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$GABA_A$/central benzodiazepine receptor and peripheral benzodiazepine receptor ligands as inducers of phenobarbital-inducible CYP2B and CYP3A

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Abstract

A sequence critical for phenobarbital (PB) induction, the PB response unit (PBRU), situated upstream of the rat *CYP2B1* and *CYP2B2* genes, includes two nuclear receptor binding sites, NR1 and NR2. When NR1 and NR2 are mutated PB responsiveness is abolished. While no nuclear receptor for which PB is an agonist ligand has yet been identified, PB is a ligand of $GABA_A$ receptors and it can displace [^{3}H] 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195) from its binding site on the peripheral benzodiazepine receptor (PBR). We assessed CYP2B levels in primary rat hepatocytes following treatment with 10 ligands of either or both of these receptors. All compounds tested were found to be CYP2B1/CYP2B2 inducers and most were CYP3A inducers. Five had not previously been described as CYP2B1/CYP2B2 inducers: bicuculline, flunitrazepam, 4′-chlorodiazepam (Ro5-4864), *N*,*N*-dihexyl-2-(4-fluorophenyl)indole-3-acetamide (FGIN 1–27) and 7-(dimethylcarbamoyloxy)-6-phenylpyrrolo-[2,1-*d*][1,5]benzothiazepine (DCPPBT). Reporter gene analysis demonstrated that CYP2B induction by these agents and other PBR or $GABA_A$ receptor ligands is mediated through the PBRU and the NR1/NR2 sites, suggesting a molecular mechanism similar to that for PB induction. The potencies for PBRU-dependent induction by 11 ligands of PBR or the $GABA_A$ receptor was evaluated. FGIN-127, DCPPBT and PK 11195 exhibited EC_{50} values for PBRU-dependent transcription activation about three orders of magnitude higher than the reported affinities of the PBR for these agents, arguing against the involvement of the PBR in PB induction. However the EC_{50} values found for the agents tested encourage further investigation on the possible involvement of the $GABA_A$ receptor in PB induction.

Keywords: Phenobarbital; Induction; $GABA_A$ receptor; Hepatocytes; CYP2B; CYP3A

1. Introduction

Cytochrome P450s (CYPs) are mainly hepatic hemoproteins with monooxygenase activity, many of which are involved in xenobiotic metabolism. While treatment with high doses of the anticonvulsant phenobarbital (PB) induces only modestly the transcription of members of the *CYP2C* and *CYP3A* subfamilies, it strikingly induces transcription of rat *CYP2B1* and *CYP2B2* [1]. A 163-bp fragment that has the properties of a multicomponent PB-dependent transcriptional enhancer was identified by our group at coordinates −2317/−2155 in the *CYP2B2* 5′-flank and termed Phenobarbital Response Unit (PBRU) [2,3]. It includes two nuclear receptor binding sites, NR1 and NR2 [4], and when both of these are mutated PB responsiveness is abolished in primary hepatocytes [5]. While several observations [6,7] suggest a role for the orphan nuclear receptor CAR in PB induction of CYP2B forms, PB is not an agonist ligand of CAR [8,9] suggesting a possible role for an upstream component in the induction process. However, CAR is known to activate

Abbreviations: CAR, constitutively active receptor or constitutive androstane receptor; DMSO, dimethylsulfoxide; FGIN 1–27, *N*,*N*-dihexyl-2-(4-fluorophenyl)indole-3-acetamide; GABA, γ-aminobutyric acid; GAPDH, glyceraldehyde phosphate dehydrogenase; PB, phenobarbital; PBR, peripheral benzodiazepine receptor; PBRU, phenobarbital response unit; PK 11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide; PVDF, polyvinylidene difluoride; PXR, pregnane X receptor; Ro5-4864, 4′-chlorodiazepam; SDS, sodium dodecyl sulphate; DCPPBT, 7-(dimethylcarbamoyloxy)-6-phenylpyrrolo-[2,1-*d*][1,5]benzothiazepine; DBI/ACBP, diazepam binding inhibitor/acyl-CoA binding protein.

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transcription in the absence of a ligand [10] and the failure of CAR-negative mice to respond to PB-like inducers [11] clearly implies that CAR is essential at some level for PB induction.

Neurodepressant barbiturates such as PB are known to bind to the barbiturate/picrotoxin site on $GABA_A$ receptor complex [12] where they are thought to exert the essential of their neurological effects by potentiating GABA-mediated hyperpolarization of post-synaptic neurons. Interestingly, there appears to be a relation between the capacity of diverse barbiturates to induce sedation through this mechanism and their capacity to induce the synthesis of PB-inducible CYP2B proteins, non-sedative barbiturates being essentially inactive as CYP2B inducers [13]. $GABA_A$ receptors were first characterised in neurons and are thought to consist of pentamers of five subunits forming a GABA-gated chloride channel [14] with allosteric modulatory binding sites for several drugs, including barbiturates and benzodiazepines [12]. So far, 19 different subunits, as well as several isoforms that are products of alternative splicing, have been identified in mammalian brain, and $GABA_A$ receptors have distinct pharmacological properties depending on which subunit is present [15]. $GABA_A$ receptor-like binding sites are present in rat hepatocytes and their activation by GABA results in prompt and marked hyperpolarization of the hepatocyte membranes, an effect abolished by the GABA antagonist bicuculline [16]. Recently, the $GABA_A$ β3 subunit was detected in rat hepatocytes [17]. In human lymphocytes, PB can also displace [^{3}H]PK 11195 from its binding site on the $GABA_A$ receptor-uncoupled PBR [18], a protein with binding sites for benzodiazepines, isoquinoline carboxamides and porphyrins [19]. The PBR is present in outer mitochondrial membranes of rat hepatocytes [20].

At least seven other PBR or $GABA_A$ receptor ligands have been shown by different groups to be PB-like inducers. These include picrotoxin [21], a $GABA_A$ receptor negative modulator, carbamazepine [22] and several benzodiazepines, such as clonazepam and diazepam [23], midazolam [24] and zolazepam [25], which bind to both PBR and $GABA_A$ receptor with different affinities. PK 11195 is an isoquinoline carboxamide derivative that binds to PBR with low nanomolar affinity [19], and at micromolar concentrations it allosterically perturbs *t*-butylbicyclophosphorothionate effects at $GABA_A$ receptors [26]. It is also a PB-like inducer [27]. Inversely, amitriptyline and the polychlorinated biphenyl mixture Aroclor 1254, known PB-like inducers, modulate GABA-stimulated chloride influx via the $GABA_A$ receptor [28,29].

These results led us to ask whether other ligands of either PBR or the $GABA_A$/central benzodiazepine receptor induce CYP2B1 and CYP2B2. By transfecting *luc* reporter constructs into primary rat hepatocytes, we also tested whether induction by ligands of these receptors was mediated by the PBRU and the NR1 and NR2 sites, which would indicate a molecular mechanism of induction similar to that for PB. Finally, we evaluated the potency of these ligands to induce transcription via the PBRU to see if it fitted reported values of affinity for either the $GABA_A$ receptor or PBR.

2. Materials and methods

2.1. Plasmid constructs

The pGL3-2B2X vector, based on the pGL3 basic vector (Promega), was used for transfection analysis. It contains 2.5 kb of the *CYP2B2* 5′-flank, including the natural promoter and the PBRU, subcloned upstream of the *luc* gene [5]. A construct from which the PBRU was deleted (pGL3-2B2X-ΔPBRU) and a double mutant in which both the NR1A and NR2B half sites were mutated (pGL3-2B2X-NR1a/NR2b) were also used [5].

2.2. Isolation and culture of primary hepatocytes, transfection and treatments with PBR or $GABA_A$ receptor ligands

Methods and materials for hepatocyte isolation and culture in serum-free medium, as well as those for liposome-mediated transfection, were as described in Paquet et al. [5], except that six-well (10 cm^2) Falcon plates (VWR) were used instead of 60 mm petri dishes. Cells (1.8×10^6 per well) were plated in serum-free modified Chee's medium [30] containing gentamicin (50 μg/ml) and incubated for 3 h to permit attachment. The cells were then washed (6.7 mM KCl, 142 mM NaCl, 10 mM Hepes, pH 7.6) and incubated for an additional 4 h in medium devoid of antibiotics and transfected. Each well received 1 μg of *luc* reporter construct and 5 ng of internal control plasmid (bearing the Renilla *luc* gene under control of the cytomegalovirus promoter/enhancer) in 15 μl of lipofectin reagent (Invitrogen). After 15 h, lipofectin-containing medium was removed, cells were washed twice and incubated an additional 24 h in presence of antibiotic. Cells were then incubated for 48 h in medium containing one of the tested agents; medium was changed after a 24 h period. Cells were finally harvested and luciferase activity was assayed using the dual-luciferase kit (Promega). PB stock solution was prepared by directly dissolving PB in culture medium, whereas stock solutions of other compounds were prepared by dissolving in DMSO. For isolation of proteins for Western blot analysis, the procedure was similar except that the transfection step was omitted. Diazepam, flunitrazepam, Ro5-4864, PK 11195, carbamazepine, picrotoxin, (+)-bicuculline and clonazepam were from Sigma–Aldrich Canada. DCPPBT and FGIN-1–27 were from Tocris Cookson and PB was from BDH.

2.3. Immunochemical detection of CYP proteins

Hepatocytes were harvested at 4 °C directly in the gel-loading buffer (125 mM Tris–HCl pH 6.8, 20% glycerol, 1.5 M 2-mercaptoethanol, 6% SDS, 0.005% bromophenol blue), frozen, boiled for three minutes and loaded on a SDS acrylamide gel. Electrophoresis was carried out for 7 h (80 V for the stacking gel; 200 V, 45 mA for the running gel) using Laemmli's buffer (25 mM Tris, 200 mM glycine, 0.01% SDS pH 8.3). Proteins were then electrotransferred overnight at 4 °C to a PVDF membrane (Hybond-P Amersham Pharmacia) according to the manufacturer's instructions. On each gel, molecular markers were run in parallel to the samples. The electrotransfer and electrophoresis apparatus were from Bio-Rad. After a 1 h incubation in blocking buffer (5% fat free dried milk, 20 mM Tris–HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20) and a rinse, membranes were incubated for 1 h with a 1:10,000 dilution of anti-PB4 antibody in TBS-Tween (20 mM Tris–HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20). The anti-PB4 polyclonal rabbit antibody [31], a gift from David Waxman, recognises both PB-inducible CYP2B1 and CYP2B2 and the constitutive CYP2B3 [32]. Immunodetection was carried out using an enhanced chemiluminescence detection kit (Lumi-Light Western blotting substrate from Roche Diagnostics) with horseradish peroxidase-labeled donkey anti-rabbit immunoglobulin as a secondary antibody (1:10,000 dilution). For detection of CYP3A1, the PVDF membrane was stripped according to the manufacturer's instructions and subjected again to the detection protocol using a rabbit polyclonal anti-CYP3A1 primary antibody (Research Diagnostics). Finally, as a loading control, the membrane was stripped once more and subjected again to the detection protocol using a rabbit polyclonal anti-GAPDH primary antibody (Novus Biologicals).

3. Results

Homogenates of hepatocytes treated with vehicle, PB, PK 11195, Ro5-4864, DCPPBT, carbamazepine, bicuculline, picrotoxin, clonazepam, flunitrazepam and FGIN 1–27 were assessed by Western blot analysis for the presence of CYP2B proteins (Fig. 1A). While the intensity of the lower band, representing constitutive CYP2B3 protein, appeared nearly constant in all samples (as did the GAPDH loading control in Fig. 1C), the intensity of the upper band, which represents both CYP2B1 and CYP2B2, was clearly greater following treatment with all PBR or $GABA_A$ receptor ligands tested. As expected, CYP2B1/CYP2B2, undetectable in controls, were strikingly induced (8.6- to 12.4-fold) following treatment with PB, carbamazepine, picrotoxin and clonazepam. The GABA-binding antagonist bicuculline induced CYP2B1/CYP2B2 to a similar extent (11.4-fold), as did flunitrazepam (8.2-fold), a classic

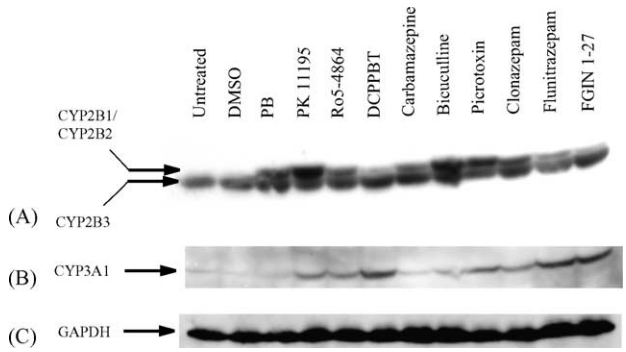

Fig. 1. Induction of CYP2B1/CYP2B2 and CYP3A proteins in primary rat hepatocytes by PBR or $GABA_A$ receptor ligands. Hepatocytes were treated for 48 h with one of ten compounds (100 μM PB, 10 μM PK 11195, 2 μM Ro5-4864, 10 μM DCPPBT, 30 μM carbamazepine, 10 μM bicuculline, 10 μM picrotoxin, 10 μM clonazepam, 10 μM flunitrazepam or 10 μM FGIN 1–27) and cell lysates were then assessed by Western blot analysis for the presence of CYP2B proteins (A). The membrane was then stripped and assessed for the presence of CYP3A1 (B); then it was stripped again and assessed for the presence of GAPDH as a loading control (C). Untreated and DMSO-treated controls are presented in the two first lanes. In (A), CYP2B1 and CYP2B2 (upper band) are not resolved; the lower band represents the constitutive form CYP2B3 [32]. Fold-induction values for CYP2B1/CYP2B2 were estimated in (A), with Scion Image analysis software using CYP2B3 as a loading control, as follows. PB: 11.1-fold; PK 11195: 13-fold; Ro5-4864: 9.8-fold; DCPPBT: 5.6-fold; carbamazepine: 8.6-fold; bicuculline: 11.4-fold; picrotoxin: 12.4-fold; clonazepam: 10.4-fold; flunitrazepam: 8.2-fold; FGIN 1–27: 5.6-fold. For CYP2B1/CYP2B2, similar results were obtained in five Westerns (except for FGIN 1–27, which was tested three times) using two sets of cellular extracts. For CYP3A1, similar results were obtained in two Westerns using two sets of cellular extracts.

benzodiazepine drug with nanomolar affinity for both the PBR and $GABA_A$ receptors [33]. FGIN 1–27 and DCPPBT, both characterised as specific nanomolar affinity PBR ligands [34,35], led to a more modest induction (5.6-fold) of CYP2B1/CYP2B2. Finally, the atypical benzodiazepine Ro5-4864, which binds with an affinity in the low nanomolar range to rodent PBR [19] and which, like PK 11195, also binds to $GABA_A$ receptors with much lower affinity [26,36,37] induced CYP2B1/CYP2B2 by about 10-fold.

Rat CYP3A1 is known to be modestly induced by PB-like inducers [1]. The membrane used in Fig. 1A was stripped and tested for the presence of CYP3A1 (Fig. 1B). CYP3A1 induction by most agents tested (PK 11195, Ro5-4864, picrotoxin, clonazepam, flunitrazepam, FGIN 1–27 and DCPPBT) was far greater than that, marginal at best, obtained with PB, bicuculline and carbamazepine. Flunitrazepam, FGIN 1–27 and DCPPBT were all weaker than PB in inducing CYP2B1/CYP2B2 yet stronger in inducing CYP3A1. Hence, there was a lack of parallelism between induction of CYP2B1/CYP2B2, on the one hand, and CYP3A1, on the other.

Homogenates of primary hepatocytes transfected with one of three reporter vectors and treated with either PB, PK 11195, Ro5-4864, carbamazepine, bicuculline, FGIN 1–27 (Fig. 2), or picrotoxin or DCPPBT (data not shown), were assessed for luciferase activity. Following treatment with

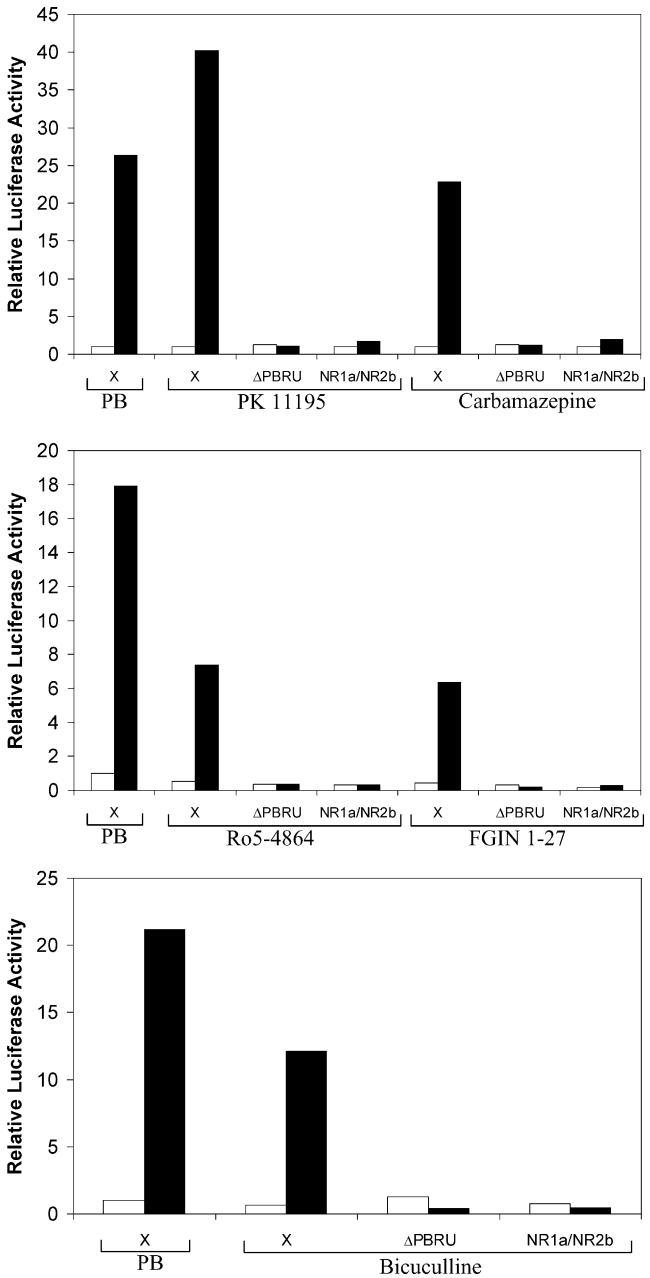

Fig. 2. Induction of PBRU-driven reporter gene transcription by $GABA_A$ and PBR receptor ligands. Primary rat hepatocytes were transfected in duplicate with one of three reporter vectors and treated for 48 h with one of six substances (100 mM PB, 10 mM PK 11195, 10 mM Ro5-4864, 50 mM carbamazepine, 10 mM bicuculline, or 10 mM FGIN 1–27). The reporter constructs used were pGL3-2B2X (labeled X), which contains an intact PBRU; pGL3-2B2X-ΔPBRU, from which the PBRU was deleted; and pGL3-2B2X-NR1a/NR2b, in which NR1A and NR2B hemisites were mutated. Luciferase activity relative to that obtained for the untreated control with the X construct, which was set at 1, is presented for a representative experiment with each substance. White bars represent untreated cells and black bars represent treated cells. Data grouped in the same panel were obtained with the same hepatocyte preparation. Substances were tested from two to five times. Induction for PB varied from 18- to 27-fold with the different hepatocyte preparations used. Since these experiments were done before completion of the dose-effect assays (Table 1), the dose used for treatments with Ro5-4864 was not optimal, yielding a lower level of induction compared to that obtained in Western blots (Fig. 1). For all substances tested, including PB, doses higher than the optimal dose-effect range yielded lower fold induction values.

Table 1
Potencies of 11 substances known to bind to the $GABA_A$ receptor, the PBR or both to induce PBRU-dependent transcription

Substance	EC_{50}
Phenobarbital (μM)	43 ± 11
Clonazepam (μM)	5.4 ± 3.9
Diazepam (μM)	6.4 ± 4.3
Carbamazepine (μM)	10.6 ± 1.7
Flunitrazepam (μM)	5.7 ± 0.5
Picrotoxin (μM)	10.0 ± 4.1
(+)-Bicuculline (μM)	7.8 ± 5.7
PK 11195 (μM)	2.7 ± 0.6
Ro5-4864 (nM)	<620
DCPPBT (μM)	9.4 ± 0.3
FGIN 1–27 (μM)	3.5 ± 2.1

EC_{50} values represent the concentration for half maximal activation of transcription of a *luc* expression vector, the transcription of which is under the control of 2.5 kb of the *CYP2B2* 5′-flank, including the natural promoter and the PBRU. Primary rat hepatocytes transiently transfected with this vector were treated for 48 h in serum free medium containing six different concentrations of each substance and luciferase activity was assessed using the Promega dual luciferase kit. EC_{50} values were evaluated by applying non-linear regression to the data points and represent the average (±S.D., except for Ro5-4864) of two to six experiments.

these substances, induction of luciferase was obtained in cells transfected with the pGL3-2B2X construct (which contains an intact rat CYP2B2 PBRU) while induction was essentially absent in cells transfected with either the pGL3-2B2X-ΔPBRU vector or the pGL3-2B2X-NR1a/NR2b vector (in which one half site of both NR1 and NR2 is mutationally inactivated). Thus, the PBRU and its NR1 and NR2 sites have an essential role for induction of the luc reporter following treatment with PBR or $GABA_A$ receptor ligands.

Table 1 presents potencies of 11 PBR or $GABA_A$ receptor ligands for induction of *luc* transcription as determined with the pGL3-2B2X reporter vector. PB had the lowest potency to induce transcription and most of the substances tested showed relatively low inducing potencies, with EC_{50} values in the micromolar range. Ro5-4864 had the highest potency with EC_{50} values in the high nanomolar range.

4. Discussion

There is a high proportion of PB-like inducers among PBR and $GABA_A$ receptor ligands. Our results showed that five such ligands not previously known to be PB-like inducers increase CYP2B1/CYP2B2 levels in primary rat hepatocytes. Seven of the agents tested also increased levels of PB-inducible CYP3A1 (Fig. 1). Lack of parallelism between induction of CYP2B1/CYP2B2 and CYP3A is observed in Fig. 1, where the three weakest CYP2B inducers were the strongest CYP3A1 inducers. This is consistent with the view that CYP2B and CYP3A proteins are induced by different mechanisms [7]. Also, eight PBR

and $GABA_A$ receptor ligands were tested for inducing transcription using a reporter vector in which transcription of the *luc* gene is under control of 2.5 kb of the natural *CYP2B2* 5′-flank. The results obtained (Fig. 2) indicate that these compounds induce CYP2B proteins at the transcriptional level and that this induction is, as for PB, mediated through the NR1 and NR2 sites of the PBRU, suggesting a common induction mechanism for all of these substances. By using this same system of transient transfection in primary hepatocytes, EC_{50} values were determined for PBRU-dependent transcriptional activation by 11 PBR and $GABA_A$ ligands.

One possible explanation for these results is that CYP induction by all tested substances is a consequence of their direct binding to nuclear receptors [7]. Arguing in this sense, the orphan nuclear receptor PXR, for which the PB-like inducer 1,4-bis[2-(3, 5-dichloropyridyloxy)]benzene (TCPOBOP) is an agonist ligand in mouse [8], activates the transcription of both *CYP3A* and *CYP2B* genes upon binding of various xenobiotics [38]. Also, several lines of evidence imply an essential role of the nuclear receptor CAR in PB-dependent induction of *CYP2B* genes [6,11]. CAR and PXR were also shown to bind each other's response elements [39]. The involvement of two different nuclear receptors could provide an explanation for the lack of parallelism observed between induction of CYP2B and CYP3A proteins (Fig. 1). In this view, molecules that are stronger inducers of CYP3A than of CYP2B1/CYP2B2 proteins would also be more effective agonists for the receptor mainly involved in activation of *CYP3A* transcription than for that mainly involved in activation of *CYP2B* transcription. This situation would be reversed for PB, which is a poor CYP3A inducer and a strong CYP2B inducer. According to this model, up to four different cellular receptors (PBR, $GABA_A$ receptor and two nuclear receptors) would share as activators or ligands a vast group of chemicals with various structures. If this hypothesis were valid, one could argue that some nuclear receptors have developed a flexible ligand binding pocket in order to mimic the binding capacities of multimeric membrane bound receptor complexes (such as $GABA_A$ receptor and PBR) with multiple binding sites for diverse classes of chemicals. In accordance with this, PXR is known to be activated by many structurally diverse chemicals [40]. By recognising lipophilic exogenous compounds binding to membrane bound receptors and limiting the duration of their potentially adverse effects via the induction of key P450 enzymes that promote their elimination from the organism, such receptors may have conferred a selective advantage to their bearers.

A second possible explanation is that the binding of these compounds to the $GABA_A$ receptor or PBR is causally involved in a process upstream of transcription activation of *CYP2B* and *CYP3A* genes by nuclear receptors via the PBRU and PXR response elements. PB was reported to displace [^{3}H]PK 11195 from its binding site on PBR with an IC_{50} around 1 mM [18], a concentration commonly used for PB-induction of CYP2B proteins in hepatocytes. However, no study has yet determined if this property is conserved in rat hepatocytes, an important concern knowing the great variability of PBR pharmacological properties between species and tissues [19]. PK 11195, a nanomolar affinity ligand of rat PBR, is a strong CYP2B1/CYP2B2 and CYP3A inducer [27]. Our work now adds three more nanomolar affinity PBR ligands, Ro5-4864, FGIN 1–27 and DCPPBT, to the list of CYP2B1/CYP2B2 inducers. However, the observation that three of these four compounds exhibited EC_{50} values for PBRU-dependent transcription activation in the micromolar range (Table 1) (about three orders of magnitude higher than the reported affinity of PBR for these agents [19,34,35]) argues against the possibility that PBR is involved in transcriptional activation of PB-inducible *CYP2B* genes.

While the reported affinities of the $GABA_A$ receptor for PB, picrotoxin and bicuculline correspond rather well to the EC_{50} values presented in Table 1, nanomolar affinities of most $GABA_A$ receptor subtypes for benzodiazepines are substantially different from their potencies in inducing PBRU-dependent transcription, which rather stand in the low micromolar range. This observation appears, at first glance, to argue against the implication of $GABA_A$ receptor in PB-like induction of CYP2B1/CYP2B2. However, the existence of a nanomolar affinity benzodiazepine binding site on $GABA_A$ receptors was shown to require the presence of both an α and a γ subunit [41,42]. In a study to evaluate which $GABA_A$ receptor subunit is present in rat hepatocytes, only the β3 subunit could be detected [17], providing evidence that the nanomolar affinity benzodiazepine binding site is not present in these cells. Furthermore, it has been observed that many benzodiazepines can bind the picrotoxin/barbiturate binding site (which is thought to be conserved even on β homopentamers [41]) on various $GABA_A$ receptor subtypes, albeit with much lower affinity (typically in the micromolar range) than the nanomolar affinity benzodiazepine binding site [36,43]. Interestingly, such low affinity values would correspond better to the EC_{50} values presented in Table 1. This low affinity benzodiazepine binding site appears to be present on β3 homopentameric $GABA_A$ receptors, as a new low affinity [^{3}H]flunitrazepam binding site is present in Ad293 cells transfected with a rat $GABA_A$ receptor β3 subunit expression vector (C. Roberge and A. Anderson, unpublished observations).

Furthermore, Ro5-4864 and PK 11195, generally described as specific PBR ligands, were also shown to bind some $GABA_A$ receptor subtypes with affinities in the high nanomolar to micromolar range, respectively [26,37]. Such affinities would fit well with the EC_{50} values presented in Table 1. Thus, with the exception of the recently developed FGIN 1–27 and DCPPBT, all tested substances have previously been reported to bind $GABA_A$ receptors with affinities grossly matching EC_{50} values from Table 1.

This observation encourages further studies to determine whether binding of PB-like inducers to hepatic $GABA_A$ receptors is causally involved in CYP2B1/CYP2B2 induction. However, if the $GABA_A$ receptor were involved in CYP2B induction, this effect would apparently not be related to the potentiation of GABA-induced chloride currents by $GABA_A$ modulatory ligands since both positive (barbiturates, benzodiazepines) and negative (picrotoxin) allosteric modulators or antagonists (bicuculline) of GABA-induced chloride currents appear to be CYP2B inducers. In any event, it is striking that all $GABA_A$ receptor ligands tested were found to be inducers of CYP2B1/CYP2B2 in primary rat hepatocytes.

If the $GABA_A$ receptor is involved in PB-induction, signal transduction would have to take place between the receptor located at the cell membrane and the nucleus. In such a case, the diazepam binding inhibitor/acyl-CoA binding protein (DBI/ACBP), a short protein interacting with both PBR and $GABA_A$ receptors [19] would be an interesting candidate for a second messenger. It has been observed in the nucleus of rat hepatocytes and it has been proposed to modulate the activity of nuclear receptors [44,45]. An alternative possibility relates to the existence of two protein kinase A phophorylation sites (serine 408 and serine 409) on $GABA_A$ β3 subunits, the phosphorylation of which enhances GABA-induced chloride currents [46]. cAMP analogs abolish induction by PB [47] and so do serine/threonine phosphatase inhibitors [48]. The latter observation suggests that induction by PB-type inducers may require dephosphorylation at an unknown site. Serine 408 and serine 409 of the $GABA_A$ β3 subunits would be interesting candidates for this unknown dephosphorylation site.

In summary, we have shown that bicuculline, Ro5-4864, flunitrazepam, FGIN 1–27 and DCPPBT, five compounds not previously known as CYP inducers, are CYP3A1 and/or CYP2B1/CYP2B2 inducers in primary rat hepatocytes. We also demonstrated the essential role of the PBRU and of intact NR1 and NR2 sites for induction of *CYP2B2* PBRU-driven *luc* transcription in a reporter vector following treatment with eight PBR or $GABA_A$ receptor ligands, suggesting a molecular mechanism similar to that for PB induction. Finally, we evaluated potencies of 11 such compounds for PBRU-dependent transcription activation. Comparison of the values obtained with reported values of the affinity of $GABA_A$ receptor subtypes for these substances encourages further studies to determinate whether binding of PB-like inducers to hepatic $GABA_A$ receptors is causally involved in CYP2B1/CYP2B2 induction.

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