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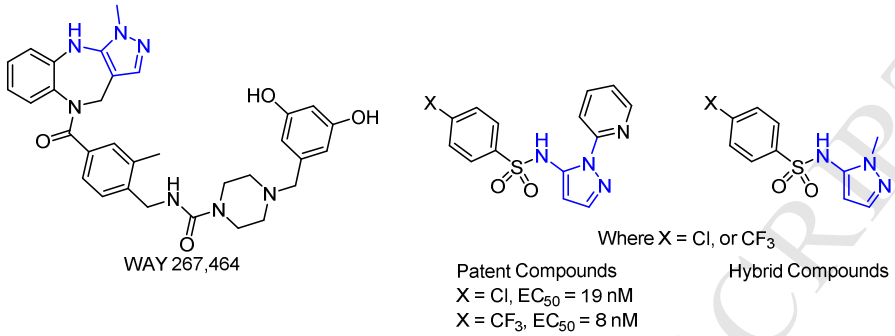
Graphical abstract



Investigation of Pyrazolo-Sulfonamides as Putative Small Molecule Oxytocin Receptor Agonists

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Abstract

The neuropeptide oxytocin has been implicated in multiple central nervous system functions in mammalian species. Increased levels have been reported to improve trust, alleviate symptoms related to autism and social phobias, and reduce social anxiety. Hoffman-La Roche published a patent claiming to have found potent small molecule oxytocin receptor agonists, smaller than the first non-peptide oxytocin agonist reported, WAY 267,464. We selected two of the more potent compounds from the patent and, in addition, created WAY 267,464 hybrid structures and determined their oxytocin and vasopressin receptor activity. Human embryonic kidney and Chinese hamster ovary cells were used for the expression of oxytocin or vasopressin 1a receptors and activity assessed via IP1 accumulation assays and calcium FLIPR assays. The results concluded that the reported compounds in the patent and the hybrid structures have no activity at the oxytocin or vasopressin 1a receptors.

Introduction

Oxytocin (OT) is a nine amino acid cyclic peptide with well-known neuromodulatory activity in complex social behaviour. Increased levels have been reported to improve trust, alleviate symptoms related to autism and social phobias, and reduce social anxiety.[1] Problems with the use of OT as a therapeutic are related to is peptidic nature, including low oral bioavailability, short half-life, and poor blood brain barrier penetration. In addition, it displays cross-talk with arginine vasopressin (AVP) receptors.[2]

Interest in the oxytocin receptor (OTR) as a potential therapeutic is reinforced by the recent patent by Hoffman-La-Roche.[3] This patent outlined a series of non-peptidic compounds based on a 5-sulfonamidopyrazole scaffold. The patent contained 26 compounds with heteroaromatic variations on the *N*1 position of the pyrazole along with various functionalized phenyl rings on the sulfonamide moiety. The exemplary drug like properties of the pyrazolo sulphonamides, coupled with their high OTR potency validates their therapeutic potential.

We chose to synthesize and explore the activity of two of the more potent molecules from this patent, **2a** and **2b** (Figure 1). EC_{50} values were reported to be 19 nM for **2a** and 8 nM for **2b** at the human oxytocin receptor (hOTR) as determined by Fluorometric Imaging Plate Reader (FLIPR) measurement of calcium influx. Such was the interest in this report that it was detailed in a patent highlight.[4] Considering the remarkable activity achieved for these compounds, and favorable physicochemical properties, we sought to investigate them further and compare them to observations made with the reported non-peptidic agonist, WAY 267,464 (**1**). After detailing improved methods of synthesis of WAY 267,464 [5-7] further studies showed that rather than being a selective OTR agonist, it was an AVP receptor antagonist, particularly at the vasopressin 1a receptor ($V_{1a}R$), (K_i of 27 nM and 230 nM for $V_{1a}R$ and OTR respectively)[8] and that this antagonism was predominately responsible for the observed *in vivo* effects.[9-11] Further structure-activity relationship and truncation studies have shown the importance of the rigid aromatic linker and the resorcinol moiety for selectivity.[8] Comparing compound **1** with sulfonamides **2** shows that in addition to the acyclic vs. cyclic structure, both contain the 5-aminopyrazole moiety that differ in *N*1-substitution, (pyridyl vs. methyl), and 5-amino substitution, (sulfonamide vs. benzyl). We aimed to synthesize lead compounds **2a-b** in addition to the hybrid structures **3a-b** and evaluate their activity at the hOTR and $hV_{1a}R$.

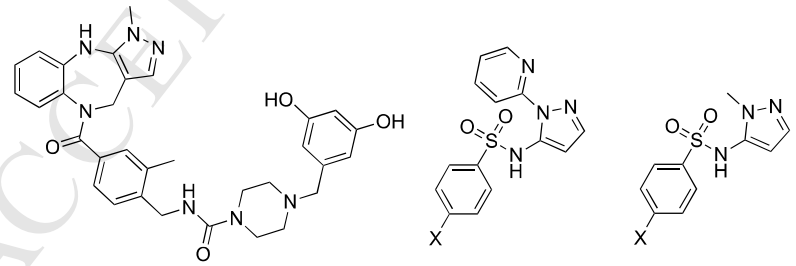


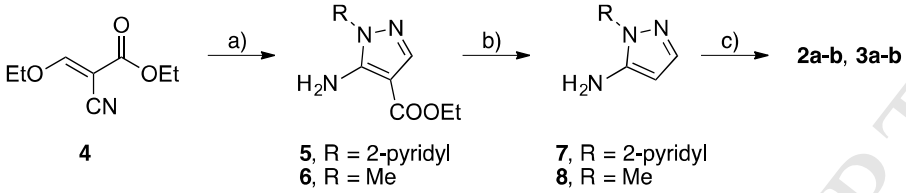
Figure 1. Lead compounds for OTR binding and targeted analogues.

Synthesis

The synthesis of target compounds **2a-b** and **3a-b** began with pyrazole formation between acrylate **4** and either 2-hydrazinopyridine or methylhydrazine to form compounds **5** and **6** respectively. Saponification and decarboxylation gave the 5-aminopyrazoles **7** and **8**.

Sulfonamide formation with the requisite sulfonyl chloride afforded lead compounds **2a-b** and their methyl analogues **3a-b** which were then subjected to biological testing.

Scheme 1: Synthesis of 2a-b and 3a-b[a]



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5, R = 2-pyridyl
6, R = Me

7, R = 2-pyridyl
8, R = Me

[a]Conditions: a) methylhydrazine (1.1 equiv.) or 2-hydrazinopyridine (1.1 equiv.), ethanol, reflux, 2 h, 68-73%; b) 1) 3 M aqueous NaOH (1.3 equiv.), ethanol, reflux, 2 h; 2) 185 °C, 85-87%; c) appropriate sulfonyl chloride (1.0 equiv.), pyridine, rt, 6 h, 23-34%.

Results and Discussion

We evaluated these compounds using HEK cells transfected with the hOTR for binding and, both agonism (Figure 2) and antagonism (Table 1). An inositol monophosphate (IP1) accumulation assay was used, which has been successfully employed previously for OTR functional studies.[8]

Competitive binding assays run on compounds **2a–b** and **3a–b** revealed a lack of binding, with an inhibition constant (K_i) of greater than 10 μM for all compounds at both the hOTR and the $hV_{1a}R$. The IP1 values obtained for compounds **2a–b** and **3a–b** at concentrations of 100 nM and 10 μM showed no difference to those obtained from the negative control (basal) (Figure 2). Due to the patent compounds being evaluated on CHO cells, we sought to verify that the HEK cells result in an accurate measurement of OT activity. It was found that OT effectively stimulated IP1 release in transfected cells in a manner that was able to be inhibited by the OTR antagonist atosiban (IC_{50}: 372 ± 97 nM), and OT did not elicit IP1 release in wild type HEK cells, suggesting its effect was specifically through action on the hOTR (Figure 2).

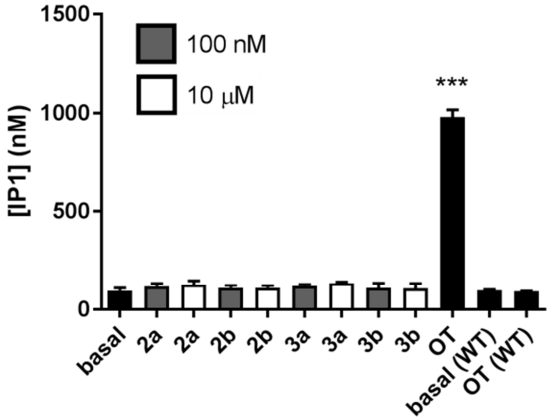

Figure 2. IP1 values obtained from treatment of HEK cells transfected with the hOTR with **2a–b**, **3a–b** (100 nM, 10 μM), vehicle (basal) and OT (10 nM (~EC_{50})), and values obtained from treatment of non-transfected HEK cells (WT) with vehicle and OT (10 nM). Values are mean ± standard error of the mean (S.E.M.) ***$p<0.001$

As these compounds did not stimulate IP1 release on their own, we investigated whether they could act as positive allosteric modulators by increasing the IP1 levels stimulated by an ~EC_{10} (3 nM) concentration of OT. They did not increase the response of the cells to OT, and in fact, at a high concentration (10 μM) a number of these compounds significantly decreased the response of OT (Figure 3). The concentration of OT used in Figure 2 is too low to allow conclusion about whether these compounds act as antagonists, so for this reason, we investigated whether they acted as antagonists by examining their effect on a concentration usually used in antagonist studies (EC_{70}). Data obtained for all compounds clearly showed no antagonism at the hOTR (Table 1).

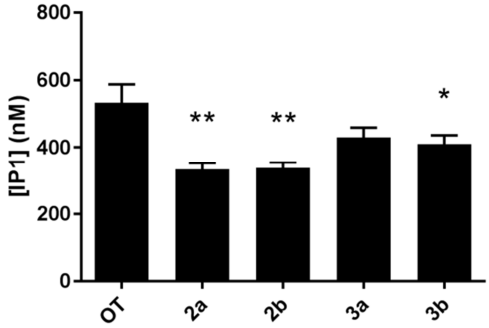

Figure 3. IP1 values obtained from treatment of HEK cells transfected with the hOTR with an ~EC_{10} concentration of OT (3 nM) with vehicle (0.1% DMSO) or **2a–b** (10 μM) and **3a–b** (10 μM). Values are mean ± S.E.M. *p<0.05, **p<0.01 compared to OT + vehicle.

Table 1. Antagonism in hOTR-transfected HEK cells

	2a	**2b**	**3a**	**3b**
Mean % at 100 nM compound[a]	89.5 ± 4.2	70.6 ± 6.2	82.5 ± 2.8	98.3 ± 3.3
Mean % at 10 μM compound[a]	78.1 ± 4.1	65.5 ± 1.5	72.5 ± 7.6	90.4 ± 2.7
pIC_{50}	<5	<5	<5	<5

[a] HEK cells transfected with the hOTR were exposed to an ~EC_{70} concentration of OT (30 nM) with compounds. Measured levels of IP1 were expressed as a percentage of the levels stimulated by 30 nM OT with a DMSO (0.1%) control. Experiments were repeated in triplicate with the mean values ± standard S.E.M;

We have previously shown that the reported hOTR agonist activity attributed to **1** was in fact antagonism of the $V_{1a}R$ [9-11] so we sought to investigate that possibility here. Additionally, we were concerned by the statement in the patent asserting, "The compounds…were also functionally tested on cell lines expressing the human vasopressin 1a and the human vasopressin 2 receptor to measure potential agonist activity and were found to be selective over the human oxytocin receptor." This statement seemed to be at odds with the title and theme of the patent, namely that the compounds were OTR agonists, however no clear description was provided as to which receptor, hOTR, $hV_{1a}R$, or hV2R, functional data provided was for. To clarify the position the same functional experiments were conducted at the $hV_{1a}R$ (Figure 4 and Table 2). Results clearly show that these compounds display no

agonism at the $hV_{1a}R$ (Figure 4). Results also conclusively showed no antagonism at the $hV_{1a}R$ (Table 2).

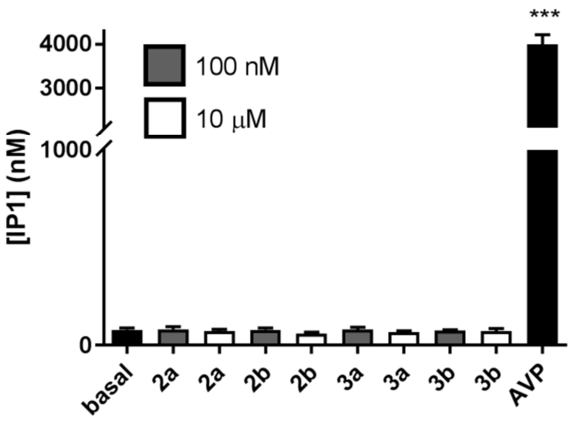

Figure 4. IP1 values obtained from treatment of HEK cells transfected with the $hV_{1a}R$ with **2a–b**, **3a–b** (100 nM, 10 μM), basal levels and AVP (10 nM). Values are mean ± S.E.M. ***p<0.001

Table 2. Antagonism in $hV_{1a}R$-transfected HEK cells

	2a	**2b**	**3a**	**3b**
Mean % at 100 nM compound[a]	85.9 ± 9.3	109.1 ± 4.9	112.0 ± 5.7	84.7 ± 1.2
Mean % at 10 μM compound[a]	80.2 ± 0.8	104.0 ± 1.9	105.2 ± 6.2	77.9 ± 2.8
pIC_{50}	<5	<5	<5	<5

[a] HEK cells transfected with the $hV_{1a}R$ were exposed to an ~EC_{70} concentration of AVP (25 nM) with compounds. Measured levels of IP1 were expressed as a percentage of the levels stimulated by 25 nM AVP with a DMSO (0.1%) control. Experiments were repeated in triplicate with the mean values ± S.E.M;

The discrepancy in our results and those reported by the patent for the same compounds were of concern and so we attempted to determine their source. The most notable difference in experimental procedure was the use of an IP1 assay as opposed to a Fluorometric Imaging Place Reader (FLIPR) Ca^{2+} flux assay. Therefore, we tested compounds **2a-b** and **3a-b** using the same assay. None of the compounds at 100 nM or 10 μM produced an increase in intracellular Ca^{2+} ($[Ca]_i$), in HEK cells expressing hOTR or $hV_{1a}R$. Pre-incubation of cells with **2a-b** and **3a-b** (10 μM) for 5 minutes did not significantly affect the potency of OT or AVP to elevate $[Ca]_i$, in cells expressing their cognate receptor, nor was the maximum effect of either OT or AVP changed (Table 3).

Table 3. Effects on $[Ca]_i$

	hOTR		$hV_{1a}R$	
	*p*EC50	Max	*p*EC50	Max
in-vehicle	9.9 ± 0.1	4.5 ± 0.1	9.8 ± 0.1	5.2 ± 0.2
2a	10.1 ± 0.1	4.5 ± 0.2	9.9 ± 0.1	5.3 ± 0.1
2b	10 ± 0.1	4.4 ± 0.2	9.8 ± 0.05	5.3 ± 0.1
3a	10.1 ± 0.1	4.0 ± 0.2	9.9 ± 0.1	5.0 ± 0.2
3b	10.0 ± 0.1	4.4 ± 0.2	9.8 ± 0.1	5.1 ± 0.2

HEK cells expressing hOTR were exposed to OT and cells expressing $hV_{1a}R$ were exposed to AVP after 5-minute pre-incubation with compounds (10 μM) or vehicle (0.1 % DMSO). Peak changes in Ca5 fluorescence, reflecting increases in $[Ca]_i$, were calculated as a fold-change compared to baseline, and a 4-parameter concentration response relationship fitted to the data to obtain a *p*EC50 in each condition. Max is a fold change in fluorescence over the basal measurement of calcium ions in the cell. Experiments were performed in duplicate with 5-6 independent replicates. Values represent the mean ± S.E.M.

A further point of difference between our methods and those used in the patent related to the cell line in which the OTR and $V_{1a}R$ were expressed. Our experiments utilized transfected HEK cells, while those in the patent were performed on transfected Chinese hamster ovary (CHO) cells. To eliminate this as a source of difference, we established stably transfected CHO cells expressing the hOTR. None of the compounds at 100 nM or 10 μM produced an increase in intracellular Ca^{2+} ($[Ca]_i$), and no significant agonist or antagonist activity at the OTR was seen in the IP1 assay performed on these cells (Figure 5, Table 4).

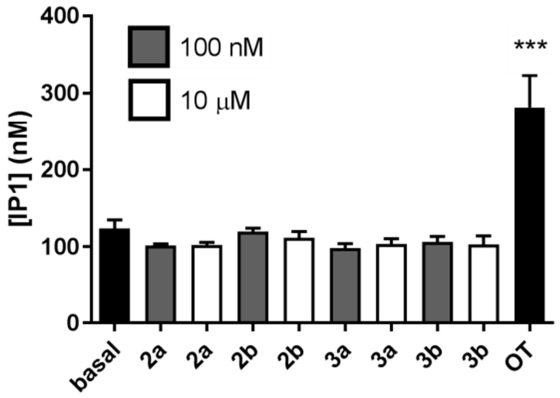

Figure 5. IP1 values obtained from treatment of CHO cells transfected with the hOTR with **2a–b**, **3a–b** (100 nM, 10 μM), vehicle (basal) and OT (10 nM). Values are mean ± S.E.M. ***p<0.001

Table 4. Antagonism in hOTR-transfected CHO cells

	2a	**2b**	**3a**	**3b**
Mean % at 100 nM compound[a]	83.9 ± 9.9	83.2 ± 6.4	90.5 ± 8.9	85.5 ± 4.0
Mean % at 10 μM compound[a]	71.1 ± 3.6	82.1 ± 4.2	94.7 ± 6.7	79.5 ± 6.4
pIC_{50}	<5	<5	<5	<5

[a] CHO cells transfected with the hOTR were exposed to an ~EC_{70} concentration of OT (30 nM) with compounds. Measured levels of IP1 were expressed as a percentage of the levels stimulated by 30 nM OT with a DMSO (0.1%) control. Experiments were repeated in triplicate with the mean values ± standard error of the mean (S.E.M).

To also examine the contribution of cell background to the differing reports of activity of these compounds at the V1aR, we established stably transfected CHO cells expressing the hV1aR. None of the compounds at 100 nM or 10 μM produced an increase in intracellular Ca^{2+} ($[Ca]_i$), and the compounds did not demonstrate agonist or antagonist activity at the hV1aR in the IP1 assay performed on these cells (Figure 6, Table 5). These results suggest that differences in cell line between the current study and the results reported in the patent literature are unlikely to be the cause of the loss of function at the hOTR and hV1aR in this study.

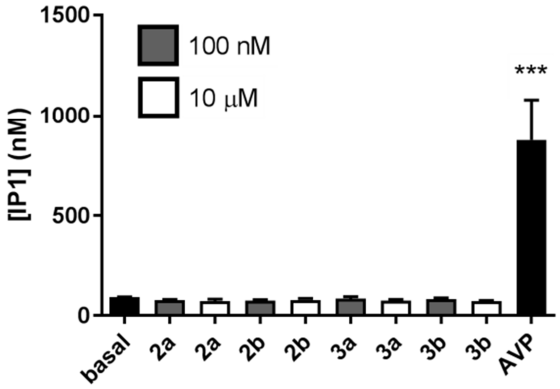

Figure 6. IP1 values obtained from treatment of CHO cells transfected with the hOTR with **2a–b**, **3a–b** (100 nM, 10 μM), vehicle (basal) and AVP (10 nM). Values are mean ± S.E.M. ***p<0.001

Table 5. Antagonism in hV1aR-transfected CHO cells

	2a	**2b**	**3a**	**3b**
Mean % at 100 nM compound[a]	103.2 ± 2.5	84.8 ± 4.3	100.1 ± 3.8	86.5 ± 1.6
Mean % at 10 μM compound[a]	116.1 ± 6.7	83.1 ± 4.0	105.2 ± 1.7	79.3 ± 0.7
pIC_{50}	<5	<5	<5	<5

[a] CHO cells transfected with the hV1aR were exposed to an ~EC_{70} concentration of AVP (25 nM) with compounds. Measured levels of IP1 were expressed as a percentage of the levels

stimulated by 30 nM OT with a DMSO (0.1%) control. Experiments were repeated in triplicate with the mean values ± standard error of the mean (S.E.M).

Conclusion

The results from this work have shown that the previously reported compounds (**2a** and **b**) and their corresponding hybrid structures (**3a** and **b**) have no notable activity at the hOTR. Furthermore, their activity at the $hV_{1a}R$ was assessed and analogous results were obtained. These results have been obtained in both CHO and HEK cells, and from both IP1 and FLIPR Ca^{2+} assays to determine functional activity. The FLIPR Ca^{2+} assays on CHO cells recapitulate the conditions in which the patent results were generated, whilst the results on HEK cells, and in the IP1 assay, acts to compliment the FLIPR assay to ensure activity of these compounds is accurately measured. Therefore, these compounds are not functionally active at the hOTR or hV1aR.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, ^{1}H and ^{13}C NMR spectra, HPLC purity analysis and additional biological data (PDF).

AUTHOR INFORMATION

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Highlights

- Patent compounds and new hybrids were synthesized
- Showed no binding or activity at the oxytocin receptor contradicting patent
- Further investigated in vasopressin which also lacked activity
- Concluded that these are not viable oxytocin agonists