

# Role of the histidine residue at position 105 in the human $\alpha 5$ containing GABA<sub>A</sub> receptor on the affinity and efficacy of benzodiazepine site ligands

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**1** A histidine residue in the N-terminal extracellular region of  $\alpha 1,2,3,5$  subunits of the human GABA<sub>A</sub> receptor, which is replaced by an arginine in  $\alpha 4$  and  $\alpha 6$  subunits, is a major determinant for high affinity binding of classical benzodiazepine (BZ)-site ligands. The effect of mutating this histidine at position 105 in the  $\alpha 5$  subunit to an arginine ( $\alpha 5$ H105R) on BZ-site pharmacology has been investigated using radioligand binding on HEK293 and L(tk-) cells and two electrode voltage clamp recording on *Xenopus* oocytes in which GABA<sub>A</sub> receptors of subtypes  $\alpha 5$ ,  $\alpha 5$ H105R,  $\alpha 4$  and  $\alpha 6$  were co-expressed with  $\beta 3\gamma 2s$ .

**2** The classical BZs, diazepam and flunitrazepam (full agonists on the  $\alpha 5$  receptor) showed negligible affinity and therefore negligible efficacy on  $\alpha 5$ H105R receptors. The  $\beta$ -carbolines DMCM and  $\beta$ CCE (inverse agonists on the  $\alpha 5$  receptor) retained some affinity but did not exhibit inverse agonist efficacy at  $\alpha 5$ H105R receptors. Therefore, the  $\alpha 5$ H105R mutation confers an  $\alpha 4/\alpha 6$ -like pharmacology to the classical BZs and  $\beta$ -carbolines.

**3** Ro15-4513, flumazenil, bretazenil and FG8094, which share a common imidazobenzodiazepine core structure, retained high affinity and were higher efficacy agonists on  $\alpha 5$ H105R receptors than would be predicted from an  $\alpha 4/\alpha 6$  pharmacological profile. This effect was antagonized by DMCM, which competes for the BZ-site and therefore is likely to be mediated *via* the BZ-site.

**4** These data indicate that the conserved histidine residue in the  $\alpha$  subunit is not only a key determinant in the affinity of BZ-site ligands on  $\alpha 5$  containing GABA<sub>A</sub> receptors, but also influences ligand efficacy.

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**Abbreviations:**  $\beta$ CCE, ethyl- $\beta$ -carboline-3-carboxylate; BZ, benzodiazepine; DMCM, dimethoxy-4-ethyl- $\beta$ -carboline-3-carboxylate; GABA,  $\gamma$ -aminobutyric acid

## Introduction

$\gamma$ -aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system. The GABA<sub>A</sub> receptor is a member of the ligand-gated ion channel superfamily (Schofield *et al.*, 1987). It is formed by the pentameric arrangement of heterogeneous membrane spanning protein subunits which form a central chloride ion channel (for reviews see MacDonald & Olsen, 1994; Rabow *et al.*, 1995; Sieghart, 1995; Whiting *et al.*, 1995). The flow of chloride ions through the GABA<sub>A</sub> receptor channel is influenced by numerous ligands in addition to GABA. For example, benzodiazepines (BZ) are able to act through a discrete binding site on the receptor to allosterically modulate the action of GABA (MacDonald & Barker, 1978; Squires & Braestrup, 1977). To date nineteen GABA<sub>A</sub> subunits have been isolated and cloned:  $\alpha 1$ – $\alpha 6$ ,  $\beta 1$ – $\beta 4$ ,  $\gamma 1$ – $\gamma 3$ ,  $\rho 1$ – $\rho 3$ ,  $\delta$ ,  $\epsilon$ ,  $\pi$  and  $\theta$  (Barnard *et al.*, 1998). Current evidence indicates that the stoichiometry of the principal GABA<sub>A</sub> subtypes include

two  $\alpha$  subunits, two  $\beta$  subunits and one  $\gamma$  subunit (Chang *et al.*, 1996). GABA binding sites are formed at the interface between the  $\alpha$  and  $\beta$  subunits (Levitan *et al.*, 1988) and the BZ-site is formed at the interface between an  $\alpha$  and  $\gamma$  subunit (Stephenson *et al.*, 1990).

A critical role of a histidine residue in BZ pharmacology was first implicated when chemical modification by an amino reagent altered binding of a BZ-site ligand in rat cortical membranes (Burch & Ticku, 1981; Maksay & Ticku, 1984). Studies on GABA<sub>A</sub> receptor subunit mutants (mainly  $\alpha 1$ ) implicate a number of residues in BZ-site ligand binding (Amin *et al.*, 1997; Buhr *et al.*, 1996; 1997a, b; Casula *et al.*, 2001; Schaerer *et al.*, 1998; Wingrove *et al.*, 1997). A histidine residue in the N-terminal extracellular region of  $\alpha 1,2,3$  and 5 subunits (position 105 in  $\alpha 5$ ) of the human GABA<sub>A</sub> receptor, which is represented by an arginine in  $\alpha 4$  and  $\alpha 6$  subunits (position 106 in  $\alpha 4$  and 100 in  $\alpha 6$ ), has been further identified as a major determinant for high affinity binding of conventional BZ-site ligands (Davies *et al.*, 1998; Wieland *et al.*, 1992). The BZ agonist diazepam has high affinity for  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\alpha 5$  containing GABA<sub>A</sub> receptors whereas  $\alpha 4$  and  $\alpha 6$  subtypes are diazepam insensitive (Hadingham *et al.*,

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1996; Wafford *et al.*, 1996). Mutation of the conserved histidine (in the  $\alpha 1$ -,  $\alpha 2$ -,  $\alpha 3$ -,  $\alpha 5$ -containing receptors) to an arginine abolished binding of diazepam (Wieland *et al.*, 1992). Conversely, mutation of the arginine residue at position 100 in the diazepam-insensitive  $\alpha 6$  subunit to a histidine bestowed diazepam sensitivity on the receptor (Wieland *et al.*, 1992).

The purpose of this study was to characterize the functional consequences of mutating the histidine residue at position 105, to an arginine, on the BZ-site pharmacology of  $\alpha 5$  receptors. Specifically, we tested the hypothesis that this mutation confers an  $\alpha 4/\alpha 6$  receptor-like pharmacology on  $\alpha 5$  receptors. Both the binding affinity and the functional efficacy of a range of BZ-site compounds were investigated using radioligand binding and two electrode voltage clamp electrophysiology.

## Methods

### Ligand binding

The binding affinity of various BZ-site ligands was determined using a conventional radioligand binding assay (as described by Wingrove *et al.*, 1997). HEK293 (Human Embryonic Kidney) cells were plated out at  $4 \times 10^6$  cells/10 cm dish in DMEM supplemented with 10% foetal calf serum. After 24 h incubation at 37°C in an atmosphere of 95% O<sub>2</sub>/5% CO<sub>2</sub>, the cells were transfected with subunit cDNA (2 µg per subunit per plate) and the vector pAdvantage (6 µg per plate) (Promega, Madison, WI, U.S.A.), to increase cDNA expression, using calcium

phosphate precipitation (Chen & Okayama, 1988). After incubation at 37°C in an atmosphere of air supplemented with 3–5% CO<sub>2</sub> for 48–72 h, the cells were harvested into phosphate buffered saline, pelleted and resuspended in assay buffer (KH<sub>2</sub>PO<sub>4</sub>, 10 mM; KCl, 100 mM; pH 7.4) then homogenized through a 27-gauge needle. In some studies, L (tk<sup>−</sup>) cells (murine fibroblasts) stably transfected with GABA<sub>A</sub> receptor subunits, as previously described (Hadingham *et al.*, 1992; 1993), were utilized, however the affinity of BZ-site ligands in these two expression systems was highly similar (unpublished observations) and therefore these data were pooled.

The radiolabelled BZ-site ligand [<sup>3</sup>H]-Ro15-4513 was incubated with the cells in a saturation assay to determine the  $K_D$ . Non-specific binding was obtained by incubation in the presence of 10 µM Ro15-4513. The assay was terminated after 90 min incubation at 4°C by filtration onto GF/B filters (Brandel, Montreal, Quebec, Canada) using a Tomtec cell harvester (Receptor Technologies Ltd., Oxon, U.K.). The filters were washed three times with 5 ml ice-cold assay buffer and dried before using liquid scintillation counting (LS6500 Scintillation System, Beckman Instruments Inc., Fullerton, CA, U.S.A.) to detect filter retained radioactivity. Displacement assays using sub- $K_D$  concentrations of [<sup>3</sup>H]-Ro15-4513 to determine the  $K_i$  of the unlabelled ligands were performed in a similar manner. The inhibition constant ( $K_i$ ) was calculated using the Cheng-Prusoff equation (Cheng & Prusoff, 1973;  $K_i = IC_{50}/(1 + [Radioligand]/K_D)$ ).

Data points were fitted by nonlinear regression analysis (Prism version 3.01; Graphpad) to give the values of  $K_D$  from the saturation assay and the  $IC_{50}$  from the radioligand displacement assay (one site competition). The  $K_i$  and the  $K_D$

**Table 1** Relative binding affinity of BZ-site ligands for human  $\alpha 5$ ,  $\alpha 5H105R$ ,  $\alpha 4$  and  $\alpha 6$  recombinant GABA<sub>A</sub> receptors

|               | $\alpha 5$           | $\alpha 5H105R$      | $K_i$ (nM) | $\alpha 4$           | $\alpha 6$           |
|---------------|----------------------|----------------------|------------|----------------------|----------------------|
| Diazepam      | 20.7<br>(15.3; 27.9) | 5% at 10 µM          |            | 8% at 10 µM          | 1% at 10 µM          |
| Flunitrazepam | 2.19<br>(1.27; 3.78) | 26% at 10 µM         |            | 2% at 10 µM          | 6% at 10 µM          |
| DMCM          | 2.01<br>(1.49; 2.72) | 12.0<br>(6.93; 20.8) |            | 27.9<br>(21.2; 36.9) | 98.0<br>(69.5; 138)  |
| $\beta$ -CCE  | 114<br>(87.9; 149)   | 1920<br>(1385; 2662) |            | 1026<br>(809; 1300)  | 3059<br>(2541; 3683) |
| $\beta$ -CCM  | 68.4<br>(52.7; 88.7) | 4434<br>(3760; 5229) |            | 1331<br>(1181; 1499) | 4403<br>(3557; 5449) |
| Abecarnil     | 8.13<br>(6.49; 10.2) | 6163<br>(4461; 8515) |            | 2002<br>(1496; 2677) | 6435<br>(4531; 9141) |
| Flumazenil    | 1.01<br>(0.72; 1.41) | 6.12<br>(4.48; 8.37) |            | 232<br>(206; 262)    | 206<br>(165; 256)    |
| Bretazenil    | 1.46<br>(1.05; 2.02) | 42.2<br>(32.7; 54.3) |            | 42.8<br>(40.8; 44.8) | 25.9<br>(12.2; 55.0) |
| FG8094        | 1.57<br>(1.01; 2.45) | 9.77<br>(8.74; 10.9) |            | 34.1<br>(28.5; 40.8) | 71.2<br>(56.4; 89.8) |
| FG8205        | 4.55<br>(3.30; 6.28) | 104<br>(81.9; 133)   |            | 431<br>(381; 488)    | 194<br>(115; 328)    |
| CL218872      | 618<br>(529; 722)    | 10% at 10 µM         |            | 8% at 10 µM          | 3% at 10 µM          |
| CGS8216       | 1.74<br>(1.50; 2.03) | 40.8<br>(35.3; 47.1) |            | 35.7<br>(28.9; 44.1) | 51.9<br>(40.1; 67.1) |
| CGS9895       | 1.04<br>(0.83; 1.30) | 31.4<br>(15.4; 63.7) |            | 54.8<br>(42.2; 71.0) | 30.3<br>(25.2; 36.6) |

Results represent displacement of specific [<sup>3</sup>H] Ro15-4513 binding and are presented as the geometric mean of the  $K_i$  with low and high errors of the mean in parentheses ( $n = 3-8$ ). The H105R mutation invariably reduced the affinity of BZ-site ligands for the  $\alpha 5$  containing GABA<sub>A</sub> receptor.

values were calculated from at least three independent experiments and expressed as mean  $\pm$  standard error of the mean. The 2-tail unpaired Student's *t*-test was used to test significance ( $P < 0.05$ ).

### Efficacy determination

The efficacies of the ligands for the BZ-site of the GABA<sub>A</sub> receptor (diazepam, flunitrazepam,  $\beta$ CCE, DMCM, Ro15-4513, flumazenil, bretazenil and FG 8094) were determined using the two electrode voltage clamp technique on oocytes expressing recombinant GABA<sub>A</sub> receptors (as described by Ebert *et al.*, 1997).

Briefly, oocytes were surgically removed from a female *Xenopus laevis* anaesthetized in a 0.4% solution of ethyl m-aminobenzoate. The oocytes (stage V and VI) were manually defolliculated in isolation medium (mM: NaCl, 108; KCl, 2; HEPES, 1; EDTA, 0.1; pH 7.9 with NaOH) followed by 5 min of mild collagenase treatment. Oocytes were then transferred to Modified Barth's Solution (mM: MBS; NaCl, 88; KCl, 1; HEPES, 10; NaHCO<sub>3</sub>, 2.4; CaCl<sub>2</sub>, 0.91; MgSO<sub>4</sub> (7H<sub>2</sub>O), 0.82; Ca(NO<sub>3</sub>)<sub>2</sub>(4H<sub>2</sub>O), 0.33; pH 7.5 with NaOH) for cDNA injection.

The cDNAs encoding the  $\alpha$ ,  $\beta$  and  $\gamma$  GABA<sub>A</sub> subunits, used in this investigation were obtained as described by Hadingham *et al.* (1993; 1995; 1996). The subunit cDNAs (engineered into the expression vector pCDM8 or pcDNA Amp) were diluted in injection buffer (mM: NaCl 88, KCl 1, HEPES 15, in sterile d.H<sub>2</sub>O, adjusted to pH 7.0 with 1 M NaOH, sterile filtered through 0.2  $\mu$ M filter) to a total cDNA concentration of 20 ng  $\mu$ l<sup>-1</sup> or 6.7 ng  $\mu$ l per subunit,  $\sim$ 15 nl of this mix was injected directly into the nucleus of the oocyte.

Oocytes were stored for up to 4 days following injection in incubation media (1 ml each of penicillin (10,000 units ml<sup>-1</sup>), streptomycin (10 mg ml<sup>-1</sup>) and gentamycin (50 mg ml<sup>-1</sup>) made up with 22 mg Na<sup>+</sup>pyruvate in 1 litre MBS). For recording, the oocytes were then impaled by the two electrodes, one electrode to voltage clamp the cell at  $-60$  mV, and the other electrode to measure the change in current following drug application. GABA was applied until the peak of the response was observed, usually 30 s or less. At least 3 min wash time was allowed between each GABA application to prevent desensitization. Concentration response curves were calculated using a non-linear squares fitting program to the equation:  $f(x) = B_{\max}/(1 + (EC_{50}/x)^n)$  where  $x$  is the drug concentration,  $EC_{50}$  is the concentration of drug eliciting a half maximal response and  $n$  is the Hill coefficient. The effects of GABA receptor modulators were examined on control GABA EC<sub>20</sub> responses with a pre-application time of 30 s. Only oocytes with an EC<sub>20</sub> within a fixed range were selected (1–6  $\mu$ M for  $\alpha 5\beta 3\gamma 2s$  and  $\alpha 6\beta 3\gamma 2s$ ; 1–12  $\mu$ M for  $\alpha 5H105R\beta 3\gamma 2s$ ) to minimize the chance of high (GABA) affinity doublet receptors with no  $\gamma$  subunit (no BZ-site) expressed by the oocyte. The efficacy of GABA receptor modulators was determined as the percentage change in the current activated by the EC<sub>20</sub> concentration of GABA using the equation: Efficacy (%) = ((current in presence of modulator/control current)  $\times$  100) – 100.

Data represent values of  $n \geq 3$  that are expressed as the mean  $\pm$  standard error of the mean. The 2-tailed unpaired Student's *t*-test was used to test significance ( $P < 0.05$ ).

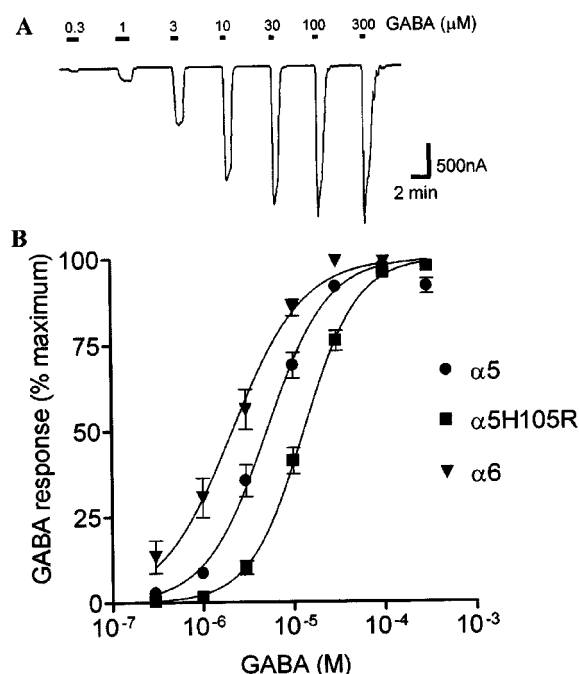
### Materials

All salts utilized for the MBS and injection buffers were obtained from BDH and were of AnalaR grade or better. The BZ-site ligands diazepam, flunitrazepam, ethyl- $\beta$ -carboline-3-carboxylate ( $\beta$ CCE), dimethoxy-4-ethyl- $\beta$ -carboline-3-carboxylate (DMCM), Ro15-4513, flumazenil, bretazenil and FG 8094 were all purchased from Sigma Chemical Company. All BZ-site ligands were dissolved in dimethylsulphoxide (DMSO), made up to a stock concentration of 2 or 10 mM and stored in a freezer ( $-20^\circ\text{C}$ ) until use. The radiolabelled BZ-site ligand [<sup>3</sup>H]-Ro15-4513 was obtained from New England Nuclear Research Products, Boston, MA, U.S.A.

## Results

### Ligand binding affinity

Radioligand binding assays were performed to investigate the effect of the  $\alpha 5H105R$  mutation on binding affinity of BZ-site ligands. Saturation binding experiments were conducted to calculate the mean values for the  $K_D$  of the [<sup>3</sup>H]-Ro15-4513 radioligand on  $\alpha 5$ ,  $\alpha 5H105R$  and  $\alpha 6$  containing GABA<sub>A</sub> receptors. Ro 15-4513 showed the greatest affinity for the  $\alpha 5$  receptor with a  $K_D$  of 1.2 nM



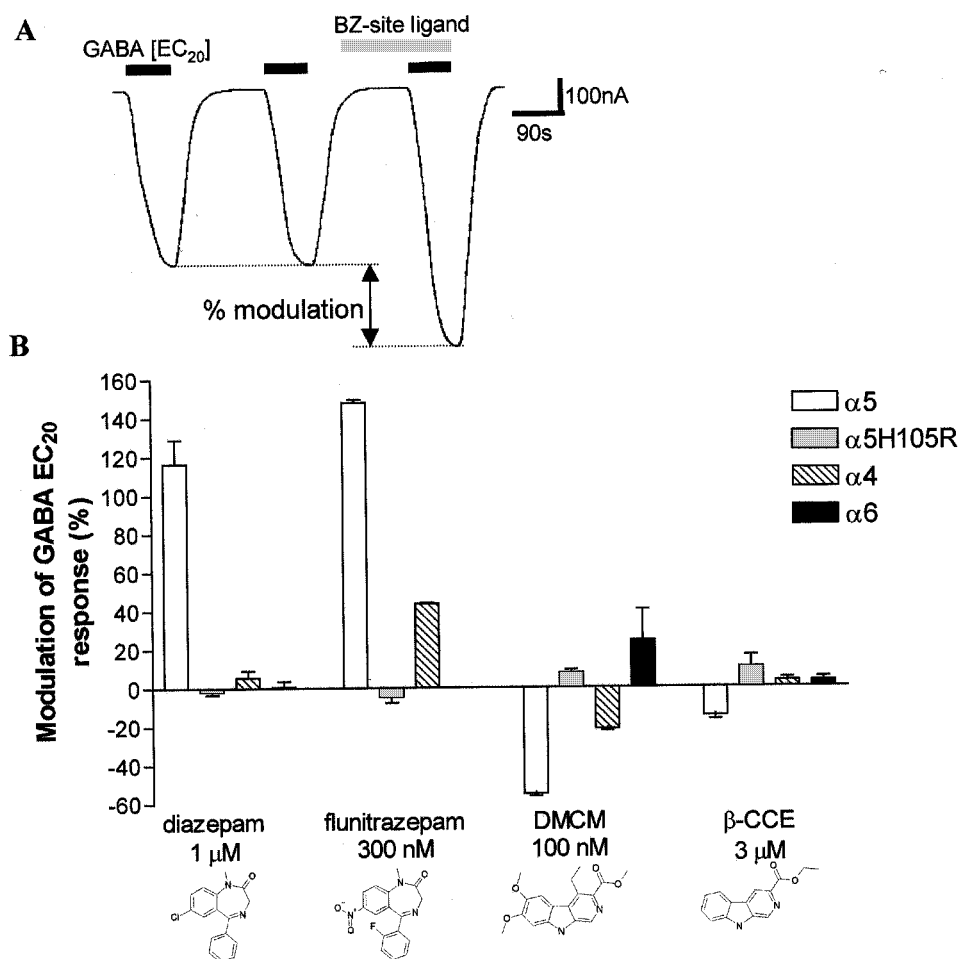
**Figure 1** Potency of GABA on human  $\alpha 5$ ,  $\alpha 5H105R$  and  $\alpha 6$  containing GABA<sub>A</sub> receptors. (A) Example of a two electrode voltage clamp recording of membrane current from *Xenopus* oocytes expressing  $\alpha\beta\gamma 2s$  GABA<sub>A</sub> receptors, showing responses to sequential bath application of increasing concentrations of GABA. (B) Graph showing the concentration dependent response to GABA (0.3–300  $\mu$ M) on  $\alpha 5$ ,  $\alpha 5H105R$  and  $\alpha 6$  containing GABA<sub>A</sub> receptors. Responses were normalized to the maximum GABA response for each oocyte and the mean  $\pm$  s.e. mean from  $n \geq 3$  oocytes is shown. Curves on all subunits showed a characteristic monophasic sigmoidal profile with similar Hill slopes.

( $pK_D = 8.92 \pm 0.04$ ), there was a significant reduction in affinity for the  $\alpha 5H105R$  receptor which had a  $K_D$  of 8.8 nM ( $pK_D = 8.08 \pm 0.09$ ) ( $P = 0.02$ ). The affinity was significantly lower again for the  $\alpha 6$  receptor with a  $K_D$  of 20.9 nM ( $pK_D = 7.68 \pm 0.08$ ) ( $P = 0.04$ ). A  $K_D$  of  $3.4 \pm 0.6$  nM has previously been reported for the  $\alpha 4$  receptor in a stably transfected Ltk cell line (Sur *et al.*, 1999).

Mean  $K_i$  values for a variety of BZ-site ligands of different structural classes were determined by displacement of specific [ $^3H$ ]-Ro15-4513 binding for  $\alpha 5$ ,  $\alpha 5H105R$ ,  $\alpha 4$  and  $\alpha 6$  containing GABA<sub>A</sub> receptors (Table 1). The classic 1,4-benzodiazepines diazepam and flunitrazepam which showed high affinity for the  $\alpha 5$  receptor ( $K_i$  of 20.7 and 2.19 nM respectively), showed a dramatic loss of affinity on mutation of the histidine residue ( $K_i$  on  $\alpha 5H105R > 10,000$  nM)

consistent with an  $\alpha 4$  or  $\alpha 6$ -like pharmacological profile ( $K_i$  also  $> 10,000$  nM).

Of the four  $\beta$ -carboline ligands examined, DMCM,  $\beta$ -CCE,  $\beta$ -CCM and abecarnil, all exhibited reduced affinity at  $\alpha 5H105R$  receptors and  $K_i$  values were consistent with an  $\alpha 4$ - or  $\alpha 6$ -like profile (Table 1). Affinities were also determined for a subset of 1,4-benzodiazepines which differ from classical ligands in that they possess an imidazobenzodiazepine core. Flumazenil, Ro15-4513, bretazenil, FG8094 and FG8205 all exhibit high affinity binding to  $\alpha 5$  receptors (1.01, 1.20, 1.46, 1.57 and 4.55 nM, respectively) and three of these compounds, flumazenil, Ro-15-4513 and FG8094 retained appreciable affinity following point-mutation of histidine 105 to arginine ( $\alpha 5H105R$  affinities 6.12, 8.80 and 9.77 nM respectively). Three additional compounds from alternative structural classes, CL218872, CGS8216 and



**Figure 2** Relative efficacy of conventional BZ-site ligands on human  $\alpha 5$ ,  $\alpha 5H105R$ ,  $\alpha 4$  and  $\alpha 6$  containing GABA<sub>A</sub> receptors. (A) Typical trace showing the consistent change in membrane current induced by an EC<sub>20</sub> concentration of GABA and modulation of this response upon bath application of a BZ-site ligand. The efficacy of the BZ-site ligand is determined as the percentage modulation in the current activated by an EC<sub>20</sub> concentration of GABA using the equation: modulation of GABA EC<sub>20</sub> response (%) = ((GABA EC<sub>20</sub> induced current in presence of the BZ-site ligand / GABA EC<sub>20</sub> induced current prior to BZ-site ligand application)  $\times$  100) - 100. A positive change is indicative of a BZ-site agonist, a negative change of an inverse agonist and no change indicates an antagonist. In the example shown, the ligand produced a small potentiation of the GABA response, consistent with partial agonist activity at the BZ-site. In subsequent figures, the efficacy of BZ-site ligands has been determined as above and then is presented as the mean  $\pm$  s.e. mean of data from  $n \geq 3$  oocytes. Only one BZ-site ligand was tested per oocyte. (B) Bar graph showing the efficacy of classical BZs (diazepam and flunitrazepam) and  $\beta$ -carboline (DMCM and  $\beta$ -CCE) on human  $\alpha 5$ ,  $\alpha 5H105R$ ,  $\alpha 4$  and  $\alpha 6$  containing GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes. All ligands were tested at a concentration 100 times their binding affinity on the  $\alpha 5$  containing GABA<sub>A</sub> receptor.

CGS9895, all displayed a loss of affinity at the mutated receptor ( $\alpha 5$   $K_i$  values 618, 1.74, 1.04;  $\alpha 5H105R > 10,000$ , 40.8, 31.4 nM), consistent with an  $\alpha 4/\alpha 6$  profile.

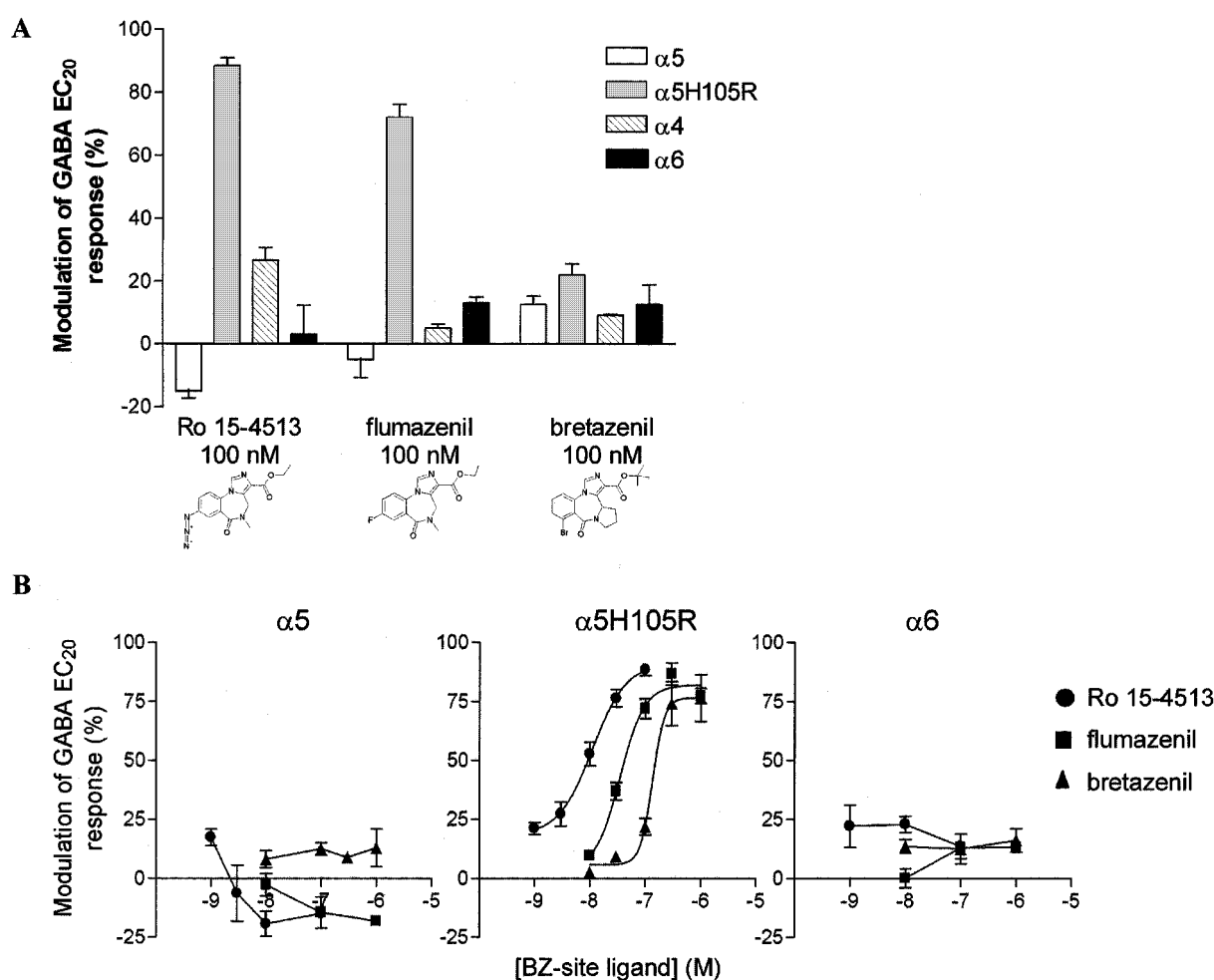
#### Effect of $\alpha 5H105R$ mutation on GABA potency

Full sequential concentration response curves to GABA (0.3–100  $\mu$ M) were constructed on  $\alpha 5$ ,  $\alpha 5H105R$  and  $\alpha 6$  containing GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes (Figure 1). All curves shared a sigmoidal relationship with similar Hill slopes ( $1.1 \pm 0.1$ ;  $1.3 \pm 0.1$ ;  $1.4 \pm 0.3$  for  $\alpha 5$ ,  $\alpha 5H105R$  and  $\alpha 6$  respectively, Figure 1B). GABA had comparable potency on  $\alpha 5$  containing GABA<sub>A</sub> receptors ( $EC_{50} = 5.1 \mu$ M;  $pEC_{50} = 5.29 \pm 0.06$ ) and  $\alpha 6$  containing GABA<sub>A</sub> receptors ( $EC_{50} = 1.1 \mu$ M;  $pEC_{50} = 5.97 \pm 0.04$ )

( $P < 0.0001$ ). The insertion of the  $\alpha 5H105R$  mutation reduced the potency of GABA ( $EC_{50} = 13 \mu$ M;  $pEC_{50} = 4.88 \pm 0.05$ ) when compared to the  $\alpha 5$  containing GABA<sub>A</sub> receptor ( $P < 0.0001$ ).

#### Effect of $\alpha 5H105R$ mutation on BZ efficacy

The ability of selected BZs to modulate an  $EC_{20}$  dose of GABA was then investigated (Figure 2A). The classical BZs, diazepam and flunitrazepam, both significantly potentiated the response to GABA being full agonists on the  $\alpha 5$  receptor ( $+116.4 \pm 12.5\%$  and  $+147.2 \pm 1.7\%$  respectively), whereas they were inactive on both  $\alpha 4$  and  $\alpha 6$  receptors (Figure 2B). Mutation of the histidine residue in  $\alpha 5$  ( $\alpha 5H105R$ ) also rendered diazepam and flunitrazepam inactive ( $+2 \pm 2\%$  and



**Figure 3** Relative efficacy of BZ-site ligands containing an imidazobenzodiazepine core on human  $\alpha 5$ ,  $\alpha 5H105R$  and  $\alpha 6$  containing GABA<sub>A</sub> receptors. (A) Bar graph showing the percentage modulation of GABA  $EC_{20}$  responses (mean  $\pm$  s.e. mean from  $n \geq 3$  oocytes) by Ro15-4513, flumazenil and bretazenil on human  $\alpha 5$ ,  $\alpha 5H105R$ ,  $\alpha 4$  and  $\alpha 6$  containing GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes (determined as described in Figure 2A). All ligands were tested at a concentration 100 times their binding affinity on the  $\alpha 5$  containing GABA<sub>A</sub> receptor. All three ligands showed partial agonist activity at the  $\alpha 5H105R$  containing GABA<sub>A</sub> receptor. (B) To determine the concentration dependence of responses to these BZ-site ligands, the effect of increasing concentrations of a BZ-site ligand (in the range  $10^{-9}$ – $10^{-6}$  M) on sequential applications of an  $EC_{20}$  concentration of GABA were determined in different oocytes. Again only one BZ-site ligand was tested at one concentration per oocyte as described in Figure 2A. The graphs show the concentration dependent response (mean  $\pm$  s.e. mean from  $n \geq 3$  oocytes) to these BZ-site ligands on  $\alpha 5$ ,  $\alpha 5H105R$  and  $\alpha 6$  containing GABA<sub>A</sub> receptors. Monophasic sigmoidal concentration-response curves for Ro15-4513, flumazenil and bretazenil were observed on  $\alpha 5H105R$  containing GABA<sub>A</sub> receptors, whereas these ligands had little effect on  $\alpha 5$  and  $\alpha 6$  GABA<sub>A</sub> receptors throughout the concentration range tested.

$-5 \pm 3\%$  respectively), consistent with a  $\alpha 4/\alpha 6$  pharmacological profile (Figure 2B).

The  $\beta$ -carbolines, DMCM and  $\beta$ CCE significantly diminished the response to GABA being inverse agonists ( $-55 \pm 1\%$  and  $-15 \pm 2\%$  respectively) at  $\alpha 5$  receptors. On mutation of the histidine residue in  $\alpha 5$  ( $\alpha 5$ H105R) the inverse agonism was significantly reduced ( $+8 \pm 2\%$  and  $+10 \pm 6\%$  respectively,  $P < 0.05$ ), more consistent with  $\alpha 4$  ( $-22 \pm 1\%$  and  $+3 \pm 2\%$  respectively) and  $\alpha 6$  ( $+21 \pm 13\%$  and  $+3 \pm 2\%$  respectively) pharmacology (Figure 2B).

The compounds Ro15-4513, flumazenil and bretazenil, an inverse agonist ( $-15 \pm 2\%$ ), an antagonist ( $-5 \pm 6\%$ ) and a weak agonist ( $+13 \pm 3\%$ ) on  $\alpha 5$  receptors, all showed significantly greater agonism on the mutant receptor ( $+89 \pm 2\%$ ,  $+72 \pm 4\%$  and  $+22 \pm 4\%$  respectively) than predicted from their weak agonism or antagonism on  $\alpha 4$  ( $+27 \pm 4\%$ ;  $+5 \pm 1\%$  and  $+9 \pm 1\%$  respectively) or  $\alpha 6$  ( $+3 \pm 9\%$ ;  $+13 \pm 2\%$  and  $+13 \pm 6\%$  respectively) receptors ( $P < 0.05$ , Figure 3A).

To investigate the unpredicted agonism seen with Ro15-4513, flumazenil and bretazenil further, different concentrations of these compounds ( $10^{-9}$ – $10^{-6}$  M) were investigated and concentration response curves were constructed. Ro15-4513, flumazenil and bretazenil were relatively inactive on  $\alpha 5$  and  $\alpha 6$  containing GABA<sub>A</sub> receptors. However, all three compounds displayed partial agonism on the  $\alpha 5$ H105R receptor (Ro15-4513,  $EC_{50} = 11$  nM,  $pEC_{50} = 7.95 \pm 0.09$ ; flumazenil,  $EC_{50} = 35$  nM  $pEC_{50} = 7.45 \pm 0.07$ ; and bretazenil,  $EC_{50} = 135$  nM,  $pEC_{50} = 6.87 \pm 0.11$ ), Figure 3B). Curves to all three compounds on the  $\alpha 5$ H105R mutant showed monophasic sigmoidal relationships indicating that the compounds are binding to a single site.

#### *Response to Ro15-4513, flumazenil and bretazenil in the presence of a ligand which competes for the BZ-site*

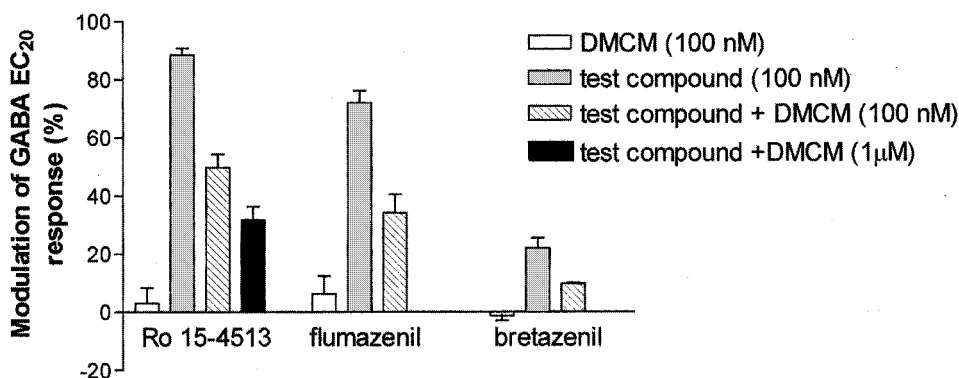
To determine whether Ro15-4513, flumazenil and bretazenil are acting *via* the BZ-site, a competition study with ligand binding to the BZ-site was conducted (Figure 4). The

conventional antagonist, flumazenil could not be used due to its agonist effect on the mutant receptor ( $+72 \pm 4\%$ ). Therefore, DMCM was selected as it has a lack of efficacy on the mutant receptor ( $+8 \pm 2\%$ ) without an accompanying loss of affinity ( $K_i = 12$  nM). DMCM was applied at an equal concentration (100 nM) to the agonists which was  $\sim 100$  times its affinity on the wild-type  $\alpha 5$  receptor. In each case the agonist response (Ro15-4513,  $89 \pm 2\%$  flumazenil,  $72 \pm 4\%$ ; bretazenil,  $22 \pm 4\%$ ) was significantly inhibited to approximately 50% (Ro15-4513, by 44% to  $50 \pm 5\%$ ; flumazenil, by 52% to  $34 \pm 6\%$ ; bretazenil, by 55% to  $10 \pm 1\%$ ,  $P < 0.05$ ) in the presence of DMCM. DMCM was also tested at a higher concentration (1  $\mu$ M) with Ro15-4513, which caused greater inhibition (by 64% to  $32 \pm 5\%$ ) indicating that DMCM inhibits binding of this compound in a concentration dependent manner.

Ro15-4513, flumazenil and bretazenil all share a common imidazobenzodiazepine core with a carboxylic acid at position-3 (Figure 3). To test the hypothesis that the presence of this core structure increases agonist properties to ligands on the  $\alpha 5$ H105R receptor, another related compound FG8094, which shares the imidazobenzodiazepine core structure, was tested. Consistent with our hypothesis, FG8094 (an inverse agonist on  $\alpha 5$ -containing receptors,  $-16.4 \pm 4.3\%$ ) produced a partial agonist response on the  $\alpha 5$ H105R containing GABA<sub>A</sub> receptor ( $+54.2 \pm 14.6\%$ ).

## Discussion and conclusions

The pharmacology of the BZ-site of GABA<sub>A</sub> receptors has been extensively studied by several groups. The main approaches used to investigate the structural requirements include site-directed mutagenesis of recombinant receptors and molecular modelling (Benson *et al.*, 1998; Boileau & Czajkowski, 1999; Casula *et al.*, 2001; Davies *et al.*, 1998; Dunn *et al.*, 1999; Renard *et al.*, 1999; Sigel & Buhr, 1997; Sigel *et al.*, 1998; Wingrove *et al.*, 1997; Wisden & Stephens, 1999; Zhang *et al.*, 1995). Characterization of the pharma-



**Figure 4** Determination of the site of action of imidazobenzodiazepines on human  $\alpha 5$ H105R containing GABA<sub>A</sub> receptors. The bar graph shows the percentage modulation of GABA  $EC_{20}$  responses (mean  $\pm$  s.e. mean from  $n \geq 3$  oocytes) by Ro15-4513, flumazenil and bretazenil on human  $\alpha 5$ H105R containing GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes (determined as described in Figure 2A, in the presence and absence of a ligand which competes for the BZ-site (DMCM,  $K_i = 12$  nM). Again only one test compound was studied per oocyte; the effect of test compounds in the presence and absence of DMCM was also determined in different oocytes as the effects of such compounds are often irreversible. DMCM was applied at an equal concentration (100 nM) to the agonists which was  $\sim 100$  times its affinity on the wild-type  $\alpha 5$  receptor and also at a higher concentration (1  $\mu$ M) with Ro15-4513.

cophore of the BZ-site has been complicated by the existence of multiple receptor isoforms, the diverse range of structures that are able to bind to the BZ-site and allosterically modulate the actions of GABA, and by the observations that efficacy cannot be predicted from binding affinity.

It is possible that site-directed mutagenesis of the histidine at position 105 in the  $\alpha 5$  subunit to an arginine may exert its effects not at the level of the BZ-site but may produce a conformational change in the receptor itself, thereby disrupting the GABA binding site. If this were the case, a large change in GABA potency would be expected. Although the potency of GABA for the  $\alpha 5$ H105R receptor (compared to the  $\alpha 5$  wild-type receptor) was reduced, the reduction in EC<sub>50</sub> was only equivalent to a shift of half a log unit. Considering the potency of GABA for a given receptor subtype can vary by up to one log unit with time after cDNA injection ( $\alpha 5$  containing GABA<sub>A</sub> receptors, day 1 EC<sub>50</sub> = 14  $\mu$ M, pEC<sub>50</sub> = 4.86  $\pm$  0.08 cf. Day 4 EC<sub>50</sub> = 3  $\mu$ M, pEC<sub>50</sub> = 5.52  $\pm$  0.11), this change in potency is not indicative of a significant alteration in the conformation of the GABA binding site of the receptor. The reason for the difference observed may be explained by the process of only selecting the oocytes with a GABA EC<sub>20</sub> value within the specified range for each receptor subtype, and these ranges can differ slightly between subunits.

The affinity and maximum efficacies of the BZs measured in oocytes were similar to those using binding techniques, suggesting that a different surrounding membrane environment and possible variations in the post-translational modifications provided by different cell types are not critical factors for the interactions of these drugs at their binding sites (Pritchett & Seeburg, 1990). The hypothesis that mutating this conserved histidine residue to an arginine confers pharmacological properties more consistent with an  $\alpha 4/\alpha 6$  isoform holds for the classical BZs and  $\beta$ -carbolines. Diazepam and flunitrazepam showed no affinity and negligible efficacy for the  $\alpha 5$ H105R receptor, consistent with previous studies (Wieland *et al.*, 1992); the lack of efficacy of these ligands can most likely be attributed to their lack of affinity for the receptor. Similarly, DMCM and  $\beta$ CCE exhibited both a loss of binding affinity and a loss of partial inverse agonist efficacy at the  $\alpha 5$ H105R receptor, consistent with the expected pharmacology of  $\alpha 4/\alpha 6$  receptors.

The compounds Ro15-4513, flumazenil, bretazenil and FG8094, which share a common imidazobenzodiazepine core, were all higher efficacy agonists on the  $\alpha 5$ H105R receptor than would have been predicted based on a simple switch from an  $\alpha 5$  to an  $\alpha 4/\alpha 6$  pharmacology. Data from previous studies with Ro15-4513 and bretazenil on  $\alpha 1$ H101R,  $\alpha 2$ H101R,  $\alpha 3$ H126R and  $\alpha 5$ H105R containing GABA<sub>A</sub> receptors (Benson *et al.*, 1998) support this.

This cannot be attributed to an increase in affinity of these ligands, since they show similar or lower affinity for the  $\alpha 5$ H105R receptor than the  $\alpha 5$  wild-type receptor. Another explanation for the unpredicted agonism with these compounds could be that they bind to another region on the mutant GABA<sub>A</sub> receptor revealed by the disruption of the BZ-site by point mutation. However, there are three reasons why this is unlikely. Firstly, the binding studies

show that [<sup>3</sup>H]-Ro15-4513 binds competitively to the same site as DMCM and  $\beta$ CCE on the  $\alpha 5$ H105R receptor. Secondly, the concentration response curves for these compounds show no evidence of a biphasic response which would be seen when a compound binds to two discrete binding sites producing different effects. Finally, DMCM was able to inhibit the effect of these compounds in a concentration dependent manner. Therefore it is likely that Ro15-4513, flumazenil and bretazenil elicit their agonist effect *via* an interaction with the BZ-site on the mutant receptor. So, even though the ligands studied bind to the same general site, the consequence on ion channel opening is not the same.

Whilst it might have been anticipated that Ro15-4513 would retain some affinity for the  $\alpha 5$ H105R receptor, given that this has been the ligand generally used to characterize diazepam-insensitive GABA<sub>A</sub> receptors, perhaps more surprising was the fact that flumazenil, FG8094 and DMCM retained significant affinity at the mutant receptor. This would not have been predicted based on their affinities for  $\alpha 4$  and  $\alpha 6$ -containing receptors and suggests that His105 is not a key determinant for high affinity binding of these ligands, implicating the importance of other residues. This theory of ligand-receptor interaction means certain residues in the BZ-site may be involved purely in the binding of the ligand to the BZ-site acting as a form of anchorage holding the ligand in the correct alignment. While other residues within the binding site do not interact with the ligand directly but facilitate the change in conformation of the receptor upon the binding of the allosteric modulator (Sigel *et al.*, 1998).

Photoaffinity labelling experiments have implicated the pendant phenyl ring of the classical BZs (e.g. diazepam) in binding to the imidazo ring of this conserved histidine residue (probably through a  $\pi$ - $\pi$  interaction; McKernan *et al.*, 1998). It has also been demonstrated that the conserved histidine residue is not required for the high affinity binding of flumazenil, Ro15-4513 and FG8094 as they still bind to the BZ-site in the presence of photoincorporated flunitrazepam (McKernan *et al.*, 1998).

>The primary objective of this study was to test the hypothesis that the  $\alpha 5$ H105R mutation confers  $\alpha 4/\alpha 6$ -containing receptor-like properties on to the  $\alpha 5$ -containing receptor. This investigation showed that the problem is more complex than first thought. The reason for this complexity is that the conserved histidine residue is directly involved in the binding of certain compounds to the BZ-site (e.g. classical BZs and some  $\beta$ -carbolines) yet for other compounds containing an imidazobenzodiazepine core (e.g. flumazenil, Ro15-4513, FG8094) the residue is not required for high affinity binding to the BZ-site but does influence the equilibrium between different conformations of the receptor.

These data support the hypothesis that GABA receptor subtypes contain common and highly conserved structural determinants that influence both the affinity and efficacy of BZ-site ligands and that can be exploited to investigate the pharmacological significance of specific GABA<sub>A</sub> receptor subtypes.

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