An optimised method to quantify target engagement of Orexin-1 receptors and correlation with biophase free concentration of a selective OX1 receptor antagonist in the rat brain

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Introduction

A key part of a Drug Discovery strategy is to ensure that compounds identified in the screening cascade are able to engage the target under investigation at relevant blood concentrations. Receptor occupancy (RO) measurements, using methods such as *in vivo/ex vivo* autoradiography, have proved extremely useful in identifying novel CNS candidate compounds due to their high translational value when used to support clinical PET studies.

Orexins are neuromodulatory peptides involved in the control of diverse physiological functions through interaction with two receptors; Orexin-1 and Orexin-2 receptors (OX1 & OX2, respectively).

AIM

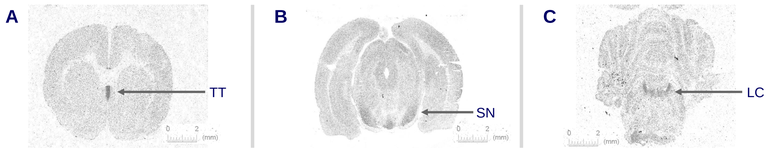
The first aim of this study was to set up the optimal experimental conditions for measuring the OX1 receptor occupancy using *ex vivo* binding to rat brain sections.

The final objective of the study was to measure OX1 RO following administration of 5 different doses (1, 3, 10, 30 and 60 mg/kg, i.p.) of the selective OX1 receptor antagonist GSK1059865 and to correlate RO with compound exposures in blood and brain.

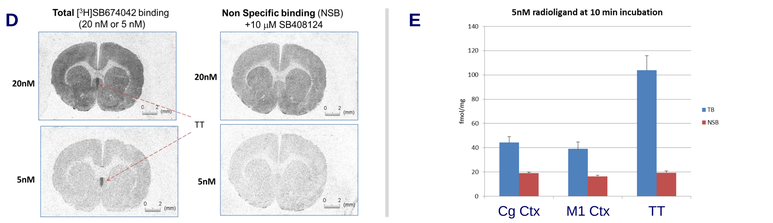
Time-course/RO: OX1 RO was also measured at 6 different time points (15min, 30min, 60min, 2hrs, 4hrs and 6hrs) following administration of a single dose (60 mg/kg, i.p.) of GSK1059865.

Results

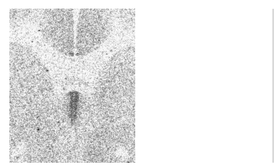
Selection of appropriate brain regions for RO studies



OX1 Receptor Distribution in rat brain sections: **a.** The highest levels of [^{3}H]SB674042 binding sites were found in the Tenia tecta (TT), located on the medial wall of the hemisphere anterior to the rostrum of the corpus callosum. Intermediate binding levels were observed in **b.** the Substantia nigra (SN) and **c.** the Locus coeruleus (LC).



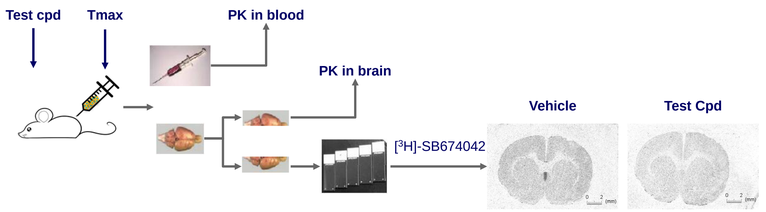
Selection of brain region and radioligand concentration: **d.** The brain region suggested for occupancy determination was Tenia tecta (TT) since it shows the highest levels of [^{3}H]SB674042 Specific Binding. Non-Specific Binding (NSB) determined in the presence of 10µM SB408124 was very low and homogeneously distributed. **e.** The radioligand concentration selected was 5nM [^{3}H]SB674042 which showed a better Specific Binding ratio (compared to 20nM).



Phospho-Imager and Beta-Imager platforms: **f.** Representative image of Tenia tecta taken with Phospho-imager (4 days exposure) or **g.** Beta-imager (12 hours exposure). Although Beta-imager requires significantly shorter exposure time, a better anatomical resolution is obtained with Phospho-imager.

Method Optimisation for *ex vivo* RO determination

The aim of these preliminary studies was to minimise the risk of compound dissociation, maintaining a reasonable amount of Specific Binding and a good signal to noise ratio. This was achieved by avoiding slice pre-incubation (generally performed to eliminate endogenous ligands) and by reducing the incubation time with radioligand to 10 min.

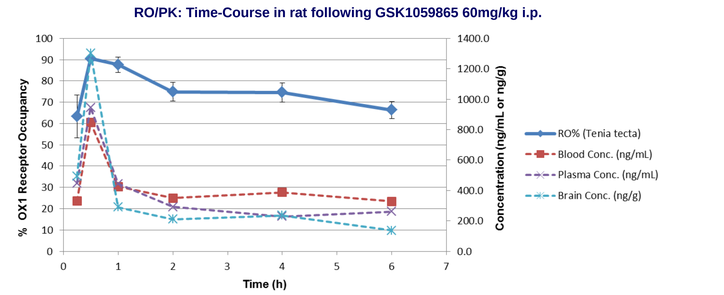


Methods: Immediately after euthanasia, the brain of treated rats was quickly removed from the skull and divided into two parts: one portion was used for determination of RO by means of *ex vivo* autoradiography and another portion was used for the determination of GSK1059865 brain exposures. The anterior brain segment was immediately frozen in pre-cooled 2-methylbutane at -30°C and cut into 14 µm thick coronal sections at the brain level corresponding to +1.60 mm from Bregma in stereotaxic coordinates (Paxinos and Watson, The Rat Brain Atlas, 1998).

The Occupancy *(O%)* of each GSK1059865-treated animal was calculated according to the following equation: $O(\%) = \left(1 - \frac{SB_T}{\overline{SB_V}}\right) \times 100$ in which SB_T is the Specific Binding (SB) in the animal treated with the test item and $\overline{SB_V}$ the mean SB of the animals treated with vehicle.

Time-course RO Study: Correlation between OX1 Receptor Occupancy and GSK1059865 (60mg/kg i.p.) exposures in Blood, Plasma and Brain

A



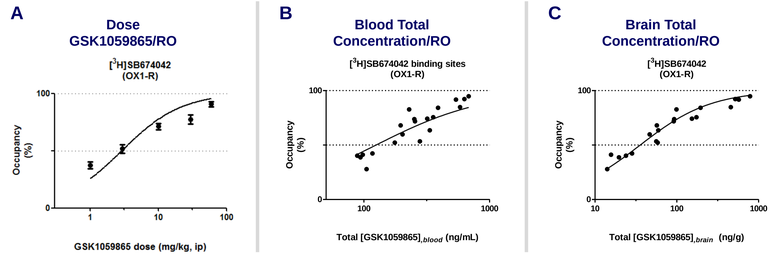
B

GSK1059865 Kinetics

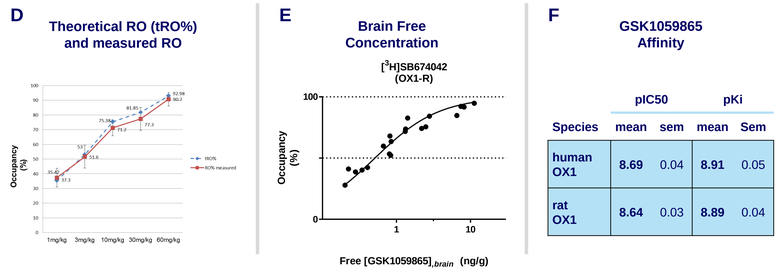
Species	K_{off} (min^{-1})	K_{on} x10^7 ($M^{-1}min^{-1}$)	t1/2 diss (min)	kinetic pK_D (n=2)	saturation pK_D (n=2)
human OX1	0.0967	11.285	7.2	9.05±0.2	8.94±0.04
rat OX1	0.0939	6.462	7.4	8.84±0	8.89±0.07

Time course RO study: **a.** Maximal RO levels (~90%) were observed at 30min and 60min following i.p. administration of GSK1059865 and relatively high levels were maintained for up to 6hrs (66.4%). RO/PK relationship between OX1 RO levels measured in Tenia tecta and compound exposures in blood, plasma and brain is shown. **b. Kinetics.** GSK1059865 showed relatively fast dissociation kinetics from OX1 receptors (T ½ dissociation time = 7.4 min).

Dose/Response Study: Correlation between OX1 Receptor Occupancy and GSK1059865 exposures in blood and brain (Total and Free)



Dose/Response RO Study: **a.** 60min after intraperitoneal (i.p.) administration, GSK1059865 dose-dependently increased OX1 RO in Tenia tecta with levels of RO (O% ± s.e.m.) of 37.3±3.2, 51.6±3.9, 71.2±2.7, 77.3±3.9 and 90.7±2.3 at the doses of 1, 3, 10, 30 and 60mg/kg i.p., respectively. Values of OC_{50} (Concentration giving 50% of maximal occupancy) were estimated in blood and brain.
b. In the blood estimated OC_{50} = 124.9 ± 12 ng/mL **c.** In the brain estimated OC_{50} = 35.72 ± 2.3 (best-fit value ± s.e.m.).



Species	pIC50 mean	pIC50 sem	pKi mean	pKi Sem
human OX1	**8.69**	0.04	**8.91**	0.05
rat OX1	**8.64**	0.03	**8.89**	0.04

Theoretical RO (tRO%) versus measured RO and Correlation between OX1 RO and Free Brain concentration: **d.** Values of RO obtained experimentally using this technique were in line with the theoretical RO values calculated on the basis of the compound exposures, affinity and the fraction unbound of the compound in the brain (fu=1.43%). **e.** Data fitting of individual occupancy values and Free GSK1059865 concentration in the brain showed a very good correlation between GSK1059865 concentration and OX1 RO in the Tenia tecta. Free Brain OC_{50} = 0.51 ± 0.033 ng/g (best-fit value ± s.e.m.) estimated compound affinity with pKi = 8.93. **f.** Compound affinity calculated from *ex vivo* RO and free OC_{50} value in the brain (pKi=8.93) was in very good agreement with the affinity value from *in vitro* studies (rat binding pKi=8.89), confirming the sensitivity of the assay.

Conclusions

- This study confirms that the OX1 RO assay described provides a robust assay for use in Drug Discovery projects to identify novel OX1-R antagonists targeting the Central Nervous System
- The data indicates that *ex vivo* autoradiography using rat brain sections can be used successfully (using optimized protocols) for OX1 RO studies, since values of RO obtained experimentally were similar to the theoretical RO in the same brain
- Dose/RO: GSK1059865 dose-dependently occupied OX1-R 60min following i.p. administration with RO levels reaching maximal RO (>90%) at 60 mg/kg
- A good PK/RO relationship between measured OX1 RO levels and compound exposures in blood and brain (Total and Free) was observed in the same animals
- Compound affinity calculated from *ex vivo* RO and free OC_{50} value in the brain (pKi=8.93) was in very good agreement with the affinity value from *in vitro* studies (rat binding pKi=8.89), confirming the sensitivity of the assay
- Time-course: Maximal RO levels (>90%) were observed at 30min/60min and relatively high levels were maintained for up to 6hrs (66.4%)
- Quantitative target engagement studies can be used to validate truly translational technologies (such as PET and pharmaco-EEG) and facilitate bridging of the preclinical/clinical interface

Methods

- Pre-incubation step (to eliminate endogenous ligands) can be skipped
- Incubation time with [^{3}H]SB674042 (60 min *at equilibrium*, RT) can be abbreviated to 10 min maintaining good Specific Binding levels
- Buffer selected: Hepes 25 mM, $CaCl_2$ 1mM, $MgCl_2$ 5mM, pH=7.4
- The radioligand concentration selected was 5nM [^{3}H]SB674042, showing a better Specific Binding ratio (compared to 20nM)
- Washing time 2 x 1min in experimental buffer at 4°C
- 4 days exposure to Phospho-imaging plates

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