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6,3′-Dinitroflavone is a low efficacy modulator of $GABA_A$ receptors

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ABSTRACT

6,3′-Dinitroflavone (6,3′-DNF) is a synthetic flavone derivative that exerts anxiolytic effects in the elevated plus maze. Based on the finding that this effect is blocked by Ro15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate) which is a specific antagonist at the benzodiazepine binding site of $GABA_A$ receptors we investigated the interaction of 6,3′-DNF with several recombinant $GABA_A$ receptor subtypes. Inhibition of [^{3}H]flunitrazepam binding to recombinant $GABA_A$ receptors in transiently transfected HEK293 cells indicated that 6,3′-DNF exhibited the highest affinity for $GABA_A$ receptors composed of $\alpha1\beta2\gamma2$ subunits and a 2–20 fold lower affinity for homologous receptors containing $\alpha2$, $\alpha3$, or $\alpha5$ subunits. Two-electrode voltage-clamp experiments in *Xenopus* oocytes indicated that 6,3′-DNF does not induce chloride flux in the absence of GABA, but exerts low efficacy inverse agonistic modulatory effects on GABA-elicited currents in the $GABA_A$ receptor subtypes $\alpha1\beta2\gamma2$ and $\alpha5\beta2\gamma2$. In the subtypes $\alpha2\beta2\gamma2$, $\alpha3\beta2\gamma2$, $\alpha4\beta2\gamma2$, $\alpha6\beta2\gamma2$ or $\alpha4\beta2\delta$ and $\alpha4\beta3\delta$, 6,3′-DNF exerts either none or very low efficacy positive modulatory effects. In contrast, 100 nM Ro15-1788 exhibited weak to moderate partial agonistic effects on each receptor investigated. These data indicate that Ro15-1788 only can antagonize the weak inverse agonist effects of 6,3′-DNF on $\alpha1\beta2\gamma2$ and $\alpha5\beta2\gamma2$ receptors, but will enhance the weak agonistic effects on the other receptor subtypes investigated. The possible mechanism of the Ro15-1788 sensitive anxiolytic effect of 6,3′-DNF is discussed.

1. Introduction

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the central nervous system and are the site of action of numerous clinically important drugs (Macdonald and Olsen, 1994; Sieghart, 1995). They control the excitability of the brain, anxiety, vigilance, muscle tone, memory and learning (Rudolph and Mohler, 2006).

$GABA_A$ receptors are composed of five subunits forming a chloride ion channel that can be opened by GABA (Ernst et al., 2003; Nayeem et al., 1994; Tretter et al., 1997). A total of 19 receptor subunits belonging to several subunit classes (6α, 3β, 3γ, one δ, one ε, one π, one θ and 3 ρ) have been identified (Barnard et al., 1998; Simon et al., 2004), each exhibiting a distinct regional distribution in the brain (Sieghart and Sperk, 2002). Depending on their subunit composition these receptors exhibit different pharmacological and electrophysiological properties (Sieghart, 1995; Sigel et al., 1990). The majority of $GABA_A$ receptors is composed of α, β and γ subunits. The classical benzodiazepines predominantly act via receptors composed of $\alpha1\beta\gamma2$, $\alpha2\beta\gamma2$, $\alpha3\beta\gamma2$ and $\alpha5\beta\gamma2$. Recent pharmacological studies using mutated mice attributed distinct behavioural effects of GABAergic compounds to $GABA_A$ receptors containing defined α subunits. Specifically, sedative effects of diazepam were attributed to $GABA_A$ receptors containing the $\alpha1$ subunit (McKernan et al., 2000; Rudolph et al., 1999), anxiolytic actions to $\alpha2/\alpha3$ containing receptors (Atack et al., 2006; Low et al., 2000; Morris et al., 2006) and anterograde amnesic effects to $\alpha1/\alpha5$ subunit containing ones (Chambers et al., 2004; Cheng et al., 2006; Savic et al., 2005). Furthermore, anticonvulsant activity was partially attributed to $\alpha1$ (Rudolph et al., 1999) and muscle relaxant effects largely to $\alpha2$-containing receptors (Crestani et al., 2001). Drugs selectively interacting with such subtypes will thus exhibit very specific actions.

The synthetic flavonoid 6,3′-DNF (see inset in Fig. 1 for structure) is a selective ligand at the benzodiazepine binding site of $GABA_A$ receptors that exhibits potent anxiolytic effects. Mild sedation was seen only at very high doses of 6,3′-DNF. No anticonvulsant or myorelaxant effects in mice or amnesic effects in rats were seen (Marder et al., 1995; Wolfman et al., 1996). In the elevated plus maze test, 6,3′-DNF i.p. injected to mice, caused a selective increase in the percentage of open arm entries and time spent in open arms (Marder et al., 1995). The anxiolytic effects triggered by 6,3′-DNF were blocked by Ro15-1788, which is a selective antagonist at the benzodiazepine binding site of $GABA_A$ receptors. Moreover, 6,3′-DNF also abolished the myorelaxant effect of diazepam which is a strong agonist at the benzodiazepine

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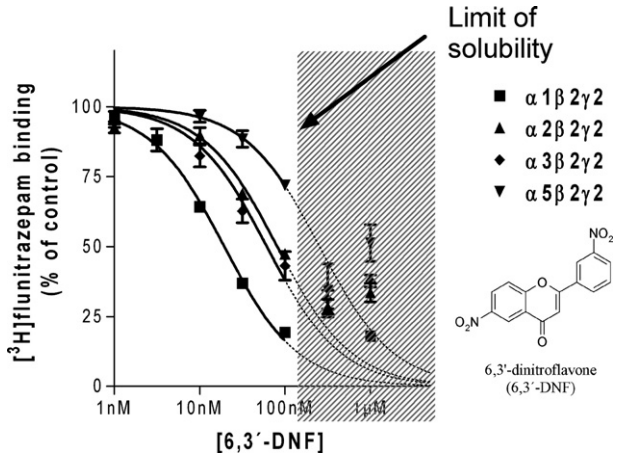

Fig. 1. Effect of 6,3′-DNF on the binding of 2 nM [^{3}H]flunitrazepam to membranes from transiently transfected HEK293 cells expressing $GABA_A$ receptors with subunit composition as indicated. Hatched area represents concentrations of 6,3′-DNF beyond maximal solubility under assay conditions. Values are mean ± S.E.M. of three determinations, triplicates each. The inset shows the structure of 6,3′-DNF.

binding site of $GABA_A$ receptors. These in vivo findings suggested that 6,3′-DNF is a partial agonist at the benzodiazepine binding site of $GABA_A$ receptors (Wolfman et al., 1996). The receptor subtype selectivity of 6,3′-DNF so far has not been investigated.

In this study we determined the affinity of 6,3′-DNF for the benzodiazepine binding site of $GABA_A$ receptor subtypes composed of α1β2γ2, α2β2γ2, α3β2γ2 and α5β2γ2 subunits by performing [^{3}H] flunitrazepam binding experiments in transiently transfected human embryonic kidney (HEK293) cells. Furthermore, we investigated the interactions of 6,3′-DNF with $GABA_A$ receptors by performing two-electrode voltage-clamp experiments in *Xenopus* oocytes that expressed recombinant $GABA_A$ receptors. In these functional tests we determined modulation of GABA-elicited currents in $GABA_A$ receptors containing the subunits α1 to α6 combined with β2 and γ2 subunits as well as the extrasynaptic α4β2δ and α4β3δ receptors. Finally we tried to attribute the in vivo effects of 6,3′-DNF to subsets of $GABA_A$ receptors.

2. Materials and methods

6,3′-DNF was synthesized by us according to the method described earlier (Marder et al., 1997).

2.1. Receptor binding studies

HEK293 cells (CRL 1573; American Type Culture Collection, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal calf serum (Bio Whittaker, Rockland, ME, USA), 2 mM L-glutamine (Gibco), 100 U/ml penicillin G and 100 μg/ml streptomycin and 1% MEM (minimal essential medium = nonessential amino acids; Gibco) in 75-cm^2 Petri dishes by the use of standard cell culture techniques. HEK293 cells were transfected with cDNAs encoding for $GABA_A$ receptor subunits α1 to α6 in combination with β2 and γ2 (all subunits from rat). Each Petri dish was transfected with a total of 21 μg of subunit cDNA at a ratio of α:β:γ = 1:1:1 using the calcium phosphate precipitation method (Chen and Okayama, 1988). 48 h after transfection cells were harvested by scraping into phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 4.3 mM Na_2HPO_4, pH 7.3). Cells were sedimented by centrifugation, supernatant was decanted and cell pellets were stored at −70 °C until usage. For inhibition studies frozen membrane-pellets were thawed and homogenized in TC50 (50 mM Tris/citrate buffer, pH 7.1), by using an Ultra-Turrax, followed by two centrifugation resuspension cycles (200,000 g for 20 min at 4 °C). 0.03 to 0.3 mg membrane protein as measured with the BCA protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as standard was incubated in a total of 1 ml TC50 containing 150 mM NaCl and 2 nM [^{3}H]flunitrazepam (74.1 Ci/mmol), (PerkinElmer Life Sciences, Waltham, MA) in the absence or presence of various concentrations of 6,3′-DNF. After incubation for 90 min at 4 °C the suspensions were rapidly filtered through Whatman GF/B filters (Whatman, UK) using a multi-channel receptor binding harvester (Brandel Inc., Gaithersburg, MD), washed three times with 3 ml of TC50 and subjected to liquid scintillation counting (Ultima Gold liquid scintillation cocktail, 2100 TR Tri-Carb Scintillation Analyser, both PerkinElmer). Data were analysed using GraphPad Prism (Graph Pad Software Inc., San Diego, CA). Ki values were calculated using Cheng Prusoff equation (Cheng and Prusoff, 1973).

2.2. Two-electrode voltage clamp

In vitro transcription of mRNA was based on the expression vectors that were also used to express the receptor protein for binding studies. After linearizing the cDNA vectors with appropriate restriction endonucleases, capped transcripts were produced using the mMESSAGE mMACHINE® T7 transcription kit (Ambion, TX). The capped transcripts were polyadenylated using yeast poly(A) polymerase (USB, OH) and were diluted and stored in diethylpyrocarbonate-treated water at −70 °C.

The methods used for isolating, culturing, injecting and defolliculating of oocytes were identical with those described by Sigel (1987) and Sigel et al. (1990) and were described elsewhere (Li et al., 2003). In short, mature female *Xenopus laevis* (Nasco, WI) were anaesthetized in a bath of ice-cold 0.17% Tricain (ethyl-*m*-aminobenzoat, Sigma, MO) before decapitation and removal of the frog's ovary. Stage 5 to 6 oocytes with the follicle cell layer around them were singled out of the ovary using a platinum wire loop. Oocytes were stored and incubated at 18 °C in modified Barths' Medium (88 mM NaCl, 10 mM HEPES–NaOH (pH 7.4), 2.4 mM $NaHCO_3$, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.34 mM $Ca(NO_3)_2$) that was supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Oocytes with follicle cell layers still around them were injected with an aqueous solution of cRNA. A total of 2.5 ng of mRNA per oocyte was injected. Subunit ratio was 1:1:5 for αβ2γ2, αβ2δ, and αβ3δ, respectively. After injection of cRNA, oocytes were incubated for at least 36 h before the enveloping follicle cell layers were removed. Collagenase-treatment (type IA, Sigma, MO) and mechanically defolliculating of the oocytes were described elsewhere (Li et al., 2003).

For electrophysiological recordings, oocytes were placed on a nylon-grid in a bath of *Xenopus* Ringer solution (XR solution, containing 90 mM NaCl, 5 mM HEPES–NaOH (pH 7.4), 1 mM $MgCl_2$, 1 mM KCl and 1 mM $CaCl_2$). The oocytes were constantly washed by a flow of 6 ml/min XR solution which could be switched to XR solution containing GABA and/or drugs. Drugs were diluted into XR solution from dimethyl sulfoxide (DMSO)-solutions resulting in a final concentration of 0.1% DMSO perfusing the oocytes. Drugs were pre-applied for 30 s before the addition of GABA, which was then coapplied with the drugs without washing in between, until a peak response was observed. This procedure ensured that a possible unspecific adsorption of the drug in the tubing occurred during the 30 s pre-application period in which 3 ml of the final drug solution passed the application system. Between two applications, oocytes were washed in XR solution for up to 15 min to ensure full recovery from desensitization. For current measurements the oocytes were impaled with two microelectrodes (2–3 MΩ) which were filled with 2 M KCl. Maximum currents measured in mRNA-injected oocytes were in the microampere range for all subtypes of $GABA_A$ receptors. To test for modulation of GABA-induced currents by the compounds 6,3′-DNF or Ro15-1788 a concentration of GABA that was titrated to trigger 3% of

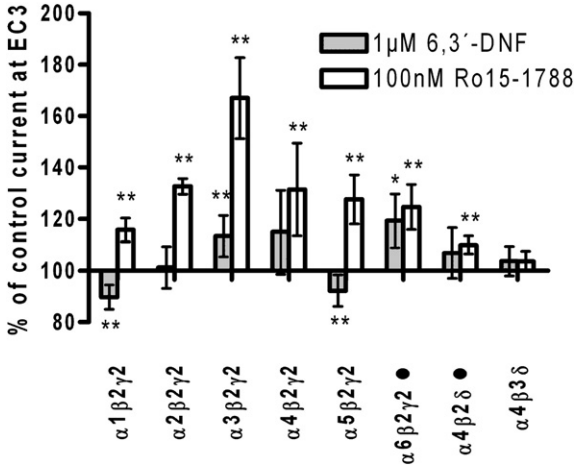

Fig. 2. Modulation of GABA currents by 1 μM 6,3′-DNF or 100 nM Ro15-1788 in receptors composed of different subunits. cRNA injected *Xenopus* oocytes were held under two-electrode voltage clamp at −60 mV. 1 μM of 6,3′-DNF or 100 nM Ro15-1788 was superfused together with a GABA concentration that elicited 3% (10% for α6β2γ2 and α4β2δ) of the maximal GABA-induced current amplitude of the respective cell. Data were individually normalized to 100% of the current in the absence of 6,3′-DNF or Ro15-1788. Values are presented as means ± S.D. of at least four oocytes from two batches. *$P<0.05$, **$P<0.01$ versus control current by *t* test. ●: EC_{10} was used instead of EC_3.

the respective maximum GABA-elicited current of the individual oocyte (EC_3). This GABA concentration was applied to the cell with various concentrations of the compounds. However, for the subtypes α4β2δ and α6β2γ2 EC_{10} was chosen to obtain reasonable signal to noise ratios. All recordings were performed at room temperature at a holding potential of −60 mV using a Warner OC-725C two-electrode voltage clamp (Warner Instruments, Hamden, CT) or a Dagan CA-1B Oocyte Clamp (Dagan Corporation, Minneapolis, MN). Data were digitised, recorded and measured using a Digidata 1322A data acquisition system (Axon Instruments, Union City, CA).

3. Results

3.1. Subtype-selective affinity of 6,3′-DNF for the benzodiazepine binding site of recombinant $GABA_A$ receptors

The affinities of 6,3′-DNF for the benzodiazepine binding sites of α1β2γ2, α2β2γ2, α3β2γ2 and α5β2γ2 $GABA_A$ receptors were determined using [^{3}H]flunitrazepam binding studies. Membranes from HEK293 cells transiently transfected with the respective $GABA_A$ receptor subunits were incubated with 2 nM [^{3}H]flunitrazepam. 6,3′-DNF dose-dependently displaced the specifically bound radiolabeled ligand up to the limit of solubility for 6,3′-DNF (approximately 100 nM at 4 °C) (Fig. 1). Ki values for 6,3′-DNF were estimated by extrapolation of binding data obtained assuming the Hill slope to be unity. Binding of 6,3′-DNF to the benzodiazepine binding site of $GABA_A$ receptors shows some selectivity for the subtype that contains the α1 subunit and low affinity for the subtype containing the α5 subunit. Ki values (95% confidence intervals) were 9.7 nM (8.7–10.8), 50.6 nM (44.0–58.2), 26.2 nM (20.7–33.3) and about 200.0 nM for α1β2γ2, α2β2γ2, α3β2γ2 and α5β2γ2, respectively.

3.2. Subtype-selective modulation of GABA-elicited currents by 6,3′-DNF in recombinant $GABA_A$ receptors

Effects of 6,3′-DNF on $GABA_A$ receptors were characterized using *Xenopus* oocytes that functionally expressed the $GABA_A$ receptor subunits α1 to α6 in combination with β2 and γ2 subunits as well as the subunit combinations α4β2δ and α4β3δ. The two-electrode voltage-clamp method was used to test whether 6,3′-DNF triggered chloride currents, modulated GABA-induced currents or antagonized the effects of diazepam in oocytes that express $GABA_A$ receptors.

Under the conditions of the experiments (20 °C) the apparent limit of solubility for 6,3′-DNF was approximately 1 μM, a concentration that did not activate chloride currents in the absence of GABA in any of the tested subtypes of $GABA_A$ receptors. During tests for modulation of GABA-elicited currents, detailed concentration response studies could not be performed because modulation of GABA-elicited currents by 6,3′-DNF was either absent or very weak for all $GABA_A$ receptor subtypes tested. However, maximum modulation of GABA-induced currents was seen at 1 μM of 6,3′-DNF in all subtypes. Maximum modulation of EC_3 (EC_{10} for α4β2δ and α6β2γ2) for the subtypes tested is presented in Fig. 2. In other experiments complete GABA dose–response curves were measured in the absence or presence of 6,3′-DNF in α1β2γ2, α2β2γ2, α3β2γ2 and α5β2γ2 receptors. 1 μM 6,3′-DNF did not significantly change the GABA EC_{50} values in these receptor subtypes (experiments not shown). This indicates that the very weak partial agonistic effect of this compound resulted in only a small shift in the GABA dose–response curves that could not be resolved in the respective semi-logarithmic plots. These experiments also indicated that the maximal GABA-elicited currents were not significantly altered in these receptors in the presence of 1 μM 6,3′-DNF (experiments not shown), as expected from the action of 6,3′-DNF via the benzodiazepine binding site of these receptors.

In parallel experiments the effects of 100 nM concentrations of Ro15-1788 on GABA evoked currents were investigated in these receptors under the same conditions. Since Ro15-1788 exhibited more pronounced positive stimulatory effects than 6,3′-DNF in most subtypes of $GABA_A$ receptors tested, tests for antagonism of 6,3′-DNF effects by Ro15-1788 were not performed in each case. In α1β2γ2 and α5β2γ2 receptors, where 6,3′-DNF and Ro15-1788 exhibited effects in different directions, however, Ro15-1788 due to its high potency not only inhibited the effects of 6,3′-DNF but also at higher concentrations elicited a positive allosteric effect.

The lack of modulation by 6,3′-DNF was not due to limited solubility or to its adsorption to the tubing since the compound antagonized the modulation of GABA-elicited currents by diazepam in a dose dependent manner (Fig. 3).

4. Discussion

In this study we extended previous evidence indicating that 6,3′-DNF exhibits a high affinity for the benzodiazepine binding site of $GABA_A$

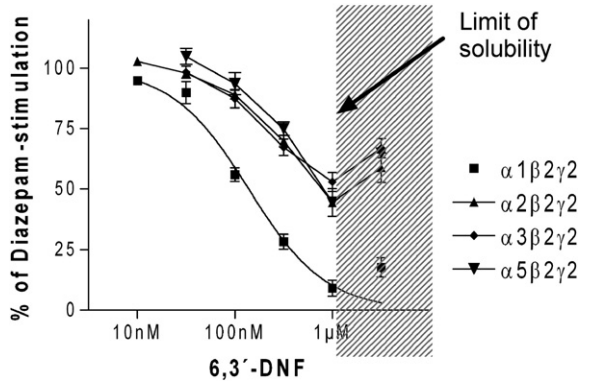

Fig. 3. Inhibition of diazepam-stimulated GABA-elicited currents by 6,3′-DNF in $GABA_A$ receptors composed of different subunits as indicated. cRNA injected *Xenopus* oocytes were held under two-electrode voltage clamp at −60 mV. Increasing concentrations of 6,3′-DNF were superfused together with 30 nM diazepam and a GABA concentration that elicited 3% of the maximal current amplitude. Hatched area represents concentrations of 6,3′-DNF beyond maximal solubility under assay conditions. Data for individual cells were normalized by using currents measured in the presence or absence of 30 nM diazepam as 100% or 0%, respectively. Values are presented as means ± S.E.M. of at least four oocytes from two batches.

receptors in brain membranes by determining the receptor subtype selectivity of this compound. We were able to demonstrate that 6,3′-DNF exhibited a high affinity (Ki of 10 nM) for recombinant receptors composed of $\alpha1\beta2\gamma2$ subunits and a 2–20 fold lower affinity for homologous receptors containing $\alpha2$, $\alpha3$, or $\alpha5$ subunits. 6,3′-DNF did not elicit currents in the absence of GABA in *Xenopus* oocytes expressing any of the recombinant $GABA_A$ receptors tested. However, 6,3′-DNF was able to slightly modulate GABA-elicited currents similar to other benzodiazepine site ligands. In $GABA_A$ receptors containing $\alpha1$ and $\alpha5$ subunits, 6,3′-DNF showed a small but significant inverse agonistic effect. In $GABA_A$ receptors containing the $\alpha2$ subunit no modulatory effect was seen, whereas in $\alpha3$ subunit containing receptors 6,3′-DNF exhibited a very weak partial agonistic effect. 6,3′-DNF was able to dose-dependently inhibit diazepam-stimulated currents in receptors composed of $\alpha1\beta2\gamma2$, $\alpha2\beta2\gamma2$, $\alpha3\beta2\gamma2$ and $\alpha5\beta2\gamma2$. For this inhibition of diazepam effects, potency and rank order of potency was consistent with aforementioned affinity data, especially when considering the different assay conditions used for these experiments. These data indicate that 6,3′-DNF is a benzodiazepine binding site ligand with a certain selectivity for $GABA_A$ receptors containing $\alpha1$ subunits, but with a very low efficacy in all receptor subtypes.

Interestingly, this compound also interacts with $\alpha4\beta2\gamma2$ or $\alpha6\beta2\gamma2$ receptors as indicated by its weak agonistic effect on $\alpha4\beta2\gamma2$ (not significant) or $\alpha6\beta2\gamma2$ $GABA_A$ receptors (significant). In receptors with the subunit combinations $\alpha4\beta2\delta$ and $\alpha4\beta3\delta$ that are predominantly located extrasynaptically, no modulation of GABA-elicited currents by 6,3′-DNF was seen (Fig. 2). This resembles the effects of the imidazobenzodiazepine Ro15-1788. To compare the properties of 6,3′-DNF with that of imidazobenzodiazepines, the effects of the prototypic benzodiazepine site "antagonist" Ro15-1788, that is still widely considered to be devoid of modulatory effects on $GABA_A$ receptors, were investigated on $GABA_A$ receptor subtypes containing different α subunits. Interestingly, this compound was a weak partial agonist on all receptor subtypes investigated. This is in line with previous findings in *Xenopus* oocytes (Rusch and Forman, 2005), cultured neurons (Weiss et al., 2002) and, to date inconsistent, in vivo findings (Savic et al., 2004; Uhlirova et al., 2004). Under our conditions, Ro15-1788 exhibited its strongest effects on $\alpha3\beta2\gamma2$ receptors in which the GABA EC_3 control current was increased to 167±16%. When the same test was performed at GABA EC_{50}, Ro15-1788 increased the control currents to approximately 125% (data not shown). This is consistent with data of June et al. (2003), who found that a saturating concentration of Ro15-1788 increased the GABA EC_{50} current of $\alpha3\beta3\gamma2$ $GABA_A$ receptors to 118±7%. At GABA concentrations as low as EC_3, modulatory effects of agonists at the benzodiazepine site are especially high; under the same conditions 1 μM diazepam stimulates this subtype to about 600% of control current. Effects of inverse agonists like DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) or FG7142 (*N*′-methyl-β-carboline-3-carboxamide) however, at EC_3 largely resemble the results of more conservative test regimens like EC_{20}. We chose EC_3 because compared to EC_{10} or EC_{20}, application of these low concentrations results in less desensitization and hence shorter recovery times of the preparation during experiments. This keeps experiments short, allows rapid experimental progress and reduces influences by drifting maximum currents which sometimes occur during the experiments that typically last for up to 45 min.

In previous studies, 6,3′-DNF exhibited potent anxiolytic but no sedative and amnesic effects and the anxiolytic effects of this compound were blocked by Ro15-1788 (Wolfman et al., 1996). This in vivo profile of 6,3′-DNF is difficult to reconcile with the in vitro activity of 6,3′-DNF we report here, at least, if assessment is based on results from studies that used genetically altered knock in or knock out mice. These studies suggest that $\alpha2/\alpha3$ containing $GABA_A$ receptors are mediating the anxiolytic effects of diazepam (Atack et al., 2006; Low et al., 2000; Morris et al., 2006). Our data show that 6,3′-DNF is an antagonist or weak partial agonist in all tested subtypes of $GABA_A$ receptors except for $\alpha1\beta2\gamma2$ and $\alpha5\beta2\gamma2$ in which the compound shows weak inverse agonistic effects. In all subtypes that show partial agonistic effects of 6,3′-DNF the efficacy of Ro15-1788 surmounts the efficacy of 6,3′-DNF. Therefore, in vivo effects that are caused via these receptors would have been increased rather than antagonized by Ro15-1788. In contrast, the inverse agonistic effects at receptor subtypes that contain $\alpha1$ or $\alpha5$ subunits are potentially blocked by Ro15-1788. So due to the very specific action of 6,3′-DNF and Ro15-1788 on the various $GABA_A$ receptor subtypes, anxiolytic in vivo effects of 6,3′-DNF that are blocked by Ro15-1788 are likely based on the partial inverse agonist effects of 6,3′-DNF on receptors containing $\alpha1$ and/or $\alpha5$ subunits.

Alternatively, anxiolysis might have been caused by an active metabolite of 6,3′-DNF exhibiting substantial agonistic action on $GABA_A$ receptor subtypes that can be blocked by Ro15-1788. The possibility, that 6,3′-DNF caused anxiolysis by antagonizing the anxiogenic action of an endogenous inverse agonist acting on $\alpha1$, $\alpha2$, $\alpha3$ or $\alpha5$ containing receptors can be excluded because anxiolytic effects of 6,3′-DNF would then have been enhanced rather than inhibited by Ro15-1788. Although it is possible that the behavioural effects of 6,3′-DNF were not mediated by its interaction with $GABA_A$ receptors, but with some other receptors in the central nervous system, this possibility is not very likely because then it has to be explained how this alternative activity of 6,3′-DNF should be blocked by Ro15-1788. We cannot exclude, however, that it is the specific spectrum of actions of 6,3′-DNF on different $GABA_A$ receptor subtypes that elicits the behavioural effects observed, by influencing the subtle excitatory/inhibitory balance in different parts of the central nervous system. In this case, Ro15-1788 might have caused its antagonistic effect on the behavioural action of 6,3′-DNF not by interfering with its action on $\alpha1$ and/or $\alpha5$ receptors but by disturbing this balance via interacting with all receptor subtypes. Further experiments will have to investigate this possibility.

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References

Atack, J.R., Wafford, K.A., Tye, S.J., Cook, S.M., Sohal, B., Pike, A., Sur, C., Melillo, D., Bristow, L., Bromidge, F., Ragan, I., Kerby, J., Street, L., Carling, R., Castro, J.L., Whiting, P., Dawson, G.R., McKernan, R.M., 2006. TPA023 [7-(1,1-dimethylethyl)-6-(2-ethyl-2*H*-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-*b*]pyridazine], an agonist selective for α2- and α3-containing $GABA_A$ receptors, is a nonsedating anxiolytic in rodents and primates. J. Pharmacol. Exp. Ther. 316, 410–422.

Barnard, E.A., Skolnick, P., Olsen, R.W., Mohler, H., Sieghart, W., Biggio, G., Braestrup, C., Bateson, A.N., Langer, S.Z., 1998. International Union of Pharmacology. XV. Subtypes of γ-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. Pharmacol. Rev. 50, 291–313.

Chambers, M.S., Atack, J.R., Carling, R.W., Collinson, N., Cook, S.M., Dawson, G.R., Ferris, P., Hobbs, S.C., O'Connor, D., Marshall, G., Rycroft, W., Macleod, A.M., 2004. An orally bioavailable, functionally selective inverse agonist at the benzodiazepine site of $GABA_A$ α5 receptors with cognition enhancing properties. J. Med. Chem. 47, 5829–5832.

Chen, C.A., Okayama, H., 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. Biotechniques 6, 632–638.

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Cheng, V.Y., Martin, L.J., Elliott, E.M., Kim, J.H., Mount, H.T., Taverna, F.A., Roder, J.C., Macdonald, J.F., Bhambri, A., Collinson, N., Wafford, K.A., Orser, B.A., 2006. α5$GABA_A$ receptors mediate the amnestic but not sedative-hypnotic effects of the general anesthetic etomidate. J. Neurosci. 26, 3713–3720.

Crestani, F., Low, K., Keist, R., Mandelli, M., Mohler, H., Rudolph, U., 2001. Molecular targets for the myorelaxant action of diazepam. Mol. Pharmacol. 59, 442–445.

Ernst, M., Brauchart, D., Boresch, S., Sieghart, W., 2003. Comparative modeling of $GABA_A$ receptors: limits, insights, future developments. Neuroscience 119, 933–943.

June, H.L., Foster, K.L., McKay, P.F., Seyoum, R., Woods, J.E., Harvey, S.C., Eiler, W.J., Grey, C., Carroll, M.R., McCane, S., Jones, C.M., Yin, W., Mason, D., Cummings, R., Garcia, M., Ma, C., Sarma, P.V., Cook, J.M., Skolnick, P., 2003. The reinforcing properties of alcohol are mediated by $GABA_{A1}$ receptors in the ventral pallidum. Neuropsychopharmacology 28, 2124–2137.

Li, X., Cao, H., Zhang, C., Furtmueller, R., Fuchs, K., Huck, S., Sieghart, W., Deschamps, J., Cook, J.M., 2003. Synthesis, in vitro affinity, and efficacy of a bis 8-ethynyl-4*H*-imidazo[1,5*a*]-[1,4]benzodiazepine analogue, the first bivalent α5 subtype selective BzR/$GABA_A$ antagonist. J. Med. Chem. 46, 5567–5570.

Low, K., Crestani, F., Keist, R., Benke, D., Brunig, I., Benson, J.A., Fritschy, J.M., Rulicke, T., Bluethmann, H., Mohler, H., Rudolph, U., 2000. Molecular and neuronal substrate for the selective attenuation of anxiety. Science 290, 131–134.

Macdonald, R.L., Olsen, R.W., 1994. $GABA_A$ receptor channels. Annu. Rev., Neurosci. 17, 569–602.

Marder, M., Viola, H., Wasowski, C., Wolfman, C., Waterman, P.G., Medina, J.H., Paladini, A.C., 1995. 6,3′-Dinitroflavone, a novel high affinity ligand for the benzodiazepine receptor with potent anxiolytic properties. Bioorganic Med. Chem. Letters 5, 2717–2720.

Marder, M., Zinczuk , J., Colombo , M.I., Wasowski , C., Viola , H., Wolfman , C., Medina , J.H., Rúveda , E.A., Paladini , A.C., 1997. Synthesis of halogenated/nitrated flavone derivatives and evaluation of their affinity for the central benzodiazepine receptor. Bioorganic Med. Chem. Letters 7, 2003–2008.

McKernan, R.M., Rosahl, T.W., Reynolds, D.S., Sur, C., Wafford, K.A., Atack, J.R., Farrar, S., Myers, J., Cook, G., Ferris, P., Garrett, L., Bristow, L., Marshall, G., Macaulay, A., Brown, N., Howell, O., Moore, K.W., Carling, R.W., Street, L.J., Castro, J.L., Ragan, C.I., Dawson, G.R., Whiting, P.J., 2000. Sedative but not anxiolytic properties of benzodiazepines are mediated by the $GABA_A$ receptor alpha1 subtype. Nat. Neurosci. 3, 587–592.

Morris, H.V., Dawson, G.R., Reynolds, D.S., Atack, J.R., Stephens, D.N., 2006. Both α2 and α3 $GABA_A$ receptor subtypes mediate the anxiolytic properties of benzodiazepine site ligands in the conditioned emotional response paradigm. Eur. J. Neurosci. 23, 2495–2504.

Nayeem, N., Green, T.P., Martin, I.L., Barnard, E.A., 1994. Quaternary structure of the native $GABA_A$ receptor determined by electron microscopic image analysis. J. Neurochem. 62, 815–818.

Rudolph, U., Mohler, H., 2006. GABA-based therapeutic approaches: $GABA_A$ receptor subtype functions. Curr. Opin. Pharmacol. 6, 18–23.

Rudolph, U., Crestani, F., Benke, D., Brunig, I., Benson, J.A., Fritschy, J.M., Martin, J.R., Bluethmann, H., Mohler, H., 1999. Benzodiazepine actions mediated by specific γ-aminobutyric acidA receptor subtypes. Nature 401, 796–800.

Rusch, D., Forman, S.A., 2005. Classic benzodiazepines modulate the open-close equilibrium in α1β2γ2L γ-aminobutyric acid type A receptors. Anesthesiology 102, 783–792.

Savic, M.M., Obradovic, D.I., Ugresic, N.D., Cook, J.M., Yin, W., Bokonjic, D.R., 2004. Bidirectional effects of benzodiazepine binding site ligands in the elevated plus-maze: differential antagonism by flumazenil and β-CCt. Pharmacol. Biochem. Behav. 79, 279–290.

Savic, M.M., Obradovic, D.I., Ugresic, N.D., Bokonjic, D.R., 2005. Memory effects of benzodiazepines: memory stages and types versus binding-site subtypes. Neural. Plast. 12, 289–298.

Sieghart, W., 1995. Structure and pharmacology of γ-aminobutyric acidA receptor subtypes. Pharmacol. Rev. 47, 181–234.

Sieghart, W., Sperk, G., 2002. Subunit composition, distribution and function of $GABA_A$ receptor subtypes. Curr. Top. Med. Chem. 2, 795–816.

Sigel, E., 1987. Properties of single sodium channels translated by *Xenopus* oocytes after injection with messenger ribonucleic acid. J. Physiol. 386, 73–90.

Sigel, E., Baur, R., Trube, G., Mohler, H., Malherbe, P., 1990. The effect of subunit composition of rat brain $GABA_A$ receptors on channel function. Neuron 5, 703–711.

Simon, J., Wakimoto, H., Fujita, N., Lalande, M., Barnard, E.A., 2004. Analysis of the set of $GABA_A$ receptor genes in the human genome. J. Biol. Chem 279, 41422–41435.

Tretter, V., Ehya, N., Fuchs, K., Sieghart, W., 1997. Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. J. Neurosci 17, 2728–2737.

Uhlirova, L., Sustkova-Fiserova, M., Krsiak, M., 2004. Behavioral effects of flumazenil in the social conflict test in mice. Psychopharmacology (Berl) 171, 259–269.

Weiss, M., Tikhonov, D., Buldakova, S., 2002. Effect of flumazenil on $GABA_A$ receptors in isolated rat hippocampal neurons. Neurochem. Res 27, 1605–1612.

Wolfman, C., Viola, H., Marder, M., Wasowski, C., Ardenghi, P., Izquierdo, I., Paladini, A.C., Medina, J.H., 1996. Anxioselective properties of 6,3′-dinitroflavone, a high-affinity benzodiazepine receptor ligand. Eur. J. Pharmacol 318, 23–30.