been described (19) and represents the phenobarbital-responsive unit of CYP2H1. The amplified fragment was inserted into the pBLCAT5 vector. Empty vectors were used as control plasmids.

Reporter gene assays: For luciferase (LUC) containing constructs, reporter gene analysis was performed according to the manufacturers protocol (Luciferase assay system, Promega, Madison, WI, USA). Cells were washed with PBS and lysed in 100 µl of reporter lysis buffer (Promega). Lysates were cleared by centrifugation and assayed for luciferase activity with the Luciferase Assay System (Promega) and β-gal activity with the substrate chlorophenol red-β-D-galactopyranoside. Luciferase values normalised to the β-gal activity in the extracts are referred to as luciferase units. For CAT containing constructs, cells were harvested using the protocol and reagents of the CAT-ELISA kit (Roche Diagnostics, Rotkreuz, Switzerland). Protein content of the cell extracts were also measured for the determination of the relative protein concentrations

using the ESL protein assay (Roche Diagnostics, Rotkreuz, Switzerland). This value was used for normalisation of specific CAT expression to total protein content.

RESULTS

SQ1 is an inducer of CYP2H1 and CYP3A37 mRNAs in LMH cells. LMH cells were maintained in chemically defined medium and in the absence of fetal calf serum at confluency for several days without visible toxicity. Under these conditions, LMH cells were treated with various doses of PB and SQ1. PB induced the mRNA of both CYP2H1 and CYP3A37 in a dose-dependent manner, as previously described (19, 20). SQ1 induced the mRNA of both genes with a nearly maximal effect at 1 μM, the induction of CYP2H1 by SQ1 was approximately one-third of the response to PB, whereas CYP3A37 mRNA was induced to equal levels by both inducers. When LMH cells were cultured in the presence of 10% fetal calf serum (FCS), PB induction was unchanged, but the SQ1 effect was much lower, showing a weak activation of CYP2H1 only at 10 μM (data not shown). In the absence of serum, SQ1 thus is the most potent inducer of CYP2H1 and CYP3A37 so far known with an effect in the low micromolar range in LMH cells (Fig. 1A).

SQ1 induction is reversed by oxysterols: If SQ1 acts through cholesterol depletion, its effect should be reversed by oxysterols. PB induction of CYP2H1 and CYP3A37 was unaffected by coincubation with 25-hydroxycholesterol (25 OHC). In sharp contrast, SQ1 induction was completely abolished by coincubation with 20 μM of 25 OHC (Fig. 1B).

Control of cholesterol depletion in LMH cells: Depletion in intracellular sterols triggers the expression of several key enzymes of cholesterol synthesis. This occurs via the activation of SREBPs (sterol response element binding proteins), which are able to bind and transactivate sterol responsive elements (SREs) located in the regulatory regions of these genes (10). To test for sterol depletion in LMH cells, we used a reporter gene construct (pSRE-luc) containing the

SRE enhancer sequence of the human HMG-CoA synthase gene in front of a luciferase gene. SQ1 resulted in a dose-dependent up-regulation of reporter gene expression in LMH cells implying the activation of endogenous chicken SREBPs already at 100 nM concentration (Fig. 2). This activation was reversed by coincubation with increasing concentrations of 25 OHC. PB had no effect on reporter gene expression (data not shown). These data indicate that SQ1 effectively blocks cholesterol production in LMH cells.

Bile acids are regulators of CYP expression: CCA and CA are the two major primary bile acids formed from cholesterol both in mammals and chicken (21, 22). CCA and CA have a regulatory role in cholesterol homeostasis by activating the bile acid receptor BAR and this affects the expression of CYP7A. CYP7A is the rate-limiting enzyme in bile acid formation, catalysing the biotransformation of cholesterol to 7α-hydroxycholesterol. LMH cells were treated with bile acids alone or in combination with SQ1 and PB. Surprisingly, the effect on CYP2H1 and CYP3A37 mRNA was opposite. CCA and CA had little effect on CYP2H1 mRNA except at 100 μM where they induce its mRNA several fold (Fig.3A). Moreover, when incubated together with SQ1 or PB, bile acids produced a dose-dependent inhibition of induction. In contrast, CYP3A37 mRNA was strongly induced by bile acids (Fig.3B). In particular, CCA (50 μM) induced higher levels of CYP3A37 mRNA than SQ1 or PB at 50 μM. CCA did not influence the effects of SQ1 or PB at concentrations below 20 μM but induced CYP3A37 mRNA in an additive fashion at higher concentrations. CA has a similar but weaker effect (Fig. 3B).

Squalestatin1 and bile acid effects act at the transcriptional level: We have defined a regulatory element in the 5' flanking region of the CYP2H1 gene (19). This element spans 264bp and is located at approximately -1600bp upstream of the transcription start site of CYP2H1 and

is responsive to PB. It contains a DR4 like motif neighbouring a putative nuclear factor-1 (NF-1) site. This motif is common to other PBRUs found in PB-regulated genes (7, 19, 23). SQ1 transactivated this element and 25 OHC completely abolished this effect (Fig. 4A). At the reporter gene level, the effect of PB also was partially inhibited by oxysterols. When bile acids were incubated with transfected cells, a weak activation of reporter gene expression was observed with CCA alone as at the mRNA level (Fig.4B). Coexposure to CCA and PB resulted in an inhibition of PB induction to the level of CCA alone.

DISCUSSION

The data presented here demonstrate that cholesterol homeostasis regulators such as bile acids and oxysterols affect both the basal and induced levels of two drug-inducible CYPs, namely CYP2H1 and CYP3A37. This is demonstrated by results obtained at the mRNA level in LMH cells, a hepatoma cell line that uniquely maintains PB-type induction of several cytochromes P450 (7, 19). In these cells, SQ1, an inhibitor of squalene formation and thereby cholesterol synthesis, is the most potent inducer of CYP2H1 and CYP3A37 so far tested. SQ1 has been chosen as an inhibitor of cholesterol biosynthesis because of its high specificity for squalene synthase, a late enzyme in the mevalonate pathway. SQ1 inhibition affects specifically the sterol production in the cell in contrast to the multiple effects of so-called “statins“ such as lovastatin (16). SQ1 induced CYP2H1, but also CYP3A37, a typical member of the CYP3A subfamily (20). SQ1 indirectly or directly activated a PBRU of 264 bp in reporter gene assays (19), imitating the effects of phenobarbital-type inducers. The fact that the effect of SQ1 on mRNA and on PBRU is reversed by oxysterols suggests an indirect mechanism. The data also suggest that SQ1 and PB are acting on CYP2H1 and CYP3A37 via a different mechanisms because the effect of PB is not reversed by oxysterols. Induction by SQ1 correlates with its inhibitory action on cholesterol biosynthesis as evidenced by activation of SREBPs, which also is fully reversed by oxysterols. We therefore hypothesise that endogenous sterols (such as hydroxycholesterol derivatives) negatively regulate the transcription of these CYP genes by interaction with the PBRU. It is of interest that no canonical SRE elements are contained in this sequence. Oxysterols transactivate the nuclear receptor LXRα which binds to a nuclear receptor hexamer repeat sequence arranged as direct repeat spaced by 4 nucleotides (DR4) (24). Indeed, the 264bp PBRU contains a DR4 element as a recognition site for CXR that is involved in PB-responsiveness (6, 19). This avian PBRU also recognises the mammalian receptors CAR and PXR which all form

heterodimers with RXR for binding to this element and in all cases, a DR4 element is of importance (6, 7). Thus, there may be competition between CXR/RXR and LXR/RXR and this could explain that the PB-induction of CYP2H1 is dependent on oxysterol levels. We are testing this hypothesis at present.

The effect of bile acids is more pronounced with CYP3A than with CYP2H1 and confirms recent results that some mammalian CYPs, e.g. CYP3A4 are induced by bile acids (17, 18). Primary bile acids are major products of cholesterol catabolism, synthesised specifically in the hepatocyte (21). These compounds have detergent properties and consequently, their accumulation is potentially deleterious for cells. Metabolism of bile acids to non-toxic, excretable products may represent an adaptable protective pathway against this toxicity (25). The concentrations of the primary bile acids used in our experiments correspond to the concentrations occurring in bile. The dose-dependent effect of bile acids on CYP3A37 induction could thus be a detoxification reaction similar to the protective effect of drug metabolism against drug toxicity. The effect of bile acids on CYP2H1 appears to be more difficult to interpret. At low concentrations (10 μM), the inducibility of CYP2H1 and CYP3A37 by SQ1 or PB is inhibited. At higher concentrations (100 μM), this effect appears to be compensated by induction of these enzymes. The inhibitory effect of bile acids differs from that of 25 OHC because it affects both SQ1 and PB induction.

The observation of cross-talk between regulators of cholesterol homeostasis and drug- and xenobiotic-mediated changes in CYP transcription may have important clinical implications. A review of the literature indeed suggests numerous so far unexplained interactions between cholesterol homeostasis and drug metabolism. Rats with high cholesterol levels in the liver, for instance rats exposed to a high cholesterol diet, or rats with spontaneous hyperlipidemia have a markedly decreased expression of basal and PB-induced CYPs as compared to controls (26, 27).

Furthermore, treatment of epileptic patients or rodents with PB results in increased plasma cholesterol and lipoproteins (see ref. 28 and references therein). It also is well established but mechanistically unexplained that patients with cholestasis benefit from treatment with PB and rifampicin (29, 30). The present studies now offer first insights into the mechanisms that underlie the interactions between cholesterol homeostasis and drug effects on CYPs.

ACKNOWLEDGMENTS

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ABBREVIATIONS

25 OHC, 25-hydroxycholesterol; CAR, constitutive active receptor; CXR, chicken xenobiotic receptor; CYP, cytochrome P450; FXR/BAR, bile acid receptor; GAPDH, glyceraldehyde 3-phosphate-dehydrogenase; HMG, 3-hydroxy-3-methylglutaryl; LMH, leghorn male hepatoma; LXR, liver X receptor; PB, phenobarbital; PBRU, phenobarbital response unit; PXR, pregnane X receptor; RXR, retinoid X receptor; SQ1, squalestatin

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FIGURE LEGENDS

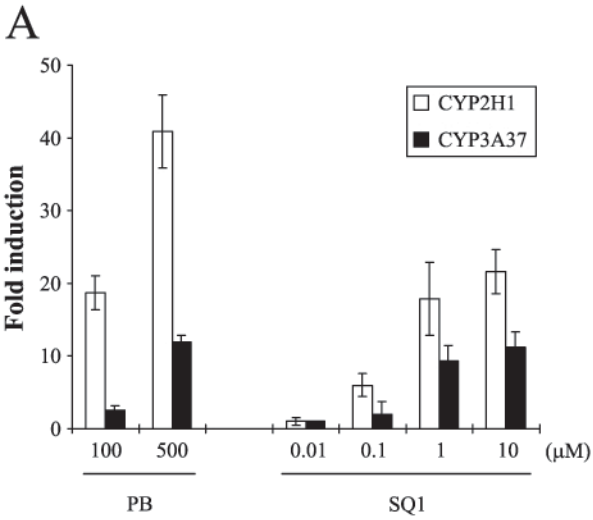
Figure 1: Effect of squalestatin1 (SQ1) on CYP2H1 and CYP3A37 mRNAs. LMH cells, cultured in the absence of serum, were treated with the indicated doses of phenobarbital (PB) or SQ1 in the absence (A) or presence (B) of 20 μM 25-hydroxycholesterol (25 OHC). After 16 hours, CYP2H1, CYP3A37 and GAPDH mRNA levels were determined by real-time PCR analysis. The relative expression of each gene is given as fold induction by comparison to untreated samples, and standardised according the expression of GAPDH mRNA. Each value represents the mean and standard deviation of at least three independent experiments.

Figure 2: Activation of a sterol responsive element (SRE)-containing reporter construct by squalestatin1 (SQ1) and its reversibility by 25-hydroxycholesterol (25 OHC). LMH cells were transfected with pSRE-luc as described in Material and Methods. After removal of fetal calf serum for 24 hours, cells were treated with increasing doses of SQ1 or a constant dose of SQ1 and increasing doses of 25 OHC. Each value represents the average and standard deviation of triplicate samples and is expressed in relative light unit standardised against an empty pluc vector. The data are derived from one representative experiment of several independent ones.

Figure 3: Effect of bile acids on CYP2H1 and CYP3A37 mRNA. LMH cells were treated with increasing doses of chenodeoxycholic acid (CCA) or a single dose of cholic acid (CA) alone or in combination with squalestatin1 (SQ1, 10μM) or phenobarbital (PB, 500μM). CYP2H1 (A) or CYP3A37 (B) mRNA levels are represented as fold induction compared to untreated samples. Each value is the average of duplicate samples. The data represent the mean and standard deviation of three independent experiments.

Figure 4: Transcriptional activation of CYP2H1 phenobarbital responsive unit (PBRU) by squalestatin 1 (SQ1) and bile acids. LMH cells were transfected with a reporter gene construct containing the 264bp phenobarbital responsive enhancer unit of CYP2H1 in front of the chloramphenicol acetyltransferase gene. The transfected cells were treated with SQ1 in the presence or absence of 25-hydroxycholesterol (25 OHC, 20μM) (A) or with chenodeoxycholic acid (CCA, 10 μM) or cholic acid (CA, 10 μM) alone or in combination with SQ1 or phenobarbital (PB) (B). The data are expressed as the mean and standard deviation of four independent experiments.

Figure 1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CYP3A37
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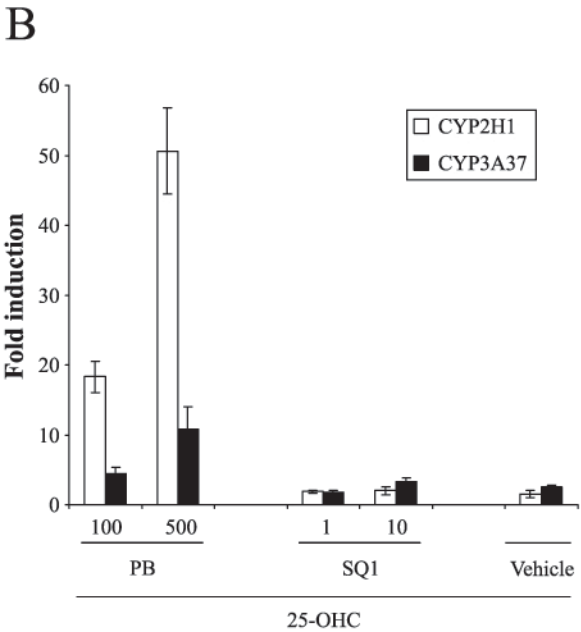
(μM)
PB
SQ1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CYP3A37
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1
10
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SQ1
Vehicle
25-OHC

Figure 2

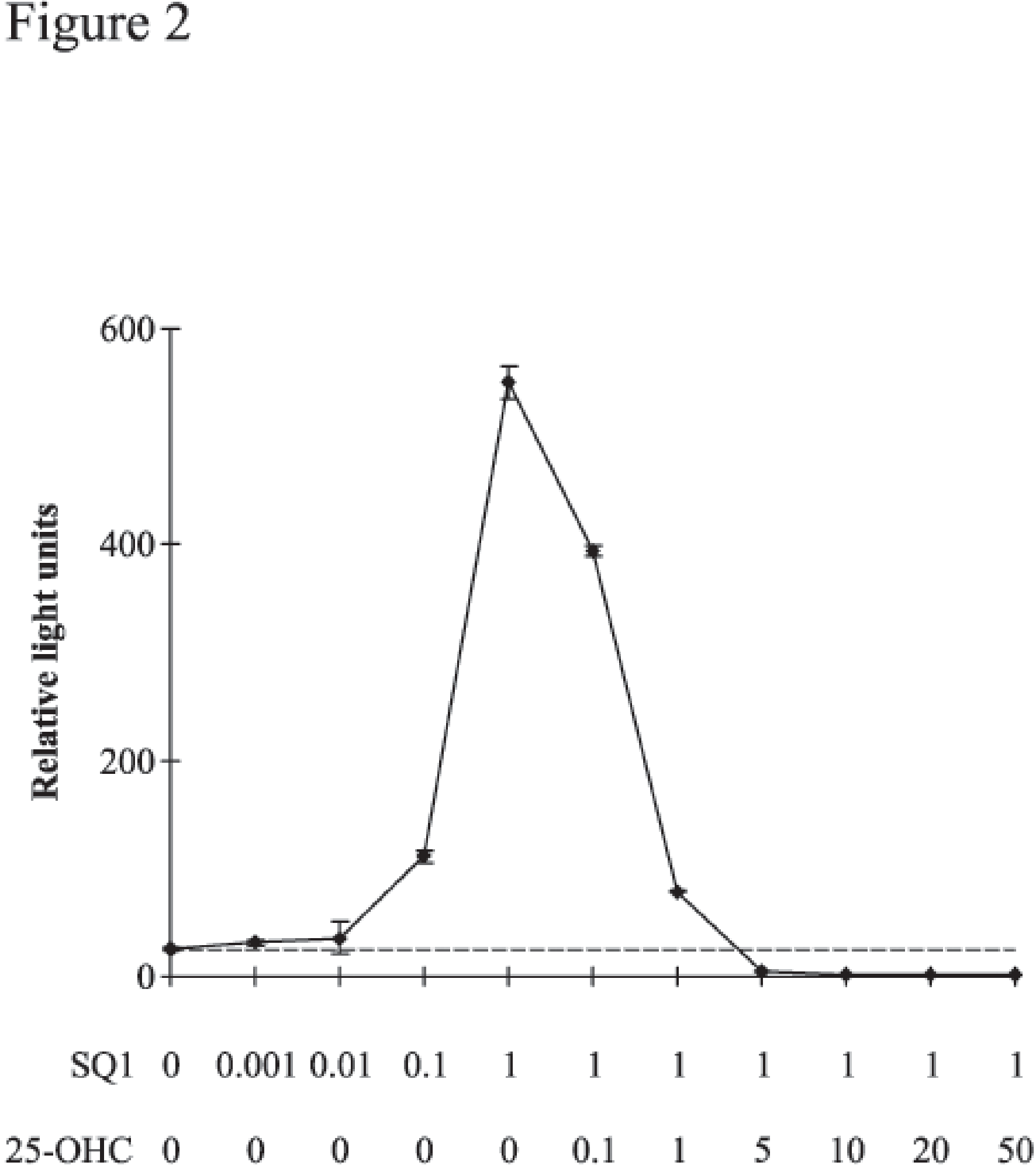
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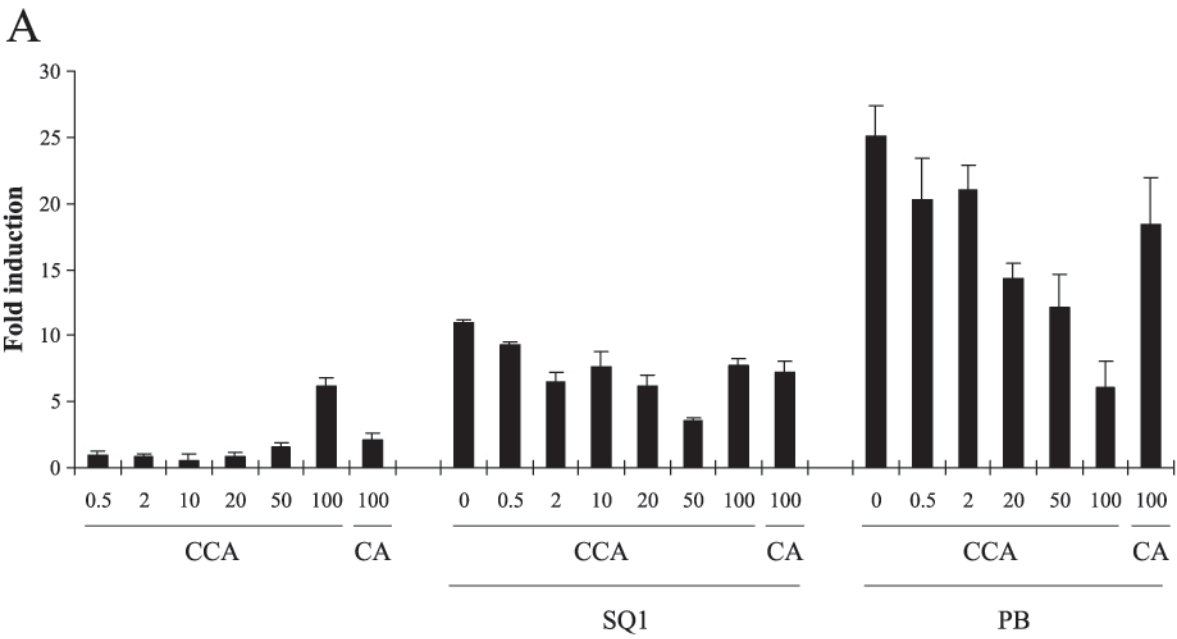
Figure 3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Fold induction
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25
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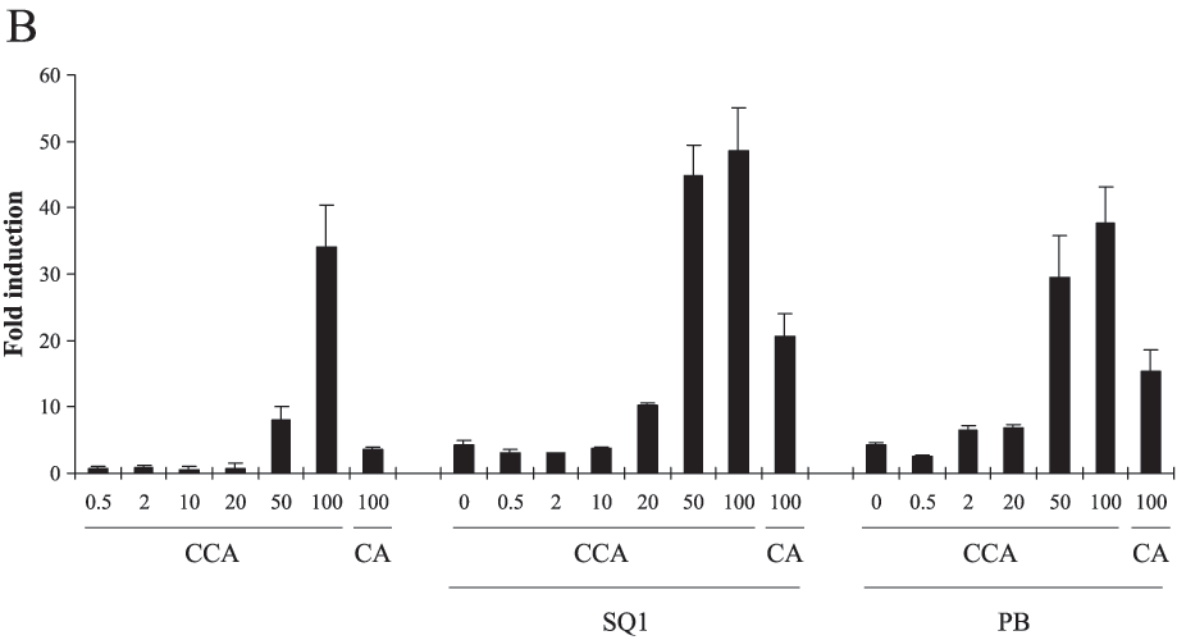
CCA CA CCA CA CCA CA
SQ1 PB


B
Fold induction
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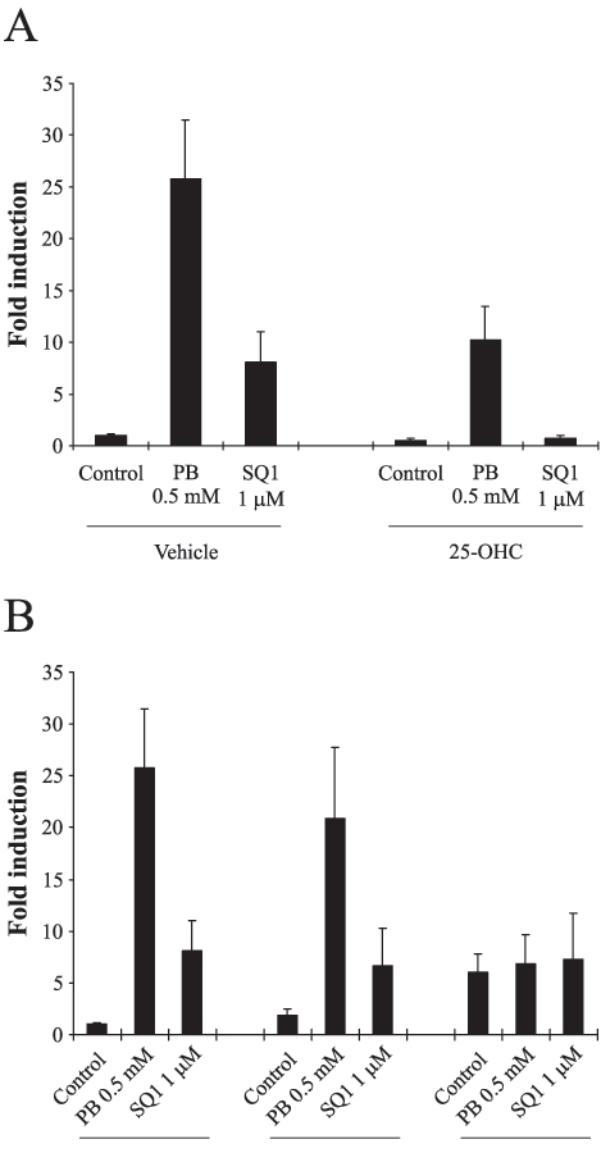
0
0.5 2 10 20 50 100 100 0 0.5 2 10 20 50 100 100 0 0.5 2 20 50 100 100
CCA CA CCA CA CCA CA
SQ1 PB

Figure 4


A
Fold induction
35
30
25
20
15
10
5
0
Control
PB 0.5 mM
SQ1 1 μM
Control
PB 0.5 mM
SQ1 1 μM
Vehicle
25-OHC
B
Fold induction
35
30
25
20
15
10
5
0
Control
PB 0.5 mM
SQ1 1 μM
Control
PB 0.5 mM
SQ1 1 μM
Control
PB 0.5 mM
SQ1 1 μM
Vehicle
CA 100 μM
CCA 100 μM