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TP003 is a non-selective benzodiazepine site agonist that induces anxiolysis via α2GABA$_A$ receptors

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HIGHLIGHTS

- TP003 has been claimed to be an α3GABA$_A$ receptor selective modulator.
- Based on TP003, claims were made that anxiolysis occurs through α3GABA$_A$ receptors.
- Here, TP003 potentiated all four benzodiazepine sensitive GABA$_A$ receptor subtypes.
- *In vivo*, it exerted anxiolysis through α2 but not α3GABA$_A$ receptors.

ABSTRACT

Benzodiazepines (BDZ), which potentiate the action of GABA at four subtypes of GABA$_A$ receptors (α1, α2, α3, and α5GABA$_A$Rs), are highly effective against anxiety disorders, but also cause severe side effects greatly limiting their clinical application. Both, preclinical studies in genetically engineered mice, and preclinical and clinical trials with subtype-selective compounds indicate that undesired effects can in principle be avoided by targeting specific GABA$_A$R subtypes. While there is general consensus that activity at α1GABA$_A$Rs should be avoided, controversy exists as to whether α2 or α3GABA$_A$Rs need to be targeted for anxiolysis. While previous experiments in GABA$_A$R point-mutated mice demonstrated a critical role of α2GABA$_A$Rs, studies solely relying on pharmacological approaches suggested a dominant contribution of α3GABA$_A$Rs. As most α1GABA$_A$R-sparing BDZ site agonists discriminate little between α2 and α3GABA$_A$Rs, these claims rest almost exclusively on a single compound, TP003, that has been reported to be a selective α3GABA$_A$R modulator. Here, we have revisited the *in vitro* pharmacological profile of TP003 and, in addition, tested TP003 in GABA$_A$R triple point-mutated mice, in which only either α1, α2, or α3GABA$_A$Rs were left BDZ sensitive. These experiments revealed that TP003 behaves as a partial, rather non-selective BDZ site agonist *in vitro* that acts *in vivo* through α1, α2, and α3GABA$_A$Rs (α5GABA$_A$R-mediated effects were not tested). With respect to anxiolysis, our results support a critical contribution of α2GABA$_A$Rs, but not of α3GABA$_A$Rs. TP003 should therefore not be considered an α3GABA$_A$R selective agent. Previously published studies using TP003 should be interpreted with caution.

1. Introduction

Benzodiazepines (BDZs) are widely used for the treatment of anxiety disorders since more than 50 years (Kalueff and Nutt, 2007; Rudolph and Knoflach, 2011; Möhler, 2012). The anxiolytic effects of basically all clinically used BDZs are accompanied by major unwanted effects including sedation, impaired motor coordination, addiction and tolerance development. Desired and undesired effects of classical BDZs result from their nonselective action on four subtypes of GABA$_A$Rs. These BDZ sensitive GABA$_A$Rs carry a high-affinity binding site formed by an interface between an α1, α2, α3 or α5 subunit and a γ2 subunit (Wieland et al., 1992). Compelling evidence both from experiments

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with $GABA_AR$ point-mutated mice and with subtype-selective compounds indicate that the different desired and undesired actions can be attributed to specific $GABA_AR$ subtypes best defined by the subtype of the α subunit included in the receptor complex. Sedation, and most of the other unwanted effects, including addiction, amnesia and possibly also part of the motor incoordination occur through α1$GABA_ARs$ (Rudolph et al., 1999; McKernan et al., 2000; Tan et al., 2010; Ralvenius et al., 2015). There is, however, no general consensus about the $GABA_AR$ subtype underlying the anxiolytic effects of BDZs. Studies performed in genetically modified mice have provided strong evidence supporting a critical role of α2 but not of α3$GABA_ARs$. These experiments employed knock-in mice carrying a histidine to arginine point mutation in their α2 or α3$GABA_ARs$ that rendered these receptors BDZ-insensitive. Analysis of these mice in different anxiety models demonstrated that diazepam-induced anxiolysis was absent in α2$GABA_AR$ point-mutated mice but fully retained in α3$GABA_AR$ point-mutated mice (Löw et al., 2000; Smith et al., 2012). In addition, Yee et al. (2005) found that the anxiolytic-like activity of diazepam was preserved in α3$GABA_AR$ knock-out mice. Furthermore, a recent study using triple point-mutated mice, in which only a single $GABA_AR$ subtype was left BDZ sensitive, revealed that selective targeting of α2 and potentially of α5$GABA_ARs$ is sufficient to produce anxiolytic actions of diazepam, while selective modulation of α3$GABA_ARs$ did not produce any anxiolytic effects (Ralvenius et al., 2015; Behlke et al., 2016). By contrast, other studies that employed $GABA_AR$ subtype-selective compounds postulated that exclusive targeting of α3$GABA_ARs$ is sufficient to produce profound anxiolytic effects in rodents and monkeys (Atack et al., 2005; Dias et al., 2005; Morris et al., 2006; Vinkers et al., 2010; Fischer et al., 2011). As most α1$GABA_AR$ sparing BDZ site agonists discriminate little between α2 and α3$GABA_ARs$, these claims rely almost exclusively on a single compound (TP003) that has been reported to be a full and highly specific agonist at α3$GABA_ARs$ (Dias et al., 2005).

In the present study, we have revisited the *in vitro* $GABA_AR$ subtype selectivity of TP003 and tested its *in vivo* pharmacological profile in triple $GABA_AR$ point-mutated mice. We found that TP003 potentiates all four BDZ sensitive $GABA_ARs$ *in vitro* with comparable efficacies. Our *in vivo* experiments revealed that TP003 elicits pronounced anxiolytic actions in the elevated plus maze test via α2 but not via α3$GABA_ARs$. Consistent with its rather promiscuous *in vitro* effects, TP003 also evoked α1$GABA_AR$-mediated sedation and α3$GABA_AR$-mediated muscle relaxation.

2. Materials and methods

2.1. Mice

Experiments were performed in wild-type mice, and in homozygous triple and quadruple $GABA_AR$ point-mutated mice (Ralvenius et al., 2015). All animals were of the 129X1/SvJ background. Triple and quadruple point-mutated mice were generated by cross-breeding of single point-mutated mice that have been described previously: α1(H101R), Rudolph et al. (1999); α2(H101R) and α3(H126R) Löw et al. (2000); α5(H105R), Crestani et al. (2002). We refer to these mice as HRRR, RHRR, and RRHR, for mice in which only α1, α2, or 3 subunits remained BDZ sensitive. RRRR defines mice in which all four BDZ sensitive subunits have been rendered insensitive. After the end of each experiment, mice were euthanized and a tail biopsy was taken to verify the genotype of the mice by PCR.

2.2. Drugs

TP003 (2′,4-difluoro-5'-[8-fluoro-7-(1-hydroxy-1-methylethyl)imidazo[1,2-a]-pyridin-3-yl]-[1,1′-biphenyl]-2-carbonitrile) was obtained from Tocris Bioscience (batch no 2, purity 98%, ^{1}H NMR and mass spectrum consistent with published structure according to Tocris data sheet). For electrophysiological experiments, TP003 was dissolved in DMSO and diluted with extracellular solution. Final DMSO concentrations were < 0.1%. For behavioral experiments, TP003 was suspended in 0.9% saline and 1% Tween80 and applied orally (p.o.).

2.3. Electrophysiology

The effects of TP003 on membrane currents through recombinant $GABA_ARs$ were studied in HEK293 cells (ATCC) transiently transfected with rat $GABA_AR$ subunits using lipofectamine LTX (Paul et al., 2014). To ensure expression of the γ2 subunit in all recorded cells, we transfected cells with a plasmid containing an eGFP preceded by an IRES in addition to the γ2 subunit, and selected only eGFP-positive cells for recordings. The transfection mixture contained (in μg): 1 αx, 1 βy, 3 γ2/eGFP. Whole-cell patch-clamp recordings were made 18–36 h after transfection at room temperature (20–24 °C) and at a holding potential of −60 mV. Recording electrodes (3.5 MOhm) were filled with a solution containing (in mM): 120 CsCl, 10 EGTA, 10 HEPES (pH 7.40), 4 $MgCl_2$, 0.5 GTP and 2 ATP. The external solution contained (in mM): 150 NaCl, 10 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.40), and 10 glucose. GABA was applied to the recorded cell using a manually controlled pulse (6–10 s) of a low sub-saturating GABA concentration (EC_{10}). EC_{10} values of GABA were determined for all subunit combinations analyzed. They were 1 μM for α1β2γ2, 5 μM for α2β3γ2, 8 μM for α3β3γ2 and 1 μM for α5β2γ2 $GABA_ARs$. EC_{50} values and Hill coefficients (n_H) were obtained from fits of normalized concentration-response curves to the equation $I_{GABA} = I_{max} \cdot [GABA]^{nH}/([GABA]^{nH} + [EC_{50}]^{nH})$. I_{max} was determined as the average maximal current elicited by 1 mM GABA. TP003 was dissolved in DMSO (final concentration < 0.1%.) and subsequently diluted with recording solution and co-applied with GABA without preincubation.

2.4. Behavioral experiments

All behavioral experiments were performed in 7–10 week old mice of either sex. Care was taken to ensure equal numbers of female and male mice in all groups. Experimenters were blinded either to the genotype or the treatment with vehicle and drug. Permission for animal experiments was obtained from the Veterinäramt des Kantons Zürich (126/2012, 257/2014 and 231/2017) prior to the start of the experiments. During all experiments we closely adhered to the ARRIVE guidelines and the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines, EU Directive 2010/63/EU for animal experiments.

Locomotor activity was assessed in an arena of 10 cm radius equipped with four pairs of light beams and photosensors. Mice were placed into the arena and locomotor activity was recorded for 120 min and analyzed between 60 and 120 min after TP003 administration. Muscle relaxation was measured using a metal horizontal wire placed 20 cm above the ground. Mice were assisted to place their forepaws on the wire and successes and failures to grab the wire with at least one hindpaw were recorded between 60 and 120 min after TP003 administration.

The elevated plus maze (EPM) was placed 50 cm above the floor and consisted of two open arms (35 cm long x 6 cm wide), two closed arms (35 cm long x 6 cm wide x 20 cm high) and one center area (5 cm × 5 cm). The open and closed arms crossed at a right angle. One hour after vehicle or drug administration, each mouse was placed in the center area of the maze facing the closed arm and allowed to explore the maze for 5 min. Mice were videotaped and videos were analyzed off-line. Time spent in open and closed arms (s), the percent open arm entries (%) and the total number of entries were measured.

2.5. Statistics

Data were analyzed using unpaired t-tests, when two independent groups of animals were compared (Fig. 2) and paired t tests when the