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SPECIAL REPORT

SB-334867-A: the first selective orexin-1 receptor antagonist

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The pharmacology of various peptide and non-peptide ligands was studied in Chinese hamster ovary (CHO) cells stably expressing human orexin-1 (OX_1) or orexin-2 (OX_2) receptors by measuring intracellular calcium ($[Ca^{2+}]_i$) using Fluo-3AM. Orexin-A and orexin-B increased $[Ca^{2+}]_i$ in CHO-OX_1 ($pEC_{50} = 8.38 \pm 0.04$ and 7.26 ± 0.05 respectively, $n = 12$) and CHO-OX_2 ($pEC_{50} = 8.20 \pm 0.03$ and 8.26 ± 0.04 respectively, $n = 8$) cells. However, neuropeptide Y and secretin (10 pM – 10 μM) displayed neither agonist nor antagonist properties in either cell-line. SB-334867-A (1-(2-Methyylbenzoxanzol-6-yl)-3-[1,5]naphthyridin-4-yl-urea hydrochloride) inhibited the orexin-A (10 nM) and orexin-B (100 nM)-induced calcium responses ($pK_B = 7.27 \pm 0.04$ and 7.23 ± 0.03 respectively, $n = 8$), but had no effect on the UTP (3 μM)-induced calcium response in CHO-OX_1 cells. SB-334867-A (10 μM) also inhibited OX_2 mediated calcium responses ($32.7 \pm 1.9\%$ *versus* orexin-A). SB-334867-A was devoid of agonist properties in either cell-line. In conclusion, SB-334867-A is a non-peptide OX_1 selective receptor antagonist.
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Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; CHO, Chinese hamster ovary; FIU, fluorescence intensity units; FLIPR, fluorometric imaging plate reader; hPYY, human PYY; NPY, human neuropeptide Y; OX_1, human orexin-1 receptor; OX_2, human orexin-2 receptor; PYY, peptide YY; pPYY, porcine PYY; SB-334867-A, (1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl-urea hydrochloride); VIP, rat vasoactive intestinal peptide

Introduction Orexin-A and orexin-B are 33 and 28 amino acid peptides respectively, which were recently isolated from the rat hypothalamus and are derived from a 130 amino acid precursor, prepro-orexin (Sakurai *et al*., 1998). Both peptides bind to two receptors, orexin-1 (OX_1) and orexin-2 (OX_2), although orexin-B displays 10 fold selectivity for OX_2 (Sakurai *et al*., 1998). In a recombinant system the binding of these ligands to either receptor is associated with an increase in intracellular calcium concentrations ($[Ca^{2+}]_i$) (Smart *et al*., 1999).

The orexin receptors are located predominantly in the hypothalamus and locus coeruleus (Sakurai *et al*., 1998; Peyron *et al*., 1998), but are also found elsewhere in the CNS (Smart, 1999; Van den Pol, 1999). The orexins have been linked to a range of physiological functions (Jerman & Smart, 2001) including the control of feeding and energy metabolism (Sakurai *et al*., 1998), modulation of neuroendocrine function (Van den Pol, *et al*., 1998; Smart, 1999), and regulation of the sleep-wake cycle (Piper *et al*., 2000). However, the study of the role of the orexins in these functions has been hampered by the lack of orexin receptor antagonists (Jerman & Smart, 2001). Therefore, as preliminary studies showed that SB-334867-A (1-(2-Methylbenzoxazol-6-yl)-3-[1,5]napthyridin-4-yl-urea hydrochloride) inhibited an OX_1-mediated calcium response (Smart, 2000), further characterization of this compound's interactions with the orexin receptors has been undertaken. Furthermore, it has recently been reported that neuropeptide Y (NPY), secretin and, to a lesser extent, several other related peptides displace orexin-A binding (Kane *et al*., 2000). Therefore, these peptides have also been tested as both agonists and antagonists at recombinant human OX_1 and OX_2 receptors expressed in CHO cells using a FLIPR-based functional assay.

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Methods *Cloning and expression of human OX_1 and OX_2 receptors in CHO cells* OX_1 and OX_2 were produced by PCR from in-house foetal and adult brain cDNA libraries respectively, using primers located across the start and stop codons. The receptors were sub-cloned into the pCDN vector (with neomycin resistance) and transfected into CHO cells using lipofectamine (Life Technologies). Clones were selected using 400 μg ml^{-1} G418 (Life Technologies) and single cell clones were produced by limiting dilution cloning.

Cell culture CHO-OX_1 and CHO-OX_2 cells were routinely grown as monolayers in MEM-Alpha medium supplemented with 10% foetal calf serum and 400 μg ml^{-1} G418, and maintained under 95%/5% O_2/CO_2 at 37°C. Cells were passaged every 3–4 days and the highest passage number used was 21.

Measurement of $[Ca^{2+}]_i$ using the FLIPR CHO-OX_1 or CHO-OX_2 cells were seeded into black walled clear-base 96-well plates (Costar UK) at a density of 20,000 cells per well in MEM-Alpha medium, supplemented as above and cultured overnight. The cells were then incubated with MEM-Alpha medium containing the cytoplasm calcium indicator, Fluo-3AM (4 μM; Teflabs, Austin, Texas) and 2.5 mM probenecid at 37°C for 60 min. The cells were washed

four times with, and finally resuspended in, Tyrode's medium containing 2.5 mM probenecid and 0.1% gelatine, before being incubated for 30 min at 37°C with either buffer alone (control) or buffer containing SB-334867-A (0.1 nM – 10 μM). The plates were then placed into a FLIPR (Molecular Devices, U.K.) to monitor fluorescence (λ_{ex} = 488 nm, λ_{EM} = 540 nm) (Smart *et al.*, 2000) before and after the addition of orexin-A or orexin-B (10 pM – 1 μM), or other peptides (100 pM – 10 μM).

Measurement of human OX_1 receptor binding CHO-OX_1 cells were seeded (17,000 cells per well) into 16-well chambers (Lab-Tek, Nalge Nunc International) and cultured overnight in MEM-Alpha medium. The cells were then incubated for 30 min at 37°C with 28 nM rhodamine green tagged orexin-A ($N^{6,10}$-RG-orexin-A) and different concentrations of competitor peptide in HEPES buffered saline containing 2.5 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 0.5% BSA. Cells were then washed in the same buffer without BSA and fixed with 4% paraformaldehyde. Prior to the fluorescence reading, cells were stained with 0.6 μM Syto 62 (Molecular Probes) for 10 min and 20°C and then analysed using a laser scanning cytometer (Compu Cyte). Cells were selected, based on their red fluorescence, by exciting the Syto probe with a 5 mW HeNe laser and collecting the emitted fluorescence with a 650 nm longpath filter. The green fluorescence from the selected cells was also measured by scanning the cells with a 20 mW Argon ion laser and collecting the emitted fluorescence with a 530 nm/30 filter.

Data analysis For the calcium studies, responses were measured as peak fluorescence intensity (FI) minus basal FI, and where appropriate were expressed as a percentage of a maximum orexin-A-induced response. Fir the binding studies, displacement of $N^{6,10}$-RG-orexin-A from OX_1 measured by monitoring green fluorescence maximal pixel intensity. Data are expressed as mean ± s.e.mean unless otherwise stated. Curve-fitting and parameter estimation were carried out using Graph Pad Prism 3.00 (GraphPad Software Inc., California, U.S.A.).

Materials Orexin-A and orexin-B were synthesized for SmithKline Beecham at California Peptides, CA, U.S.A. All other peptides were supplied by Bachem, U.K. SB-334867-A and $N^{6,10}$-RG-orexin-A were manufactured at SmithKline Beecham. All cell culture media were obtained from Life Technologies, Paisley, U.K.

Results Orexin-A and orexin-B caused a concentration-dependent increase in $[Ca^{2+}]_i$ in CHO-OX_1 cells (Figure 1) with pEC_{50} values of 8.38 ± 0.04 and 7.26 ± 0.05 respectively, (n = 12). Similarly, both peptides increased $[Ca^{2+}]_i$ in CHO-OX_2 cells (Figure 1), with pEC_{50} values of 8.20 ± 0.03 and 8.26 ± 0.04 respectively (n = 8). However, NPY, secretin, pPYY, [Leu^{31},Pro^{34}]hPYY, hPYY(3-36), NPY free acid, human pancreatic polypeptide and VIP were inactive at both receptors at all concentrations (100 pM – 10 μM) tested (Figure 1 and data not shown). In keeping with this, in the binding studies, orexin-A and orexin-B had pK_i values of 7.79 ± 0.04 and 6.93 ± 0.02 (n = 4) respectively at OX_1, whereas NPY and secretin displayed no specific binding to OX_1 receptors at concentrations up to 10 μM.

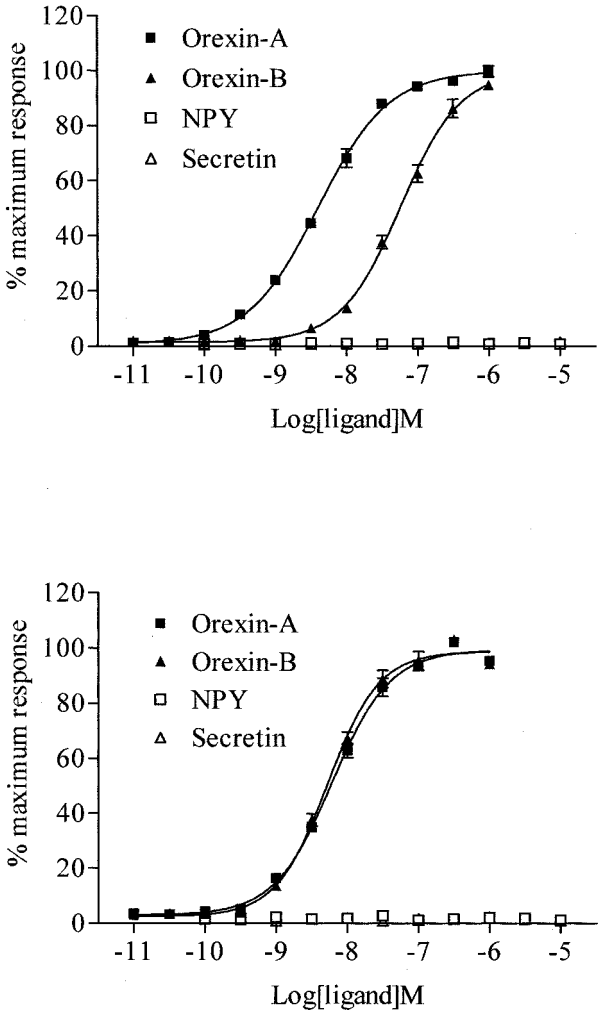

Figure 1 Orexins cause a concentration-dependent increase in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was monitored using Fluo-3AM in CHO cells stably expressing human OX_1 (upper panel or OX_2 receptors (lower panel) before and after addition of orexin-A (10 pM – 1 μM), orexin-B (10 pM – 1 μM), NPY B (100 pM – 10 μM), or secretin B (100 pM – 10 μM). Responses were measured as peak increase in fluorescence minus basal and are given as mean ± s.e.mean, where n = 12.

In CHO-OX_1 cells SB-334867-A (100 pM – 10 μM) inhibited the orexin-A (10 nM) and orexin-B (100 nM)-induced calcium responses in a concentration-dependent manner, with apparent pK_B values of 7.27 ± 0.04 and 7.23 ± 0.03 (n = 8), but had no effect on the calcium response elicited by UTP (3 μM), which activated an endogenous purinergic receptor (Figure 2). SB-334867-A also inhibited OX_2-mediated calcium responses, but with lower affinity, causing a 32.7 ± 1.9 and 22.0 ± 4.0% inhibition at 10 μM of the orexin-A (10 nM) and orexin-B (10 nM)-induced responses respectively. SB-334867-A was devoid of agonist properties in either cell-line (data not shown). SB-334867-A also displaced $N^{6,10}$-RG-orexin-A binding at human OX_1 receptors, with a pK_i of 7.17 ± 0.04 (n = 4). Neither NPY, secretin nor any of the related peptides antagonized the orexin-A (10 nM)-induced calcium responses in either CHO-OX_1 or CHO-OX_2 cells (data not shown).

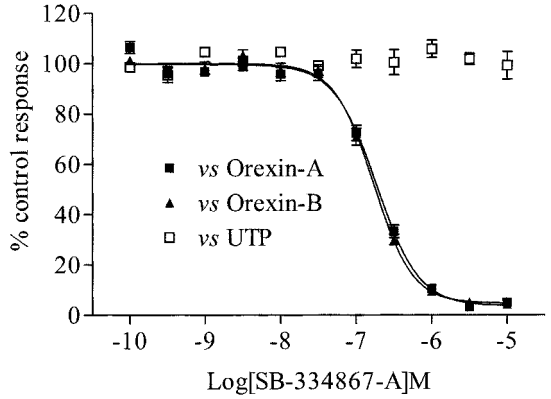

Figure 2 SB-334867-A inhibits human OX_1 receptor-mediated calcium responses in a concentration-dependent manner. CHO-OX_1 cells were preincubated with SB-334867-A (10 pM – 10 μM) for 30 min and then $[Ca^{2+}]_i$ was monitored using Fluo-3AM before and after addition of orexin-A (10 nM), orexin-B (100 nM) or UTP (3 μM). Responses were measured as peak increase in fluorescence minus basal and are given as mean ± s.e.mean, where $n = 8$.

Discussion The orexins are a recently discovered family of neuropeptides (Sakurai *et al*., 1998) which have been linked to a wide range of physiological functions (Smart, 1999), although the elucidation of the mechanisms involved has been hampered by the lack of selective orexin receptor antagonists (Jerman & Smart, 2001). We have demonstrated in a recombinant system that SB-334867-A is an OX_1-selective receptor antagonist. Furthermore, we have shown that NPY, secretin and related peptides were neither agonists nor antagonists at recombinant human OX_1 or OX_2 receptors.

In the present study orexin-A and orexin-B caused a concentration-dependent increase in $[Ca^{2+}]_i$ in CHO cells expressing either OX_1 or OX_2 with potencies similar to those reported previously (Smart *et al*., 1999). Orexin-A was equipotent at OX_1 and OX_2, whilst orexin-B displayed moderate selectivity for OX_2, consistent with the literature (Sakurai *et al*., 1998; Smart *et al*., 2000). Furthermore, the potencies at OX_1 were also in keeping with the affinities of orexin-A and orexin-B in the fluorescence-based binding assay.

It has previously been reported that NPY, secretin and related peptides displaced radiolabelled orexin-A binding with moderate to high affinity (Kane *et al*., 2000). However, in the present study, at equivalent concentrations, none of these peptides displayed any agonist-like activity, nor inhibited the orexin-A induced calcium response, in either CHO-OX_1 or CHO-OX_2 cells. Moreover, NPY and secretin, the two peptides with the highest affinity in the earlier study (Kane *et al*., 2000), did not display any affinity for OX_1 in the present binding studies. These discrepancies might be explained, at least in part, by the fact that the previous study (Kane *et al*., 2000) used porcine secretin to define the non-specific binding in crude hypothalamic membranes and thus the specificity of the assay may be questionable. Alternatively, recent evidence suggesting the possibility of a third orexin receptor which couples to adenylate cyclase rather than phospholipase C has been reported (Nanmoko *et al*., 2000). Therefore, it is possible that NPY and the other peptides were binding to this receptor in the Kane *et al*. (2000) study, although this putative receptor has only been proposed in PC12 cells to date (Nanmoko *et al*., 2000).

In the present study SB-334867-A was shown to bind to recombinant human OX_1 receptors with nanomolar affinity, and also to inhibit the OX_1-mediated calcium response at similar concentrations. SB-334867-A also inhibited the OX_2-mediated calcium response, but only at considerably higher concentrations. These inhibitory effects were specific for the orexin receptors as SB-334867-A had no effect on the calcium response elicited by the activation of a purinergic receptor endogenously expressed by CHO cells. Furthermore, SB-334867-A had no appreciable affinity for over 50 G-protein coupled receptors and ion channels in a CEREP screen (Porter, unpublished observations). As agonist-induced receptor desensitization also results in inhibition of orexin receptor-mediated calcium responses (Smart *et al*., 1999; 2000), it is important to note that SB-334867-A was devoid of agonist-like activity in both CHO-OX_1 and CHO-OX_2 cells. Taken collectively these data demonstrate that SB-334867-A is a selective OX_1 antagonist and thus may be a useful tool for studying the physiological tole of the orexins. Indeed, preliminary reports have recently described how SB-334867-A inhibited natural and orexin-A induced feeding in rats (Arch, 2000) and inhibited orexin-A induced arousal behaviours (Upton, 2000).

In conclusion, we have identified and characterized the first selective OX_1 receptor antagonist, SB-334867-A. Furthermore, we have demonstrated that NPY, secretin and related peptides do not interact with either recombinant human OX_1 or OX_2 receptors.

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