Supplemental Methods

Selectivity Profiling

The selectivity of TRV130 was profiled against a panel of GPCR, ion channel and enzyme targets using Binding, Kinase, Non-kinase Enzyme and Cell-based Assays (Cerep, Potiers, France)The following receptors and ion channels represent a subset investigated : ***(human)*** Adenosine A_1, A_{2A} A_{2B} and A_3, Adrenergic α_{1A}, α_{1B}, α_{2A}, α_{2B}, α_{2C}, β_1 β_2 and β_3, Angiotensin-II AT_1, AT_2, Bradykinin B_2, Cannabinoid CB_1 and CB_2, Chemokines CXCR2, CXCR4 Cholecystokinin CCK_1 (CCK_A) and CCK_2 (CCK_B), Corticotropin Releasing Factor (CRF_1), Dopamine D_1, and D_{2Short}, D3, Endothelin ET_A and ET_B, GABA A_1 and B ($_{1b}$), Glucagon, Glycine (strychnine insensitive, Histamine H_1 and H_2, H_3 and H_4, kainite, Melanocortin MC_4, Melatonin MT_1 (ML_{1A}), Muscarinic M_1, M_2 and M_3, Neurokinin NK_2 and NK_3, Neuropeptide Y_1 and Y_2, Neurotensin NTS_1 (NT_1), NMDA, Opioid δ_2DOP), Opioid μ(MOP), Opioid NOP (ORL1), Prostanoid TP (TXA2/PGH2), Serotonin 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B} , 5-HT_3 , 5-HT_{5a} , 5-HT_6 and 5-HT_7, TNFα, Vasoactive intestinal peptide $VPAC_1$ (VIP_1) and V_{1a}, Norepinephrine transporter, Dopamine transporter, Serotonin 5-HT transporter, APJ (apelin), Angiotensin-converting enzyme ACE; ***(rat)*** , AMP A, Benzodiazepine BZD, Serotonin 5-HT_{1B}, Opioid κ (KOP), Ca^{2+} channel (L verapamil site), K+ channels K_V and SK_{Ca} Na^+ channel (site 2), Cl^- channel (GABA-gated); ***(murine)*** Somatostatin sst (non-selective). In addition, interactions at 29 enzyme (including kinases) targets were evaluated for activity.

Antagonist Study in the Mouse 56°C Hot-Plate Test

The opioid antagonist naloxone, was used to probe the receptor pharmacology mediating the antinociceptive effect ofTRV130 in the mouse 56°C hot-plate test. Morphine-induced antinociception was studied in parallel. The antinociceptive agents were used at doses near their 50% effect level, x mg/kg for morphine and x mg/kg for TRV130, both administered s.c.. The antagonists were administered at doses and times determined in preliminary studies to be appropriate: naloxone, X mg/kg s.c., 20 min prior to TRV130. Antinociception was assessed 30 min after the administration of morphine or TRV130.

Mouse gastrointestinal motility (GIT) studies

Mice were acclimated to the vivarium for at least 48 hours prior to behavioral testing. Animals were allowed access to food and water ad libitum until the night prior to the experiment, when the animal's food intake was withheld. It is necessary to fast the animals so that the GI tract is empty and an accurate measurement of charcoal meal transit can be measured. Animals were allowed free access to water the night before the experiment. On the day of the experiment, animals were weighed and identified with a unique number (permanent marker) on the tail. A total of 117 mice were used in these experiments (n = 6-11/group). Animals were administered vehicle, morphine, or TRV130 subcutaneously 10 minutes before they received a charcoal meal, which consisted of charcoal, flour, and water (1:2:6, w:w:v). A volume of 0.3 ml of the charcoal meal was administered orally. Twenty-five minutes after the charcoal meal was administered, the animals were euthanized by CO2 asphyxiation. After the animal was euthanized, the small intestine was removed from the gastro-duodenal junction to the cecum. The length of the intestine and the distance travelled by the charcoal meal was measured[1].

Data Analysis – GIT studies

The distance that the charcoal travelled in the intestine was determined for each animal and expressed in centimeters. Data was analyzed according to the following formula: %GIT = (distance the charcoal travelled / total length of the small intestine) x 100. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA).

Radioligand binding membrane preparation

HEK-293 cells with stable expression of the human MOR were harvested by centrifugation at 400xg for 30min at 4°C, washed once with a balanced salt solution, re-pelleted, and the pellet flash frozen in liquid nitrogen. The cell pellets were stored at -80°C until processed for membranes. Pellets were resuspended in buffer (50 mM HEPES, 2 mM EDTA pH 7.4 containing fresh protease inhibitors - Complete Brand protease tablets from Roche Diagnostics (Indianapolis, IN) and subjected to nitrogen cavitation with a Parr Cell Disruption Bomb (Parr Instrument Co., Moline, IL) at 1000 psi for 20 min on ice. Ruptured cells were sedimented at 500g for 10 min at 4°C and the supernatant containing cellular membranes was washed twice at 48,000g for 15 min. cell pellets were re-suspended at 4°C in 10 volumes of ice-cold buffer A and cavitation, placed on ice. To remove large particles, a low speed centrifugation (500xg for 30 min at 4°C) was performed, followed by high-speed centrifugation (48,000xg for 45 min at 4°C), resuspension in buffer plus protease inhibitor cocktail, and a final high speed centrifugation at (48,000g for 45 min at 4°C). A dounce homogenizer was used to resuspend the final pellet using ice-cold buffer. The membrane suspension was passed through a 23G needle, aliquoted, and stored at -80°C. Total protein concentration of the membrane preparation was determined with a Coomassie Plus Reagent Kit from Pierce Biotechnology (Rockford, IL) using bovine serum albumin as the standard.

Data Analysis – Radioligand binding

Apparent binding affinities, K_i = IC_{50}/ (1+ [Ligand]/K_d) were performed using the nonlinear iterative curve-fitting computer program GraphPad PRISM (GraphPad Software San Diego, CA). The association and dissociation rate constants of unlabeled ligands were determined using a previously described methods [2-3] in which association of a radiolabeled ligand is measured in the presence of a fixed concentration(s) of unlabeled test ligand. The model assumes that the ligands bind in a competitive manner according to simple bimolecular reactions.

References

1. Raehal, K.M., J.K.L. Walker, and L.M. Bohn, *Morphine Side Effects in β-Arrestin 2 Knockout Mice.* Journal of Pharmacology and Experimental Therapeutics, 2005. **314**(3): p. 1195-1201.
2. Motulsky, H.J. and L.C. Mahan, *The kinetics of competitive radioligand binding predicted by the law of mass action.* Molecular Pharmacology, 1984. **25**(1): p. 1-9.
3. Hoare, S.R.J. and T.B. Usdin, *Tuberoinfundibular Peptide (7-39) [TIP(7-39)], a Novel, Selective, High-Affinity Antagonist for the Parathyroid Hormone-1 Receptor with No Detectable Agonist Activity.* Journal of Pharmacology and Experimental Therapeutics, 2000. **295**(2): p. 761-770.