

FULL-LENGTH ORIGINAL RESEARCH

Functional characterization of the antiepileptic drug candidate, padsevonil, on GABA_A receptors

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Funding information

Studies reported here were funded by UCB
Pharma.

Abstract

Objective: The antiepileptic drug candidate, padsevonil, is the first in a novel class of drugs designed to interact with both presynaptic and postsynaptic therapeutic targets: synaptic vesicle 2 proteins and γ -aminobutyric acid type A receptors (GABA_ARs), respectively. Functional aspects of padsevonil at the postsynaptic target, GABA_ARs, were characterized in experiments reported here.

Methods: The effect of padsevonil on GABA-mediated Cl⁻ currents was determined by patch clamp on recombinant human GABA_ARs ($\alpha 1\beta 2\gamma 2$) stably expressed in a CHO-K1 cell line and on native GABA_ARs in cultured rat primary cortical neurons. Padsevonil selectivity for GABA_AR subtypes was evaluated using a two-electrode voltage clamp on recombinant human GABA_ARs ($\alpha 1-5/\beta 2/\gamma 2$) in *Xenopus* oocytes.

Results: In recombinant GABA_ARs, padsevonil did not evoke Cl⁻ currents in the absence of the agonist GABA. However, when co-administered with GABA at effective concentration (EC)₂₀, padsevonil potentiated GABA responses by 167% (EC)₅₀ 138 nmol/L) and demonstrated a relative efficacy of 41% compared with zolpidem, a reference benzodiazepine site agonist. Similarly, padsevonil demonstrated GABA-potentiating activity at native GABA_ARs (EC)₅₀ 208 nmol/L) in cultured rat cortical neurons. Padsevonil also potentiated GABA (EC)₂₀ responses in GABA_ARs expressed in oocytes, with higher potency at $\alpha 1$ - and $\alpha 5$ -containing receptors (EC)₅₀ 295 and 281 nmol/L) than at $\alpha 2$ - and $\alpha 3$ -containing receptors (EC)₅₀ 1737 and 2089 nmol/L). Compared with chlordiazepoxide—a nonselective, full GABA_AR agonist—the relative efficacy of padsevonil was 60% for $\alpha 1\beta 2\gamma 2$, 26% for $\alpha 2\beta 2\gamma 2$, 56% for $\alpha 3\beta 2\gamma 2$, and 41% for $\alpha 5\beta 2\gamma 2$; no activity was observed at benzodiazepine-insensitive $\alpha 4\beta 2\gamma 2$ receptors.

Significance: Results of functional investigations on recombinant and native neuronal GABA_ARs show that padsevonil acts as a positive allosteric modulator of these receptors, with a partial agonist profile at the benzodiazepine site. These properties may confer better tolerability and lower potential for tolerance development compared with classic benzodiazepines currently used in the clinic.

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KEYWORDS

benzodiazepine, drug-resistant epilepsy, patch clamp, SV2 proteins

1 | INTRODUCTION

Padsevonil, a novel antiepileptic drug (AED) candidate, is the first in a class of drugs with both presynaptic and postsynaptic mechanism of action. The rationale for the design and development of such a drug was the observation that in nonclinical models, co-administration of levetiracetam (LEV), a synaptic vesicle 2A (SV2A) ligand together with agents acting on γ -aminobutyric acid type A (GABA_A) receptors (GABA_ARs) led to enhanced protection against seizures, without any detrimental effect on safety.¹ Consequently, it was hypothesized that a single molecular entity acting simultaneously on these presynaptic and postsynaptic targets may translate into improved clinical efficacy in the treatment of patients with epilepsy, particularly those with drug-resistant epilepsy.

The selective interaction of Padsevonil with its molecular targets, as intended in the rational drug discovery program, has been confirmed in both in vitro and in vivo studies.² Specifically, Padsevonil was designed to bind to the presynaptic SV2 proteins with high affinity and to postsynaptic GABA_ARs, with low-to-moderate affinity. In radioligand displacement studies, Padsevonil has been shown to bind to SV2A with nanomolar affinity (pKi 8.5) that is approximately 100- and 2000-fold greater than that of brivaracetam (BRV) and LEV, respectively.² Another major differentiating factor between Padsevonil and the SV2A selective ligands LEV and BRV is that Padsevonil also binds with high affinity to the B and C isoforms of SV2 proteins (pKi 7.9 and 8.5, respectively).² Padsevonil also demonstrates different binding properties to SV2A in that it displays significantly slower dissociation kinetics, translating into longer target occupancy.² Furthermore, unlike LEV and BRV, the interaction between Padsevonil and SV2A is not affected by UCB1244283, a positive allosteric modulator of SV2A, suggesting a different binding site for Padsevonil in the SV2A protein.²⁻⁴ At recombinant GABA_ARs, Padsevonil displayed low-to-moderate affinity (pKi 6.4) for the benzodiazepine (BZD) binding site. This binding profile has been confirmed in receptor occupancy studies in vivo (mice), where Padsevonil exhibited SV2A occupancy at low doses (median effective dose [ED₅₀] 0.2 mg/kg), and BZD site occupancy at higher doses (ED₅₀ 36 mg/kg).² Overall, these observations support the unique target profile of Padsevonil that should translate into presynaptic modulation of neurotransmitter release and the postsynaptic potentiation of GABAergic inhibitory transmission.

Key Points

- Padsevonil, an antiepileptic drug candidate, was rationally designed to interact with presynaptic (SV2) and postsynaptic (GABA_A receptor) targets
- Padsevonil binds with low-to-moderate affinity to recombinant and native GABA_A receptors
- Padsevonil acts as a partial agonist at the benzodiazepine site of GABA_A receptors

The objective of the experiments reported here was to fully characterize the functional effects of Padsevonil on different GABA_AR subunits in recombinant and native neuronal systems using electrophysiologic techniques.

2 | MATERIAL AND METHODS**2.1 | Animals**

All experiments were conducted in compliance with guidelines issued by the ethics committee for animal experimentation according to Belgian law. All efforts were made to minimize animal pain and discomfort.

For patch-clamp recordings in rat primary cortical neurons, pregnant female Wistar rats were obtained from Charles River (Lyon, France) and embryonic animals were surgically removed at embryonic days E17-E18. Animals were housed in a holding room under a 12-hour light–dark cycle with lights on at 06:00 hours. Temperature was maintained at 20–24°C, relative humidity at 40%–70%, and the rate of air replacement was at least 15 times per hour. Animals had on-demand access to standard dry pellet food and tap water.

2.2 | Compounds

Padsevonil ((4R)-4-(2-chloro-2,2-difluoroethyl)-1-[[2-(methoxymethyl)-6-(trifluoromethyl)imidazo[2,1-b][1,3,4]thiadiazol-5-yl]methyl]pyrrolidin-2-one) was synthesized at UCB Pharma. Zolpidem was obtained from Tocris (Ref 0655; B5A/91713), GABA from Sigma (Ref A2129; B045K00721), chlordiazepoxide from Sequoia Research (Cat. No. SRP02170c), and Ro15-4513 from Tocris (Cat. No. 1997). All stock solutions were prepared at a maximum of 10 mmol/L in dimethyl sulfoxide (DMSO, 100%) and stored at –20°C. When

required, the compounds were diluted into the extracellular medium yielding a final DMSO concentration of 0.1%–0.5%.

2.3 | Patch-clamp recordings on recombinant human GABA_A receptors

GABA_AR Cl⁻ currents were recorded using automated patch clamp from recombinant human GABA_ARs consisting of the following subunits: α 1 (accession number NM000806), β 2 (NM021911), and γ 2 (NM198904) stably expressed in a CHO-K1 cell line. Full details have been described previously.⁵ Briefly, CHO cells expressing these subunits were cultured in Ham's F-12 medium with glutamine (GIBCO, Carlsbad, CA, USA) containing 400 μ g/mL G418 (GIBCO), 250 μ g/mL Zeocin (Invitrogen), 10% fetal bovine serum, and 1% penicillin/streptomycin (Lonza). Cells were harvested using Accumax treatment (Sigma) and allowed to recover for 90 min at room temperature before recording started.

For patch-clamp recording, the extracellular solution contained 135 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L MgCl₂, 11 mmol/L glucose, and 10 mmol/L HEPES (adjusted to pH 7.4 with NaOH 5 N), and intracellular solution 100 mmol/L KCl, 40 mmol/L KF, 2 mmol/L MgCl₂, 11 mmol/L EGTA, 10 mmol/L glucose, and 10 mmol/L HEPES (adjusted to pH 7.2 with KOH 1 N).

Currents were recorded using the automated patch-clamp platform PatchXpress 7000A (Molecular Devices), with the holding potential set to -60 mV. Current traces were recorded at room temperature by patch-clamp amplifier (Multiclamp 700A Computer-Controlled Patch Clamp Dual Headstage Amplifier, Molecular Devices) at a sampling rate of 2 KHz. Data were analyzed using DataXpress 2 software (version 2.0.4.2; Molecular Devices). To measure the effects of an allosteric modulator binding to the BZD site of GABA_ARs, a low concentration of agonist is needed to potentiate the drug response. For these experiments, 5 μ mol/L GABA was used, a concentration that mediates 20% of GABA_AR activation (EC₂₀). To quantify the potentiation of GABA_AR Cl⁻ currents by Padsevonil relative to zolpidem, the experimental protocol described by Ghisdal et al was followed.⁶ Briefly, potentiation of currents following application of 5 μ mol/L GABA and 10 or 30 μ mol/L Padsevonil is determined and compared with the maximum potentiation elicited by application of 5 μ mol/L GABA and 1 μ mol/L zolpidem in the same cell after washout.

2.4 | Two-electrode voltage clamp recordings on recombinant human GABA_A receptors in *Xenopus* oocytes

Different subunits of the human GABA_AR, including α 1 (NM000806), α 2 (NM000807), α 3 (NM000808), α 4

(NM000809), α 5 (NM000810), β 2 (NM021911), and γ 2 (BC059389) were cloned into pcDNA3.1(+) (Invitrogen). GABA_AR subunit mRNA for oocyte injections were prepared by RD-Biotech (Besançon, France). *Xenopus leavis* oocytes (stage V–VI), dissected and defolliculated, were purchased from EcoCyte Bioscience (Castrop-Rauxel, Germany). Microinjections of mRNA for the GABA_AR subunits (α 1-5: β 2: γ 2; ratio 1:1:5.5) were performed with the automated system Roboocyte (Multi Channel Systems MCS, Reutlingen, Germany) using a total volume ~50 nL mRNA dissolved in RNase-free water (Ambion). Injected oocytes were kept at 17°C for 3–6 days in Barth's solution containing 88 mmol/L NaCl, 2.4 mmol/L NaHCO₃, 1 mmol/L KCl, 0.33 mmol/L Ca(NO₃)₂, 0.41 mmol/L CaCl₂, 0.82 mmol/L MgSO₄, 5 mmol/L Tris/HCl, supplemented with penicillin (100 UI/mL)/streptomycin (100 μ g/mL) (Whittaker Cambrex) (adjusted to pH 7.4 with NaOH 1 N).

Two-electrode voltage clamp (TEVC) recordings were performed with the automated Roboocyte system (Multi Channel Systems MCS) using standard recording protocols. Oocytes were impaled and voltage clamped at a holding potential of -60 mV. After impalement, the oocytes were rinsed with normal frog ringer buffer for 60 s, and allowed to stabilize for a further 60 s. Normal frog ringer solution contained 115 mmol/L NaCl, 2.5 mmol/L KCl, 1.8 mmol/L CaCl₂, 10 mmol/L HEPES (adjusted to pH 7.2 with NaOH 5 N). For each oocyte, GABA was applied twice for 20 s with a 6 min interval to evoke inward Cl⁻ currents and was used as the control GABA current. The concentration–response curve was started by a 6-min pre-incubation of Padsevonil (1 nmol/L to 30 μ mol/L) followed by co-application of GABA and Padsevonil for 20 s.

Drugs were applied via a liquid dispenser (Gilson GX271) coupled to a peristaltic pump (Gilson MINIPULS 3). The perfusion rate of oocytes in the 96-well plates was set at approximately 3 mL/min. All solutions were prepared freshly before each experiment. During measurements, oocytes were perfused with normal frog ringer solution.

GABA-evoked Cl⁻ currents were analyzed by the Roboocyte Software version 2.2 (Multi Channel Systems MCS). The magnitude of the potentiation of the currents by the drug was calculated based on the ratio I(GABA + drug)/IGABA where I(GABA + drug) is the current response evoked by co-application of drug and GABA and IGABA represents the current amplitude evoked by GABA alone.

Dose–response curves were analyzed using GraphPad Prism software and EC₅₀ values calculated by nonlinear regression analysis using a sigmoidal dose–response equation, where $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X))})$. Data were normalized to maximum potentiation values of the drug.

2.5 | Patch-clamp recordings of native GABA_A receptor currents in rat primary cortical neurons

Rat cortical neurons were prepared as described previously.⁶ Briefly, neocortex was removed from Wistar embryonic rats (E17-E18) of either sex, dissociated in 0.25% trypsin, and triturated in complete neurobasal-based medium composed of neurobasal medium, 2% B27 serum-free supplement, 0.5 mmol/L glutamax (all from Invitrogen), 10% horse serum, and 1% penicillin/streptomycin (Lonza, Basel, Switzerland). Coverslips coated with poly-D-lysine and laminin (Sigma) were seeded, and neurons (40 000-50 000 cells/mL) grown in complete neurobasal-based medium. Cultures were maintained for 15 days at 37°C in a 5% CO₂/95% air incubator.

Voltage clamp recordings of GABA_AR currents were performed on primary cortical neurons (days in-vitro, 10-14) in tight-seal whole-cell patch-clamp configuration. Patch pipettes were pulled from 1.5 mm (inside diameter) borosilicate glass capillaries (Harvard Apparatus, MA, USA) and had a resistance of 4-5 MΩ when filled with intracellular solution composed of 140 mmol/L KCl, 10 mmol/L EGTA, 2 mmol/L MgCl₂, 2 mmol/L Na₂-ATP, and 10 mmol/L HEPES (adjusted to pH 7.2 with KOH). Currents were recorded at room temperature. Neuronal preparations were continuously perfused at a rate of ~1 mL/min with extracellular solution composed of 140 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L MgCl₂, 1.3 mmol/L CaCl₂, 12.5 mmol/L HEPES, 10 mmol/L glucose, 0.001 mmol/L tetrodotoxin, and 0.01 mmol/L CGP 35 348, a GABA_B receptor antagonist (adjusted to pH 7.3 with NaOH). The junction potential between the intracellular and extracellular solutions was measured at 4 mV and compensated for by on-line subtraction. Series resistances were compensated by 70% on the amplifier (Axopatch 200B, Molecular Devices). After seal formation, the pipette capacitive current was cancelled, and following breakthrough, the whole-cell capacitive current was cancelled. Currents were filtered at 100 kHz and digitized at 5 kHz using a Digidata 1322A converter and the acquisition program pClamp (both Molecular Devices). According to the Cl⁻ equilibrium potential measured at -1 mV, the holding membrane potential was set at -70 mV for recording GABA-activated Cl⁻ currents. Control of drug application, data acquisition, and data analysis was achieved using pClamp software (Molecular Devices).

Padsevonil was diluted in the extracellular solution and applied by a rapid microperfusion system (VC83, ALA Scientific Instruments). GABA was applied at 2 μmol/L (concentration previously determined as EC₁₀) for 10 s to evoke the basal inward GABA_AR Cl⁻ currents, followed by a series of GABA applications together with Padsevonil at increasing concentrations (0, 0.1, 1, 10, and 30 μmol/L). Each GABA application in the protocol was followed by a 4-min washout period to allow recovery from receptor desensitization. The intensity of GABA-evoked currents was measured at the peak.

3 | RESULTS

3.1 | Padsevonil is a positive allosteric modulator of GABA_A receptors with a partial agonist profile

The functional activity of Padsevonil on recombinant GABA_ARs was determined using patch-clamp recordings from the human α1β2γ2 GABA_AR stably expressed in a CHO cell line, for which the pharmacological profile has been previously established.⁶ The effects of Padsevonil (0.01-30 μmol/L) on the receptor was first evaluated in the presence of the agonist, GABA, at EC₂₀ (a concentration that produces 20% of the maximum response) set to 100%. Padsevonil potentiated the agonist response by 167%, with an EC₅₀ of 138 nmol/L (Figure 1A), but at concentrations up to 30 μmol/L, it did not evoke Cl⁻ currents in the absence of GABA, confirming that it modulates GABA_AR activity through an allosteric site on the receptor (Figure 1B). The effects of Padsevonil on the maximal activation of the receptor were also evaluated using GABA at a concentration of 30 μmol/L (EC₉₀), confirming that Padsevonil does not overstimulate the receptor at high GABA concentrations (data not shown).

To evaluate the relative efficacy of Padsevonil binding to the BZD site of the GABA_AR, the effects of Padsevonil and zolpidem were tested sequentially on the same cell. Zolpidem, a full agonist at the BZD site, potentiates GABA (EC₂₀) with a maximal efficacy at 1 μmol/L, defined as the maximal response (100%). At concentrations of 10 and 30 μmol/L, Padsevonil stimulated the GABA EC₂₀ response to 41% and 35% of that of zolpidem, respectively, indicating that Padsevonil elicits a partial agonist response on human α1β2γ2 GABA_ARs in comparison with zolpidem (Figure 1C).

3.2 | Padsevonil is a nonselective modulator of benzodiazepine-sensitive GABA_A receptors

The selectivity of Padsevonil for specific GABA_AR subtypes was evaluated in three sets of experiments using TEVC. In the first, activity of Padsevonil at different recombinant GABA_ARs containing BZD-sensitive subunits was measured. In a transient expression system in *Xenopus* oocytes, mRNA encoding α1, α2, α3, and α5 was expressed in the presence of β2/γ2 subunits. Dose-response values of Padsevonil (1-30 μmol/L) on the different receptor subtypes are shown in Figure 2. Padsevonil potentiated GABA responses (EC₂₀) at receptors containing α1 and α5 subunits with similar potency (EC₅₀ 295 and 281 nmol/L; Table 1), whereas potentiation of receptors with α2 and α3 subunits was noted at higher

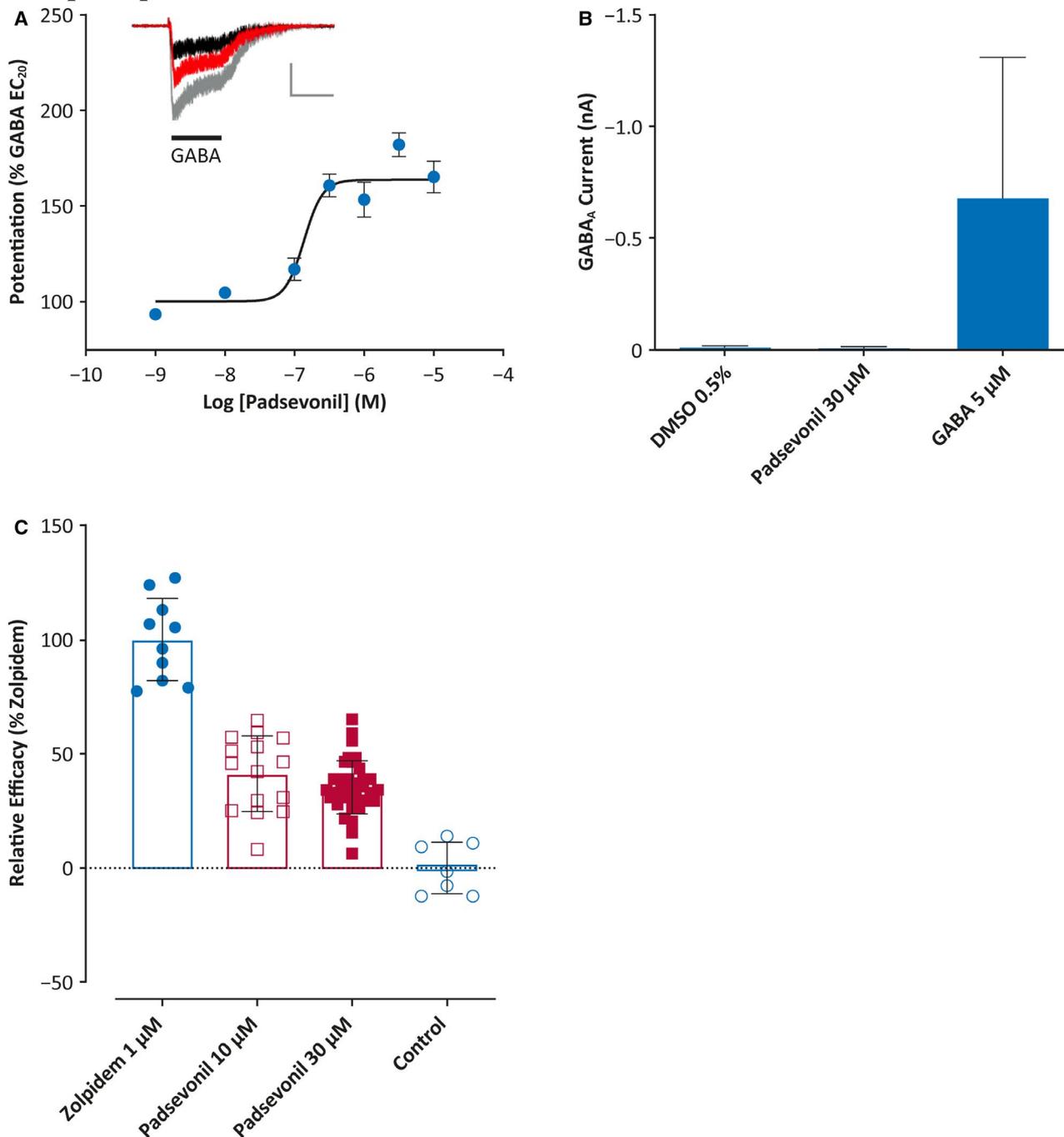


FIGURE 1 Functional properties of padsevonil on recombinant γ -aminobutyric acid type A (GABA_A) receptors. A, Patch-clamp recordings of human recombinant GABA_A receptor ($\alpha 1\beta 2\gamma 12$) currents in CHO cells. Padsevonil (1 nmol/L–10 μ mol/L) was tested in the presence of GABA at EC₂₀ (5 μ mol/L) ($n = 7$ –15 cells; \pm SEM). Inset: Representative GABA_A currents recorded from the same cell under control conditions (black), with padsevonil 10 μ mol/L (red) and with 1 μ mol/L zolpidem (gray). Horizontal bar represents 5 s and vertical bar 0.5 nA. B, Effects of padsevonil and GABA on GABA_A receptor currents ($n = 4$ –7 cells; \pm standard error of the mean [SEM]). C, Relative efficacy of padsevonil compared with zolpidem in potentiating GABA-mediated (EC₂₀) Cl⁻ currents in human $\alpha 1\beta 2\gamma 12$ GABA_A receptors ($n = 7$ –35 cells; \pm SEM). Error bars are \pm SEM

concentrations (EC₅₀ 1737 and 2089 nmol/L) (Figure 2A, Table 1).

In the second set of experiments, the relative efficacy of Padsevonil across different receptor combinations was evaluated using chlordiazepoxide as a comparator. Chlordiazepoxide is a nonselective full agonist of different

GABA_AR subtypes; its relatively low affinity and water solubility enables easy washout before subsequent incubations with other drugs being tested.⁷ The efficacy of Padsevonil was evaluated using GABA at EC₂₀ and the comparison with chlordiazepoxide (10 μ mol/L) showed a relative efficacy of $60 \pm 25\%$ for $\alpha 1$, $26 \pm 13\%$ for $\alpha 2$, $56 \pm 27\%$ for $\alpha 3$,

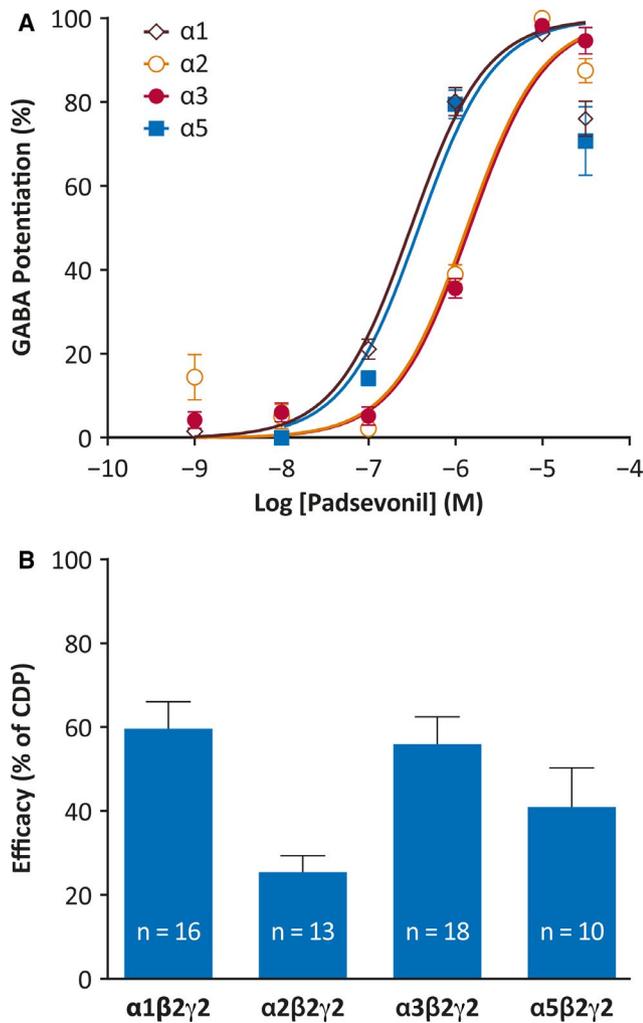


FIGURE 2 Padsevonil selectivity for γ -aminobutyric acid type A (GABA_A) receptor subtypes as determined by two-electrode voltage clamp recordings (TEVC) on human recombinant GABA_A receptors expressed in *Xenopus* oocytes. A, Dose–response of padsevonil (1 nmol/L–30 μ M/L). Data are shown as mean \pm SEM (n = 6–26 cells). B, Relative efficacy of padsevonil (10 μ M) compared with chlordiazepoxide (CDP, 10 μ M/L) on GABA_A receptor subtypes. Data are shown as mean \pm SEM. SEM, standard error of mean

and $41 \pm 29\%$ for $\alpha 5$ (Figure 2B). These results suggest that Padsevonil acts as a nonselective partial agonist at the BZD site of GABA_ARs.

In the third set, the activity of Padsevonil on GABA_ARs containing the $\alpha 4$ subunit was evaluated. These receptors are reported to be expressed at synaptic and extrasynaptic locations and are insensitive to BZDs. Results confirmed that GABA_ARs comprising $\alpha 4\beta 2\gamma 2$ subunits expressed in *Xenopus* oocytes are potentiated by the reference compound Ro 15-4513 (1 μ M/L)⁸ but are insensitive to zolpidem, a BZD site ligand (Figure 3). Padsevonil (10 and 30 μ M/L) did not potentiate the GABA (EC₂₀) response on $\alpha 4\beta 2\gamma 2$ receptors, confirming the lack of interaction with BZD-insensitive GABA_AR subtypes.

3.3 | Padsevonil modulates the activity of native GABA_A receptors in cultured neurons

The activity of Padsevonil on native GABA_ARs expressed in rat primary neuronal cultures was evaluated using manual

TABLE 1 Characteristics of the interaction of Padsevonil with human recombinant and native rat GABA_A receptors

	Function EC ₅₀ (nmol/L)	Function relative efficacy (%)
Human recombinant receptors		
α1β2γ2	138 ± 29 (n = 22) ^a 295 ± 38 (n = 26) ^b	41 ± 4 (n = 15) ^a 60 ± 6 (n = 16) ^b
α2β2γ2	1737 ± 258 (n = 12) ^b	26 ± 4 (n = 13) ^b
α3β2γ2	2089 ± 227 (n = 17) ^b	56 ± 6 (n = 18) ^b
α4β2γ2	No potentiation	Not evaluated
α5β2γ2	281 ± 73 (n = 6) ^b	41 ± 10 (n = 10) ^b
Primary rat cultured neurons		
Primary cortical neurons	208 ± 99 (n = 8) ^a	Not evaluated

Note: Values are mean \pm standard error of the mean.

^aPotentiation of GABA EC₂₀ measured by patch clamp. Relative efficacy of Padsevonil (10 μ M/L) is normalized toward zolpidem (1 μ M/L).

^bPotentiation of GABA EC₂₀ measured by two-electrode voltage clamp. Relative efficacy of Padsevonil (10 μ M/L) is normalized toward chlordiazepoxide (10 μ M/L).

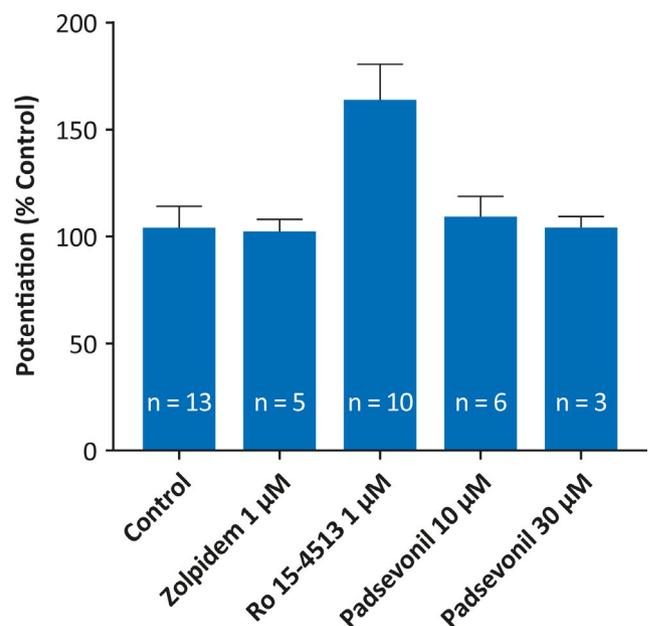


FIGURE 3 Effects of padsevonil, zolpidem, and the reference compound Ro 15-4513 on potentiation of the γ -aminobutyric acid (GABA; control) response on $\alpha 4\beta 2\gamma 2$ receptors expressed in *Xenopus* oocytes. Currents were recorded using two-electrode voltage clamp in the presence of GABA at EC₂₀. Error bars are mean \pm SEM. SEM, standard error of mean

patch-clamp recordings to measure whole-cell GABA_AR currents. In the presence of GABA at EC₂₀, Padsevoniol produced a concentration-dependent potentiation of activity at neuronal GABA_ARs (Figure 4A); pEC₅₀ was calculated to be 6.68. These results also confirm the previous observation that Padsevoniol acts via an allosteric site on native receptors.

To characterize the diversity of GABA_ARs expressed in cultured neurons (10–14 days *in vitro*), quantitative polymerase chain reaction (qPCR) analysis was performed

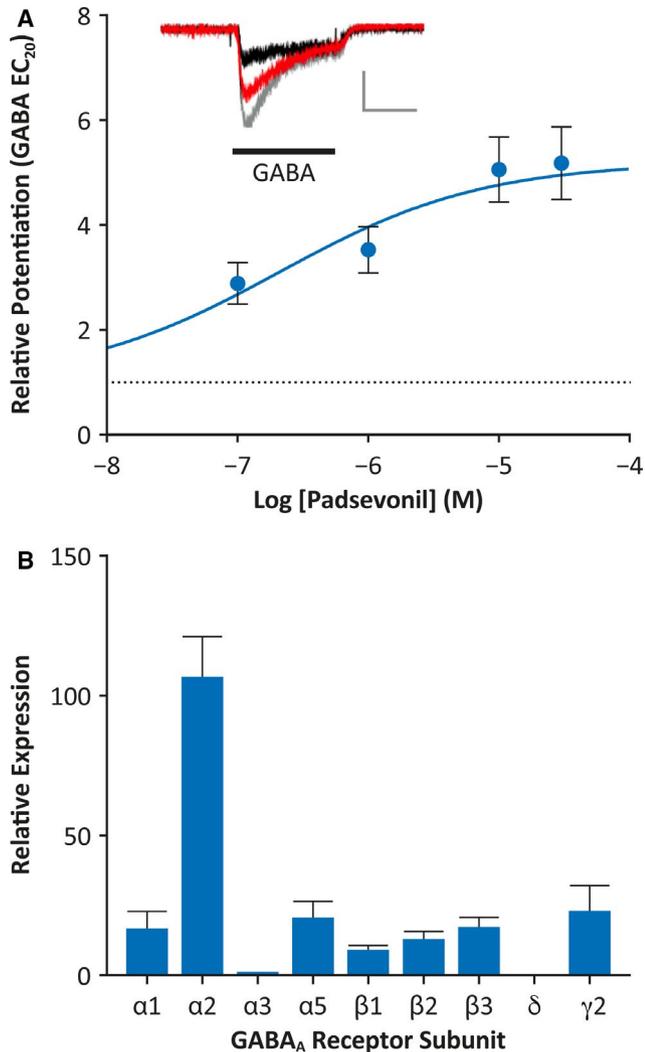


FIGURE 4 Effects of padsevoniol on native γ -aminobutyric acid type A (GABA_A) receptors in cultured neurons. A, Dose–response of padsevoniol on native GABA_A receptor currents in rat primary cortical neurons (days *in vitro* [DIV] 10–14). Data have been normalized to the basal evoked current with GABA at EC₁₀ (2 μ mol/L) ($n = 8$ neurons; mean \pm SEM). Inset: Representative traces of GABA_A receptor currents evoked under control conditions (in black), with padsevoniol 10 μ mol/L (in red) and with zolpidem 1 μ mol/L (in gray) in the same cortical neuron (DIV 11). Horizontal bar represents 5 s and vertical bar 1 nA. B, quantitative polymerase chain reaction analysis of GABA_A receptor subunits in rat primary cortical neurons (average of three different cultures; DIV 10–14). Data have been normalized to the maximal levels (mean \pm standard deviation)

(Figure 4B). With focus on the BZD-sensitive subtypes, results showed that most subunits were expressed except for the α 3 and δ subunits.

4 | DISCUSSION

Padsevoniol is an AED candidate rationally designed to bind with high affinity to SV2 proteins and with low-to-moderate affinity to the BZD binding site of the GABA_AR.² The focus of the studies reported here was on the functional aspects of the interaction between Padsevoniol and the BZD site of the GABA_AR to gain further insight into the molecular mechanism of action of Padsevoniol.

Patch-clamp experiments on recombinant GABA_ARs revealed that Padsevoniol had no intrinsic activity at these receptors—in the absence of the endogenous agonist GABA, Padsevoniol did not elicit Cl⁻ currents. However, when applied in the presence of GABA, Padsevoniol potentiated the agonist response by 167%. These results indicate that similar to many BZDs, Padsevoniol acts as a positive allosteric modulator of the GABA_AR. Such modulators bind to the GABA_AR at a site distinct from the agonist (GABA) binding site, in a pocket at the interface between the extracellular domains of α and γ subunits.⁹ Drug binding leads to a conformational change in the receptor that increases the apparent affinity for channel gating by GABA, which can bind simultaneously to two sites at the interfaces between α and β subunits.^{10,11} Different BZDs show varying levels of potency or activity at the GABA_AR, acting as full, partial, or inverse agonists or antagonists at the BZD site.¹² Padsevoniol was designed specifically to function as a partial agonist, given that in animal models, partial agonists were reported to be associated with fewer sedative adverse effects and lack of development of tolerance and dependence with long-term administration compared with classic BZDs such as diazepam, which act as full agonists.¹³ Strategies to reduce BZD limitations have included development of GABA_AR subtype-selective agents or agents with low relative efficacy to limit receptor activation.¹⁴ In experiments reported here, the relative efficacy of Padsevoniol was compared with that of zolpidem, a reference compound that acts as a full agonist at the BZD site with a maximal response defined as 100%. Results showed that the relative efficacy of Padsevoniol was 41%, confirming that it acts as a partial agonist.

As part of the ligand-gated ion channel superfamily, GABA_ARs are organized as pentameric membrane-spanning proteins surrounding a central pore that forms the ion channel.¹⁵ So far, 19 mammalian genes encoding GABA_AR subunits, forming eight subunit classes, have been cloned.¹⁵ The large number of subunits assembling in a variety of configurations gives rise to specific GABA_AR subtypes, with different cellular and subcellular distribution, and importantly, different pharmacological profiles.^{11,12} Notably, not all

pentameric configurations are sensitive to BZDs. Receptors containing an α/γ subunit interface, with $\alpha 1$, 2, 3, or 5 and predominantly $\gamma 2$ subunits, form a high affinity binding site for BZDs.^{9,16} In contrast, receptor subtypes containing $\alpha 4$ or $\alpha 6$ subunits are described as BZD-insensitive because of a substitution mutation where a conserved histidine residue, which confers BZD sensitivity, is replaced by an arginine residue.¹⁷⁻¹⁹ Given these differences in sensitivity, Padsevonil activity at BZD-sensitive and BZD-insensitive GABA_AR subtypes was determined. Results showed that Padsevonil binds to all BZD-sensitive GABA_AR subtypes; however, it displays approximately sixfold higher potency for receptors with $\alpha 1$ or $\alpha 5$ subunits than for those with $\alpha 2$ or $\alpha 3$ subunits. Similar to results described above, the relative efficacy of Padsevonil at these GABA_AR subtypes was lower than that of chlordiazepoxide, a reference compound that acts as a nonselective full agonist of different GABA_AR subtypes, confirming that Padsevonil displays a partial agonist profile. Results further showed that Padsevonil does not activate GABA_AR subtypes containing the $\alpha 4$ subunit. The BZD-insensitive receptor subtypes containing $\alpha 4$ or $\alpha 6$ subunits are predominantly extrasynaptic and are thought to mediate tonic inhibitory currents, distinguishable from phasic synaptic transmission mediated by postsynaptic GABA_AR subtypes.^{12,16} Tonic inhibition is a consequence of persistent activation of GABA_AR dispersed over the neuronal surface by low levels of ambient GABA, and is important for regulating the overall excitability of neurons; phasic inhibition on the other hand is spatially and temporally discrete, allowing the rapid, point-to-point synaptic communication used to control the flow of specific signals.^{11,16,20} Phasic inhibition also plays a central role in the generation and maintenance of neuronal network rhythmicity, and consequently, synchronization of inhibitory/excitatory networks, especially under conditions of hypersynchronicity observed during seizure activity.^{16,21} Results of experiments reported here suggest that Padsevonil may preferentially modulate the phasic inhibition mediated by postsynaptic GABA_AR. Finally, experiments performed on rat primary cortical neurons indicated that Padsevonil has a similar potency for native and recombinant GABA_AR, with native receptors mainly represented by the $\alpha 1$ -, $\alpha 2$ -, and $\alpha 5$ -containing receptor subtypes in this neuronal system.

The properties of Padsevonil described above may confer advantages over classic BZDs used in the clinic. Specifically, the low-to-moderate affinity binding combined with the partial agonist profile could limit the level of occupancy and activation of GABA_AR in the brain. Although sufficient to contribute to antiseizure activity, the risk of developing side effects and tolerance could be reduced. Nonclinical studies have shown that Padsevonil is highly active in models of drug-resistant epilepsy²² at doses that lead to >95% SV2A occupancy and <20%

occupancy on GABA_AR.² The potential of Padsevonil to induce tolerance was evaluated in the validated pentylenetetrazol (PTZ)-induced clonic seizure threshold test, where the ability of AEDs to increase the seizure threshold is evaluated after acute and repeated dosing.²³ With both acute and repeated (twice daily for 4 consecutive days) dosing, Padsevonil increased the threshold for PTZ-induced seizures to the same extent, indicating that tolerance was not developed.²² In contrast, diazepam, a full agonist at the BZD site, showed a reduced ability to increase the PTZ threshold after repeated dosing.²² Clinical evidence also supports the observation that partial agonists may be associated with a lower risk of tolerance. Clobazam, a partial agonist at the BZD site, has been used for the treatment of patients with Lennox-Gastaut syndrome for many years.^{24,25} Furthermore, in a long-term extension trial, sustained seizure control was observed at stable dosages over a 3-year period, indicating lack of tolerance development.^{26,27} Abecarnil, another partial agonist, has shown efficacy in the treatment of patients with photosensitive epilepsy without development of tolerance.²⁸

In conclusion, Padsevonil is a novel AED candidate with a unique mechanism of action, interacting with both presynaptic and postsynaptic targets. Via its dual impact on neuronal activity, dampening excitation, and enhancing inhibition, it could potentially curtail neuronal hyperactivity during seizures more rapidly and strongly than currently available AEDs. It has shown robust efficacy in patients with highly treatment-resistant focal epilepsy in a Phase IIa proof-of-concept trial.²⁹ Experiments reported here have shed further light on the functional effects of Padsevonil on GABA_AR. It is notable, however, that the activity of Padsevonil on GABA_AR during epileptogenesis or under conditions of chronic epilepsy remains to be evaluated. GABA_AR undergo major plasticity during epileptogenesis, with changes involving modulation of the expression of receptor subunits and functional alterations.^{30,31} Furthermore, it has been shown that paradoxically, in brain tissue obtained from patients with drug-resistant epilepsy, GABA can cause depolarization resulting in excitatory signals,³² an observation attributed to disruption of Cl⁻ homeostasis.³³ The impact of Padsevonil on such excitatory currents needs further evaluation. Additional experiments based on relevant ex or in vivo models, for example, brain slices from animals with chronic epilepsy, are also required to provide a better understanding of Padsevonil activity on GABA_AR that have gone plasticity changes due to pathophysiologic conditions.

ACKNOWLEDGMENTS

The authors thank Anne Vandendriessche and Nadine Noel for their contribution to the automated patch-clamp recordings. The authors also thank Fabien Debailleul, PhD, UCB

Pharma, Brussels, Belgium for overseeing the development of the manuscript, and Azita Tofighy for providing writing support, funded by UCB Pharma.

CONFLICT OF INTERESTS

All authors were employees of UCB Pharma at the time of research. MW has now retired, RMK is currently employed by OncoArendi Therapeutics SA, Warsaw, Poland, and PG by Pi life sciences consultancy, Ghent, Belgium. The authors confirm having read the Journal's position on ethical publication and affirm that this report is consistent with Journal guidelines.

DATA AVAILABILITY STATEMENT

Data from nonclinical studies are outside the scope of UCB's data sharing policy.

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How to cite this article: Niespodziany I, Ghisdal P, Mullier B, et al. Functional characterization of the antiepileptic drug candidate, padsevonil, on GABA_A receptors. *Epilepsia.* 2020;61:914–923. <https://doi.org/10.1111/epi.16497>