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Evidence that the atypical 5-HT_3 receptor ligand, [^{3}H]-BRL46470, labels additional 5-HT_3 binding sites compared to [^{3}H]-granisetron

[2]Lucinda J. Steward, Jian Ge, Kim R. Bentley, *Peter C. Barber, †Anthony G. Hope, †Jeremy J. Lambert, †John A. Peters, **Thomas P. Blackburn & [1]Nicholas M. Barnes

Department of Pharmacology, The Medical School, University of Birmingham, Edgbaston, Birmingham, B15 2TT; *Department of Pathology, The Medical School, University of Birmingham, Edgbaston, Birmingham, B15 2TT; †Department of Pharmacology and Clinical Pharmacology, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY and **SmithKline Beecham Pharmaceuticals, Psychiatry Research Department, Coldharbour Lane, Harlow, Essex, CM19 5AD

1 The radioligand binding characteristics of the ^{3}H-derivative of the novel 5-HT_3 receptor antagonist BRL46470 were investigated and directly compared to the well characterized 5-HT_3 receptor radioligand [^{3}H]-granisetron, in tissue homogenates prepared from rat cerebral cortex/hippocampus, rat ileum, NG108-15 cells, HEK-5-HT_3As cells and human putamen.

2 In rat cerebral cortex/hippocampus, rat ileum, NG108-15 cell and HEK-5-HT_3As cell homogenates, [^{3}H]-BRL46470 bound with high affinity (K_d (nM): 1.57 ± 0.18, 2.49 ± 0.30, 1.84 ± 0.27, 3.46 ± 0.36, respectively; mean ± s.e. mean, $n = 3$–4) to an apparently homogeneous saturable population of sites (B_{max} (fmol mg^{-1} protein): 102 ± 16, 44 ± 4, 968 ± 32 and 2055 ± 105, respectively; mean ± s.e.mean, $n = 3$–4) but failed to display specific binding in human putamen homogenates.

3 In the same homogenates of rat cerebral cortex/hippocampus, rat ileum, NG108-15 cells, HEK-5-HT_3As cells and human putamen as used for the [^{3}H]-BRL46470 studies, [^{3}H]-granisetron also bound with high affinity (K_d (nM): 1.55 ± 0.61, 2.31 ± 0.44, 1.89 ± 0.36, 2.03 ± 0.42 and 6.46 ± 2.58 respectively; mean ± s.e.mean, $n = 3$–4) to an apparently homogeneous saturable population of sites (B_{max} (fmol mg^{-1} protein): 39 ± 4, 20 ± 2, 521 ± 47, 870 ± 69 and 18 ± 2, respectively; mean ± s.e.mean, $n = 3$–4).

4 Competition studies with a range of structurally different 5-HT_3 receptor ligands indicated that in both rat cerebral cortex/hippocampus and rat ileum homogenates, [^{3}H]-BRL46470 binding exhibited a pharmacological profile consistent with the labelling the 5-HT_3 receptor with compounds competing with Hill coefficients close to unity.

5 In HEK-5-HT_3As cell homogenates, [^{3}H]-BRL46470 and [^{3}H]-granisetron associated rapidly ($(3.84 \pm 0.4)10^6$ M^{-1}s^{-1} and $(5.85 \pm 0.2)10^6$ M^{-1}s^{-1}, respectively, mean ± s.e.mean, $n = 3$–4) in an apparently monophasic manner. Following the establishment of equilibrium, both [^{3}H]-BRL46470 and [^{3}H]-granisetron at a saturating concentration ([^{3}H]-BRL46470 approximately 16 nM; [^{3}H]-granisetron approximately 18 nM) and at a sub-K_d concentration (approximately 1 nM for both radioligands) dissociated biphasically in HEK-5-HT_3As cell homogenates (saturating concentration; [^{3}H]-BRL46470 $4.05 \times 10^{-3} \pm 2.53 \times 10^{-3}$ s^{-1} and $5.83 \times 10^{-5} \pm 0.91 \times 10^{-5}$ s^{-1}; [^{3}H]-granisetron $3.20 \times 10^{-3} \pm 1.70 \times 10^{-3}$ s^{-1} and $18.58 \times 10^{-5} \pm 4.19 \times 10^{-5}$ s^{-1}: sub-K_d concentration; [^{3}H]-BRL46470 $2.47 \times 10^{-3} \pm 1.18 \times 10^{-3}$ s^{-1} and $9.30 \times 10^{-5} \pm 2.59 \times 10^{-5}$ s^{-1}; [^{3}H]-granisetron $65.91 \times 10^{-3} \pm 22.14 \times 10^{-3}$ s^{-1} and $49.96 \times 10^{-5} \pm 12.26 \times 10^{-5}$ s^{-1}, mean ± s.e.mean, $n = 4$–8) when induced by a 300 fold dilution in ice-cold Tris/Krebs.

6 In conclusion, the present study provides evidence that [^{3}H]-BRL46470 specifically labels the 5-HT_3 receptor in rat cerebral cortex/hippocampus, rat ileum, NG108-15 cell and HEK-5-HT_3As cell homogenates, but fails to label the 5-HT_3 receptor expressed in human putamen. Whilst the pharmacological profile of the site labelled by [^{3}H]-BRL46470 is directly comparable to that labelled by [^{3}H]-granisetron, [^{3}H]-BRL46470 consistently labelled approximately twice the density of sites compared to [^{3}H]-granisetron in the same tissue homogenates prepared from rat cortex/hippocampus, rat ileum, NG108-15 cells and HEK-5-HT_3As cells.

Keywords: 5-HT_3 receptor; [^{3}H]-BRL46470; [^{3}H]-granisetron; 5-HT_3 As receptor subunit; NG108-15 cells; rat brain; human brain

Introduction

In common with some other 5-HT_3 receptor antagonists, BRL46470 displays antipsychotic and anxiolytic potential in various animal models (Blackburn *et al.*, 1993; Piper *et al.*, 1991; for review see Bentley & Barnes, 1995). However, in some of these behavioural paradigms, BRL 46470 behaved atypically in that it lacked the bell-shaped dose-response curve which is characteristic of some 5-HT_3 receptor antagonists (Costall *et al.*, 1988; for reviews see Costall *et al.*, 1990; Barnes *et al.*, 1992a; Bentley & Barnes, 1995). Apart from the 5-HT_3 receptor, however, BRL46470 displays little or no affinity for a variety of other neurotransmitter receptors (Blackburn *et al.*, 1993; Newberry *et al.*, 1993).

In radioligand binding experiments, whilst most of the 5-HT_3 receptor ligands currently investigated appear to label a similar density of binding sites in brain tissue (Barnes *et al.*, 1992b), Wong and colleagues (1993) have recently demonstrated differences in the density of labelled 5-HT_3 receptors using structurally different radioligands. Thus, [^{3}H]-quipazine

[1]Author for correspondence.
[2]Present address: Department of Pharmacology, Faculty of Medicine, University of Alberta, Edmonton, Canada T6G 2H7.

labelled a higher density of sites than [^{3}H]-granisetron and [^{3}H]-GR65630 in rat cortex homogenates and approximately twice the density of sites labelled by [^{3}H]-GR65630 in rabbit ileum. However, these differences in the density of sites recognised by the two radioligands, were not apparent when labelling the 5-HT$_3$ receptor expressed in NG108-15 cells. Other differences in the binding characteristics of 5-HT$_3$ receptor ligands have also been reported. For instance, when the 5-HT$_3$ receptor expressed in rat brain or NG 108-15 cells is labelled with [^{3}H]-(S)-zacopride, agonists fail to generate steep competition curves with Hill coefficients greater than unity (Barnes *et al.*, 1992b; Barnes & Barnes, 1993). This contrasts with data generated with other 5-HT$_3$ receptor radioligands (e.g. [^{3}H]-granisetron, [^{3}H]-LY278,584, [^{3}H]-GR67330; Barnes *et al.*, 1992b; Barnes & Barnes, 1993) and has led to the hypothesis that [^{3}H]-(S)-zacopride may label a different site on the 5-HT$_3$ receptor complex (Barnes *et al.*, 1992b; Barnes & Barnes, 1993). Given the atypical behavioural profile of BRL46470, in the search for other differences by which ligands interact with the 5-HT$_3$ receptor, the current studies investigated the binding characteristics of [^{3}H]-BRL46470 in direct comparison to [^{3}H]-granisetron (Figure 1) in various tissue preparations (rat cerebral cortex/hippocampus, rat ileum, NG108-15 cells, HEK-293 cells stably transfected with the 5-HT$_3$-As receptor subunit (HEK-5-HT$_3$-As cells) and human putamen). A preliminary report of some of the present findings has been communicated to the British Pharmacological Society (Steward *et al.*, 1994).

Methods

Cell culture

Neuroblastoma x glioma cells of the clone NG108-15 (mouse neuroblastoma x rat glioma cells; MacDermot *et al.*, 1979) were cultured and harvested as described previously (Barnes & Barnes, 1993). HEK-5-HT$_3$-As cells (Hope *et al.*, 1993; Downie *et al.*, 1995) were cultured in minimal essential medium containing 10% v/v foetal calf serum, supplemented with streptomycin (40 μg ml^{-1}), penicillin (40 units ml^{-1}) and geneticin (0.75 mg ml^{-1}) and harvested between passage 3 and 40. Pelleted cells were frozen at –80°C prior to assay (less than 2 months).

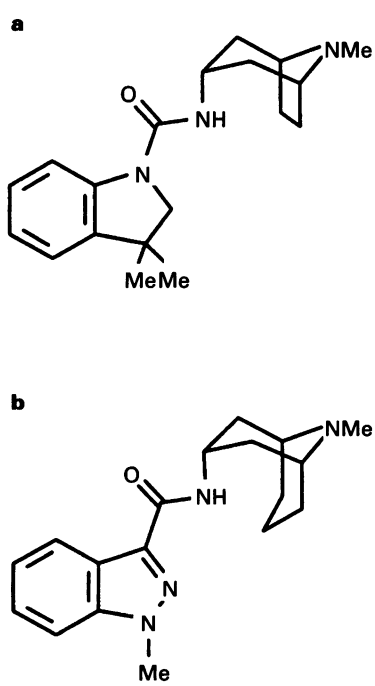

Figure 1 Chemical structures of BRL46470 (a) and granisetron (b).

Source of brain and gut tissue

Female Wistar rats (200–300 g; Birmingham bred) were killed by cervical dislocation and the cerebral cortex/hippocampus rapidly dissected and frozen at –80°C. Rat ileum was isolated from the gastrointestinal tract and the muscularis layer was finely chopped with scissors before being frozen at –80°C. Human putamen was obtained at autopsy from male and female patients who had died without neurological or psychiatric disorders (38–82 years), within 36 h of death and frozen at –80°C before assay.

Preparation of tissue

Frozen tissues were thawed and processed in ice-cold Tris/Krebs buffer (mM: Tris 50.0, NaCl 118.0, KCl 4.75, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.0, final pH 7.4) as described previously (Barnes *et al.*, 1992b) except that in studies using NG108-15 and HEK-5-HT$_3$-As cells, the homogenates were centrifuged only once and that the rat ileum homogenate was filtered through gauze and the filtrate used as the binding homogenate. The protein content of homogenates was estimated by the Bio-Rad Coomassie brilliant blue method (Bradford, 1976), with bovine serum albumin used as the standard. Final tissue homogenate concentrations were 0.2–0.3 mg protein ml^{-1} and 50–75 mg original wet weight ml^{-1} for saturation and competition studies with rat cortex/hippocampus, respectively, were 0.9–1.3 mg protein ml^{-1} and 100 mg original wet weight ml^{-1} for saturation and competition studies with rat ileum, respectively, 0.03–0.06 mg protein ml^{-1} for studies performed with NG108-15 and HEK-5-HT-As cells (although 0.3–1.7 mg protein ml^{-1} for kinetics studies with HEK-5-HT-As cells) and 100 mg original wet weight ml^{-1} for saturation studies with human putamen homogenates.

Competition and saturation radioligand binding

Radioligand binding assays were performed essentially as described previously (Barnes *et al.*, 1992b). [^{3}H]-BRL46470 binding was allowed to proceed at 37°C for 45 min prior to filtration as described for [^{3}H]-granisetron (Barnes *et al.*, 1992b).

Determination of the association and dissociation rates of [^{3}H]-BRL46470 and [^{3}H]-granisetron binding

The reaction mixture consisted of HEK-5-HT$_3$-As cell homogenate (1700 μl) plus 200 μl of radioligand ([^{3}H]-BRL46470 or [^{3}H]-granisetron, final concentration approximately 1 or 16–18 nM) and 100 μl of vehicle (Tris/Krebs buffer) or competing drug (ondansetron, final concentration 10 μM). For the association studies, immediately upon addition of the radioligand to the reaction mixture, 50 μl aliquots of the reaction mixture were removed and filtered at timed intervals under vacuum through pre-wet Whatman GF/B filters (0.1% v/v polyethyleneimine in Tris/Krebs) and immediately washed with ice-cold Tris/Krebs buffer (2 × 3.5 ml) using a Millipore filtration unit. Radioactivity remaining on the filters was assayed by liquid scintillation spectroscopy. For the dissociation studies, following the establishment of equilibrium (60 min at room temperature), dissociation was initiated by either 300 fold dilution by the addition of 50 μl of the reaction mixture to 15 ml of ice-cold Tris/Krebs (in some experiments ice-cold Tris/Krebs plus 5-HT (10 μM)), or the addition of unlabelled BRL 46470 (final concentration, 10 μM) or unlabelled granisetron (final concentration, 10 μM) to the reaction mixture and at timed intervals aliquots were removed and rapidly filtered, washed and radioactivity assayed as described for the association studies.

Drugs

Alosetron (SmithKline Beecham Pharmaceuticals), atropine (sulphate; Sigma), BRL46470 (HCl; (endo-N-(8-methyl-8-azabicyclo[3,2,1]oct-3yl)-2, 3-dihydro-3, 3-dimethyl-indole-1-carboxamide; SmithKline Beecham Pharmaceuticals), clozapine (Sandoz), cocaine (HCl; Research Biochemicals Inc (RBI)), geneticin (Gibco), granisetron (HCl; SmithKline Beecham Pharmaceuticals), GR 65630 (3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone; Glaxo Laboratories), hexamethonium (bromide; Sigma), 5-HT (maleate; Sigma), MDL 72222 (1αH, 3α, 5αH-tropan-3-yl-3,5-dichlorobenzoate; Merrell-Dow), mepyramine (maleate; May and Baker), 2-methyl-5-HT (maleate; RBI), metoclopramide (HCl; RBI), *meta*-chlorophenylbiguanide (HCl; mCPBG; RBI), naloxone (HCl; Sigma), paroxetine (HCl; SmithKline Beecham Pharmaceuticals), penicillin (Gibco), phentolamine (mesylate; RBI), phenylbiguanide (Aldrich), propranolol (HCl; RBI), quipazine (dimaleate; RBI), SDZ 206–830 ((3-α-homotropanyl)-1-methyl-5-fluoroindole-3-carboxylic acid; Sandoz), streptomycin (Gibco), tropisetron (HCl; Sandoz), (**R**)-zacopride (HCl, Centre de Recherche Delalande) and (**S**)-zacopride (HCl, Centre de Recherche Delalande) were dissolved in distilled water and diluted in Tris/Krebs buffer. Ondansetron (hydrochloride dihydrate, Glaxo Laboratories) was supplied in aqueous solution (2 mg ml^{-1}) and diluted with Tris/Krebs buffer. (+)-Tubocurarine (Wellcome) was supplied in aqueous solution (10 mg ml^{-1}) and diluted with Tris/Krebs buffer. SCH23390 (HCl; **R**(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; RBI) and (–)-sulpir-

Table 1 Summary of saturation binding data obtained with [^{3}H]-BRL46470 (0.1–17.6 nM) and [^{3}H]-granisetron (0.1–13.8 nM), in a variety of tissue preparations (non-specific binding defined by ondansetron, 10 μM)

	Tissue preparation				
	Rat cerebral cortex hippocampus	*Rat ileum*	*NG108-15 cells*	*HEK cells (5-HT$_3$-As)*	*Human putamen*
B_{max} (fmol mg^{-1} protein)					
[^{3}H]-BRL46470	102±16	44±4	968±32	2055±105	NRSB
[^{3}H]-granisetron	39±4	20±2	521±47	870±69	18±2
K_d (nM)					
[^{3}H]-BRL46470	1.57±0.18	2.49±0.30	1.84±0.27	3.46±0.36	–
[^{3}H]-granisetron	1.55±0.61	2.31±0.44	1.89±0.36	2.03±0.42	6.76±2.58
Hill coefficient					
[^{3}H]-BRL46470	1.06±0.06	1.14±0.09	0.98±0.10	1.02±0.08	–
[^{3}H]-granisetron	1.08±0.13	1.10±0.10	1.00±0.09	0.97±0.05	0.99±0.06

Data represent mean±s.e.mean, n=3–4. NRSB, no reproducible specific binding.

Table 2 The affinities and Hill coefficients of various compounds competing for the binding of [^{3}H]-BRL46470 (1 nM) to rat cerebral cortex/hippocampus and rat ileum homogenates

	Rat cortex/hippocampus			*Rat ileum*		
Compound	*pKi*	*Hill coefficient*	n	*pKi*	*Hill coefficient*	n
SDZ 206-830	9.06±0.17	1.02±0.14	6	9.10±0.04	0.79±0.01	6
(S)-zacopride	9.02±0.13	1.13±0.12	4	9.27±0.03	1.00±0.02	3
Quipazine	8.96±0.12	0.80±0.10	5	9.07±0.04	1.03±0.03	5
BRL46470	8.80±0.21	0.83±0.06	7	8.65±0.07	0.75±0.02	4
Alosetron	8.50±0.03	0.83±0.13	3	8.66±0.02	0.89±0.01	4
*m*CPBG	8.32±0.04	0.97±0.12	4	8.90±0.01	1.12±0.06	4
Tropisetron	8.31±0.21	0.91±0.14	5	8.42±0.06	1.14±0.05	4
Granisetron	8.29±0.06	1.09±0.17	4	8.56±0.04	1.01±0.10	4
GR 65630	8.12±0.16	0.85±0.12	3	ND		
(R)-zacopride	7.96±0.17	0.84±0.18	6	7.92±0.01	1.20±0.05	3
Ondansetron	7.33±0.20	0.98±0.12	8	8.14±0.01	1.03±0.14	4
MDL72222	7.52±0.13	0.82±0.11	4	7.50±0.04	1.00±0.03	5
Renzapride	7.17±0.15	0.88±0.17	6	7.56±0.01	1.04±0.04	3
PBG	6.87±0.07	1.00±0.12	4	6.21±0.02	1.00±0.07	4
(+)-Tubocurarine	6.58±0.20	0.89±0.15	7	5.70±0.03	1.18±0.07	5
Clozapine	6.57±0.23	0.95±0.21	5	6.02±0.03	1.18±0.07	5
5-Hydroxytryptamine	6.66±0.19	1.08±0.15	6	5.80±0.02	1.06±0.13	4
2-Methyl-5-HT	6.25±0.20	1.03±0.21	8	5.79±0.12	1.43±0.14	3
Metoclopramide	5.70±0.13	0.87±0.15	8	5.92±0.01	1.06±0.07	3
Cocaine	5.61±0.19	1.05±0.13	7	4.59±0.02	0.71±0.32	3
Mepyramine	5.54±0.20	1.06±0.32	4	ND		
Atropine	5.51±0.28	0.94±0.12	6	ND		
Paroxetine	5.32±0.08	1.23±0.06	3	ND		
Phentolamine	4.77±0.06	1.19±0.15	6	ND		
SCH23390	4.70±0.15	0.93±0.13	7	ND		
Propranolol	4.63±0.46	0.95±0.18	4	ND		
Naloxone	3.71±0.14	0.76±0.10	3	ND		
Hexamethonium	3.15±0.01	1.00±0.00	3	ND		

ND, not determined. Data represent mean±s.e.mean.

ide (RBI) were dissolved in a minimum quantity of glacial acetic acid, made to volume in distilled water and diluted in Tris/Krebs buffer. [^{3}H]-granisetron (84.5 or 84.8 Ci mmol^{-1}, NEN) and [^{3}H]-BRL46470 (80 Ci mmol^{-1}, Amersham), were supplied in ethanol and diluted in Tris/Krebs buffer. All drugs and reagents were used as received.

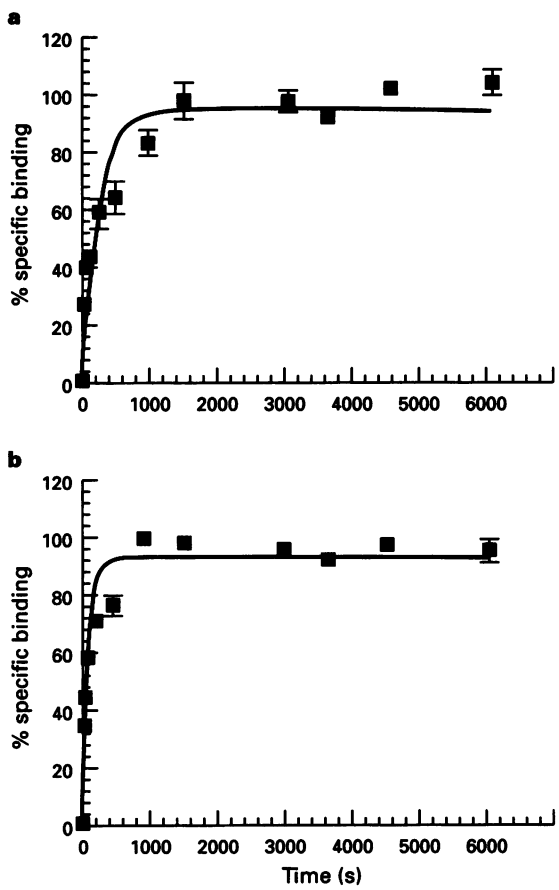

Figure 2 Association of (a) [^{3}H]-BRL46470 (1.2–1.4 nM, $n=4$) and (b) [^{3}H]-granisetron (1.4–1.5 nM, $n=3$) specific binding in homogenates of HEK-5-HT$_3$-As cells (non-specific binding defined by ondansetron, 10 μM) at room temperature. Data represent mean ± s.e.mean.

Data analysis

Saturation and competition radioligand binding data were analysed by computer assisted iterative curve fitting according to the logistic equation as described previously (Barnes & Barnes, 1993).

Kinetic radioligand binding data were analysed by computer assisted iterative curve fitting according to the equations: association; $B = B_{eq}(1\text{-}e^{-(k12L+k21)t})$ or dissociation from one site, $B = B_0$ (e^{-k21t}) or dissociation from two sites, $B = B1_0$ ($e^{-k1^{21}t}$) + $B2_0$ ($e^{-k2^{21}t}$); where B = bound radioligand, B_{eq} = bound radioligand at equilibrium, B_0 = bound radioligand at time 0 (one site dissociation), $B1_0$ = bound radioligand to site 1 at time 0 (two site dissociation), $B2_0$ = bound radioligand to site 2 at time 0 (two site dissociation), L = free radioligand concentration, t = time, $k12$ = association rate and $k21$ = dissociation rate (one site dissociation), k_1^{21} = dissociation rate from site 1 (two site dissociation), k_2^{21} = dissociation rate from site 2 (two site dissociation).

The affinities of competing compounds (K_i) were calculated by use of the Cheng Prusoff (1973) equation.

Results

Comparison of [^{3}H]-BRL46470 and [^{3}H]-granisetron saturation binding in various tissue homogenates

In saturation studies, [^{3}H]-BRL46470 (0.1–17.6 nM) labelled an apparently homogeneous population of saturable specific binding sites (non-specific binding defined by ondansetron, 10 μM) in homogenates prepared from rat cerebral cortex/hippocampus, rat ileum, NG108-15 cells and HEK-5-HT-As cells (Table 1) but failed to display reproducible specific binding in homogenates of human putamen (Table 1).

[^{3}H]-granisetron (0.1–13.8 nM) also labelled an apparently homogeneous population of saturable specific binding sites (non-specific binding defined by ondansetron, 10 μM) in the same homogenates prepared from rat cerebral cortex/hippocampus, rat ileum, NG108-15 cells and HEK-5-HT-As cells, as were used for the [^{3}H]-BRL46470 studies, but the densities of

Table 3 Dissociation kinetics of [^{3}H]-BRL46470 ([^{3}H]-B; 1.1–1.4 and 14.5–18.4 nM) and [^{3}H]-granisetron ([^{3}H]-G; 1.4–1.5 and 15.7–21.6 nM) in HEK-5-HT$_3$-As cell homogenates (after a 60 min equilibrium period) induced by a 300 fold dilution in ice-cold Tris/Krebs or a 300-fold dilution in ice-cold Tris/Krebs in the presence or absence of 5-HT (10 μM; assays performed on same homogenate preparations), or by competition with BRL46470 (B; final concentration, 10 μM) or granisetron (G; final concentration, 10 μM)

Initiation of dissociation/ Radioligand	*Fast* (10^{-3} s^{-1})	prop^n(%)	*Slow* (10^{-5} s^{-1})	prop^n(%)	n
'Infinite dilution'					
[^{3}H]-B (16 nM)	4.05 ± 2.53	42 ± 3	5.83 ± 0.91	58 ± 03	8
[^{3}H]-B (1 nM)	2.47 ± 1.18	41 ± 1	9.30 ± 2.59	59 ± 03	4
[^{3}H]-G (18 nM)	3.20 ± 1.70	51 ± 13	18.58 ± 4.19	49 ± 13	4
[^{3}H]-G (1 nM)	65.91 ± 22.14	29 ± 10	49.96 ± 12.26	71 ± 10	4
'Infinite dilution' ± 5-HT (10 μM)					
[^{3}H]-B (15 nM) + 5-HT	2.35	42	6.11	51	1
[^{3}H]-B (15 nM)–5-HT	2.36	38	6.25	62	1
Competition					
[^{3}H]-B (15 nM) + B	14.44	45	15.23	55	1
[^{3}H]-B (15 nM) + G	10.27	40	18.05	60	1

Data represent mean ± s.e.mean, except data from addition of 5-HT to buffer, or competition-induced dissociation which is representative of a single experiment which was repeated once with similar results.

labelled sites were consistently lower (Table 1). Furthermore, [3H]-granisetron also labelled an apparently homogeneous population of saturable specific binding sites in the human putamen homogenates (non-specific binding defined by ondansetron, 10 μM; Table 1).

Pharmacological characterization of [3H]-BRL46470 binding in homogenates prepared from rat cerebral cortex/hippocampus and ileum

In competition studies using homogenates of rat cerebral cortex/hippocampus or rat ileum, [3H]-BRL46470 (0.7–1.3 nM) displayed a pharmacological profile consistent with the labelling of the 5-HT$_3$ receptor (Table 2), with specific [3H]-BRL46470 binding representing approximately 50% of total binding. All competition curves displayed Hill coefficients near to unity (Table 2).

Competition for [3H]-granisetron binding by BRL46470 in homogenates prepared from NG108-15 cells and human putamen

BRL46470 competed with 5 fold lower affinity for [3H]-granisetron (0.9–1.0 nM) specific binding in human putamen homogenates (p*Ki* 7.81±0.10, Hill coefficient 1.03±0.12, mean±s.e.mean, $n=3$) compared to the binding in NG108-15 cell homogenates (p*Ki* 8.54±0.07, Hill coefficient 0.87±0.13, mean±s.e.mean, $n=3$). [3H]-granisetron specific binding represented approximately 95 and 50% of the total binding in the NG108-15 cell and human putamen homogenates, respectively.

Association rates of [3H]-BRL46470 and [3H]-granisetron binding to homogenates prepared from HEK-5-HT$_3$-As cells

[3H]-BRL46470 (1.2–1.4 nM) specific binding in HEK-5-HT$_3$-As cell homogenates (non-specific binding defined by ondansetron 10 μM) associated rapidly ((3.84±0.4) 10^6 M^{-1} s^{-1}, mean±s.e.mean, $n=4$) and in an apparently monophasic manner (Figure 2). Equilibrium was reached within 25 min and specific binding remained at a constant level for at least 100 min (Figure 2). Specific [3H]-granisetron (1.4–1.5 nM) binding (non-specific binding defined by ondansetron, 10 μM) also associated rapidly ((5.84±0.2) 10^6 M^{-1} s^{-1}, mean±s.e.mean, $n=3$) in an apparently monophasic manner reaching equilibrium within 16 min and remaining at a constant level for at least 100 min (Figure 2).

Dissociation rates of [3H]-BRL46470 and [3H]-granisetron binding to homogenates prepared from HEK-5-HT$_3$-As cells

Dissociation of specific [3H]-BRL46470 (14.5–18.4 and 1.2–1.5 nM) binding in HEK-5-HT$_3$-As cell homogenates, induced by 300-fold dilution in ice cold Tris/Krebs, appeared to be biphasic (Table 3; Figure 3). Similarly [3H]-granisetron (15.7–21.6 and 1.4–1.5 nM) dissociation, induced by 300 fold dilution, appeared to be biphasic (Table 3; Figure 3). The dissociation of [3H]-BRL46470 (15.1 nM) with the addition of 5-HT (10 μM) to the 'infinite' (300 fold) dilution buffer, was also biphasic with fast and slow dissociation rates similar to those for [3H]-BRL46470 dissociating in the absence of 5-HT (Table 3). Dissociation of [3H]-BRL46470 (15.0 nM) induced by the addition of the competing drugs, BRL 46470 (10 μM) or granisetron (10 μM) produced similar dissociation rates (Table 3), with [3H]-BRL46470 dissociating in an apparently biphasic manner.

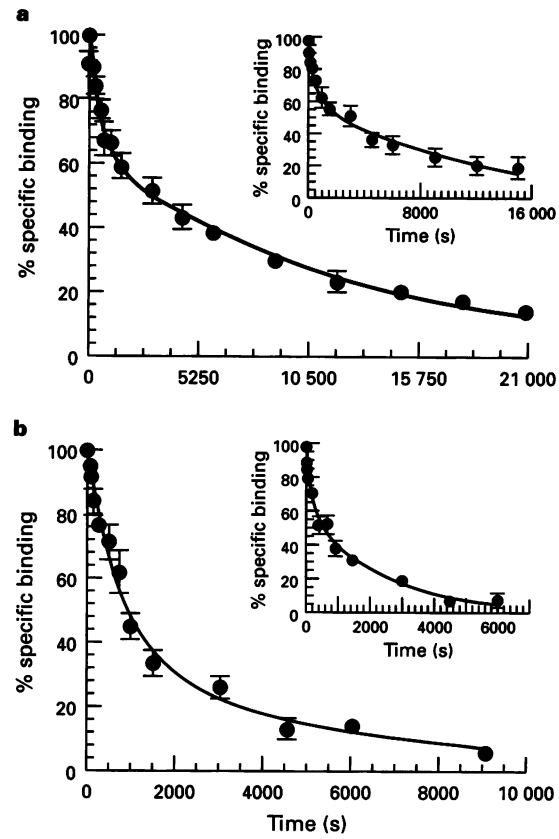

Figure 3 Dissociation of (a) [3H]-BRL46470 (14.5–18.4 nM; inset 1.15–1.4 nM) and (b) [3H]-granisetron (15.7–21.6 nM; inset 1.4–1.5 nM) specific binding in homogenates of HEK-5-HT$_3$-As cells (non-specific binding defined by ondansetron, 10 μM) at 4°C. Dissociation was initiated at time = 0, by a 300 fold dilution in ice-cold Tris/Krebs. Data represent mean±s.e.mean, $n=4$–8. For dissociation constants for [3H]-BRL46470 and [3H]-granisetron see Table 3.

Discussion

The pharmacological profile of specific [3H]-BRL46470 binding was consistent with its labelling the 5-HT$_3$ receptor in homogenates prepared from rat cerebral cortex/hippocampus and ileum; thus generally, the affinities of a range of structurally diverse competing compounds were similar to those previously reported with other 5-HT$_3$ receptor antagonist radioligands (e.g. Hoyer & Neijt, 1987; 1988; Kilpatrick *et al.*, 1987; Barnes *et al.*, 1990; 1992b; Sharif *et al.*, 1991).

In rat cerebral cortex/hippocampus, rat ileum, NG108-15 and HEK-5-HT$_3$-As cell homogenates, both [3H]-BRL46470 and the well characterized selective 5-HT$_3$ receptor radioligand [3H]-granisetron (e.g. Nelson & Thomas, 1989), appeared to label an apparently homogeneous population of saturable sites defined by the selective 5-HT$_3$ receptor antagonist, ondansetron (Butler *et al.*, 1988). [3H]-BRL46470, however, consistently labelled approximately twice the density of sites in the same homogenates compared to [3H]-granisetron. Wong and colleagues (1993) also demonstrated differences in the densities of binding sites labelled by the 5-HT$_3$ receptor radioligands, [3H]-quipazine, [3H]-granisetron and [3H]-GR65630 in rat cerebral cortex and rabbit ileal homogenates. In their studies however, differences in the binding densities between the radioligands were not apparent with the NG108-15 cell preparation. Furthermore, since in the present study [3H]-BRL46470 labelled approximately twice the density of sites than [3H]-granisetron labelled in homogenates prepared from HEK-293 cells stably transfected with the 5-HT$_3$-As subunit, which form functional presumably homomeric 5-HT$_3$ receptors when transfected into *Xenopus* oocytes (Hope *et al.*, 1993; Downie *et al.*, 1995) and HEK-293 cells (Werner *et al.*, 1993; Sepulveda & Lummis, 1994), it would appear that [3H]-BRL46470 was labelling an additional site(s) on the 5-HT$_3$ receptor complex, no specific [3H]-BRL46470 or [3H]-granisetron binding was detected in HEK-293 cells not transfected with the 5-HT$_3$-As subunit (data not shown).

The reason underlying the 'additional' [3H]-BRL46470 binding is not easily explained, as in competition studies in rat cerebral cortex/hippocampus, unlabelled granisetron displaced [3H]-BRL46470 binding with a Hill coefficient near to unity. It may have been expected that since [3H]-BRL46470 labels approximately twice the density of sites as [3H]-granisetron in rat cerebral cortex/hippocampus homogenates, unlabelled granisetron would either not compete for all the specific [3H]-BRL46470 binding or would compete in a biphasic manner in this preparation, however this was not the case. It may be of relevance, that the 5-HT$_3$ receptor complex is likely to possess two agonist recognition sites. These sites are likely to possess a similar pharmacology and therefore the ability of [3H]-BRL46470 to label both sites, whereas [3H]-granisetron only has access to one of the sites, would suggest an explanation for the higher density of sites labelled by [3H]-BRL46470, including the 'additional' binding to presumably homomeric 5-HT$_3$ receptors comprised of the 5-HT$_3$-As receptor subunit. It may therefore be possible that the competition by granisetron for one of the [3H]-BRL46470 recognition sites is competitive but the binding of granisetron to one of the sites induces the dissociation of [3H]-BRL46470 from the 'second' site by negative cooperativity.

In order to investigate whether granisetron displaces [3H]-BRL46470 binding from at least one of the sites by negative cooperativity and also to study further the binding properties of [3H]-BRL46470, the kinetics of [3H]-BRL46470 binding were examined. [3H]-BRL46470 and [3H]-granisetron associated rapidly to the 5-HT$_3$ receptor expressed in HEK-5-HT$_3$-As cell homogenates in an apparently monophasic manner. Following a 300 fold dilution in Tris/Krebs buffer, the dissociation of both approximately 16 and 1 nM [3H]-BRL46470 from HEK-5-HT$_3$-As cell homogenates was markedly biphasic. This indicated that [3H]-BRL46470 was binding to two different sites or affinity states, and initially appeared to provide a plausible explanation for the differences in the densities detected in the saturation binding studies. However, [3H]-granisetron (approximately 1 and 18 nM) dissociation from HEK-5-HT$_3$-As cell homogenates, initiated by a 300 fold dilution, also appeared to be biphasic although the difference between the 'fast' and 'slow' dissociation rates was not as pronounced as for [3H]-BRL46470. The biphasic dissociation of [3H]-granisetron had not been reported previously (Nelson & Thomas, 1989), but may be explained by differences in experimental conditions such as the lower temperature used in the present study to initiate dissociation (allowing a more accurate determination of the faster dissociation component), as well as the different preparations expressing the 5-HT$_3$ receptor (HEK-5-HT$_3$-As cells compared with rat brain; present study versus Nelson & Thomas, 1989). The biphasic manner of [3H]-BRL46470 dissociation from the 5-HT$_3$ receptor, may be explained by the radioligand binding to the receptor in an ordered manner i.e. with the ligand molecule binding first having to dissociate last. Subsequently, the effect of addition of a second specific ligand would be expected to decrease the dissociation rate. However, the addition of 5-HT to the 300 fold dilution had no apparent effect on the dissociation rates and thus the biphasic nature of [3H]-BRL46470 dissociation, suggesting that this was not the case. This was also inferred from the similar dissociation rates for [3H]-BRL46470 induced by competition with either excess unlabelled BRL46470 or granisetron. The biphasic nature of both [3H]-BRL46470 and [3H]-granisetron may indicate the presence of different affinity states of the 5-HT$_3$ receptor. In common with the competition studies, the dissociation studies would appear to fail to explain the density differences detected with the two radioligands since no evidence of negative cooperativity with respect to the competition for [3H]-BRL46470 binding by granisetron was demonstrated.

The different densities of 5-HT$_3$ receptor in the various preparations labelled by [3H]-BRL46470 and [3H]-granisetron, are unlikely to be due to different lipophilicity values of the radioligands since the clog P values of the unlabelled drugs are 2.00 and 2.85 for granisetron and BRL46470, respectively. These values indicate that both compounds are relatively lipophilic and therefore a differential ability of the compounds to interact with the 5-HT$_3$ receptor protein in the lipid membrane environment is unlikely.

Apparent differences in binding density values resulting from different radioligands may be explained by inaccuracies in the determination of the specific activity. However the specific activity of [3H]-BRL46470 (synthesized and supplied by Amersham), was determined on 3 separate occasions and consistently found to be approximately 80 Ci mmol^{-1}. In addition, two different batches of [3H]-granisetron were used in the present study (with similar results), and furthermore, upon inquiry the specific activity of the second batch of [3H]-granisetron (supplied by NEN/DuPont) was confirmed to be 84.5 Ci mmol.$^{-1}$.

Recent studies concerning the radiolabelling of the dopamine D$_2$ receptor, have shown similar discrepancies with respect to the density of binding sites using different radioligands. Using a recombinant D$_2$ receptor preparation, Seeman and colleagues (1992) demonstrated that [3H]-emonapride and [3H]-raclopride, labelled 1.8–2 fold more binding sites than [3H]-spiperone. It was proposed that the radioligands [3H]-emonapride and [3H]-raclopride may be binding to two sites on the recombinant D$_2$ receptor, or that the receptor is dimerising and that [3H]-spiperone is labelling the dimeric form. In addition, Wong and colleagues (1992) demonstrated a similar discrepancy in the density of dopamine D$_2$ receptors in human striatum using the technique of positron emission topography, and the radioligands [11C]-raclopride and [11C]-methylspiperone. It is possible that a similar explanation could be provided for the present data using [3H]-BRL46470 and [3H]-granisetron. It may therefore be relevant that the nicotinic acetylcholine receptor, which is structurally related to the 5-HT$_3$ receptor, also forms dimers (Chang & Bock, 1977; Conti-Tronconi *et al.*, 1982). Furthermore, the presence of two agonist binding sites on the 5-HT$_3$ receptor complex (discussed above) may provide a parallel to the anomaly with the D$_2$ receptor binding.

It remains to be determined whether the apparent additional 5-HT$_3$ receptors labelled by [3H]-BRL46470 has any connection with functional differences with respect to the behavioural pharmacology of unlabelled BRL46470. Unfortunately, ^{3}H derivatives of other 5-HT$_3$ receptor antagonists that fail to display bell-shaped response curves in behavioural models (e.g. Alosetron; Smith *et al.*, 1993) are not yet available to allow further investigation of this relationship.

In human putamen homogenates, [3H]-granisetron labelled an apparently homogeneous population of specific binding sites defined by ondansetron, concordant with our previous findings (Bufton *et al.*, 1993). [3H]-BRL46470, however, failed to reproducibly label specific sites in the same human putamen homogenates. Consistent with these data, BRL46470 competed with approximately 5 fold lower affinity for [3H]-granisetron binding in human putamen homogenates compared to the [3H]-granisetron binding in NG108-15 cell homogenates. The lower affinity of BRL 46470 for the human 5-HT$_3$ receptor compared to that expressed in NG108-15 cells, combined with the relatively low density of 5-HT$_3$ receptors in human putamen, may account for the inability of [3H]-BRL46470 to label reproducibly 5-HT$_3$ receptors in the human putamen under the present experimental conditions.

It is also of interest that 5-HT$_3$ receptor agonists competed for [3H]-BRL46470 binding in rat cerebral cortex/hippocampus and ileum with Hill coefficients close to unity. This contrasts with data generated with most radioligands which label the 5-HT$_3$ receptor (e.g. Hoyer & Neijt, 1987; 1988; Kilpatrick *et al.*, 1987; Barnes *et al.*, 1992b; Barnes & Barnes, 1993) although is comparable to previous studies investigating the labelling of the 5-HT$_3$ receptor with [3H]-(R/S)-zacopride and [3H]-(S)-zacopride (Barnes *et al.*, 1988; 1990; 1992b; La-

porte *et al.*, 1992; Barnes & Barnes, 1993). It would appear unlikely, however, that the failure of agonists to compete for [^{3}H]-BRL46470 with steep competition curves (Hill coefficient >1), underlies the different density of sites labelled by this radioligand relative to [^{3}H]-granisetron, since [^{3}H]-(S)-zacopride labels a similar density of 5-HT$_3$ receptor recognition sites in rat brain and NG108-15 cells compared to [^{3}H]-granisetron (Barnes *et al.*, 1992b; Barnes & Barnes, 1993).

In summary the ^{3}H-derivative of the 5-HT$_3$ receptor antagonist BRL46470, appeared to label selectively a saturable population of specific binding sites with a pharmacological profile consistent with that of the 5-HT$_3$ receptor. However, the density of specific sites recognised by this radioligand (defined by ondansetron) in various tissue preparations (rat cerebral cortex/hippocampus, NG108-15 cells, HEK-5-HT$_3$-As cell homogenates), were consistently higher (approximately double), when compared to the density of specific sites labelled by the well characterized 5-HT$_3$ receptor radioligand, [^{3}H]-granisetron. The inability of [^{3}H]-BRL46470 to radiolabel the 5-HT$_3$ receptors expressed in human putamen, highlights the inter-species differences with respect to the pharmacology of the 5-HT$_3$ receptor and the subsequent necessity of investigating the affinity of prospective drugs for clinical development using receptors of human origin.

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