**Supplemental Digital Content 5**

**Measurement of the HepG2 cells ATPase activity**

For these experiments, the cells were plated and grown in 25 cm2 plates (20 flasks) and treated with fentanyl for 1 hour (2 ng/ml) (n=10 each: 10 controls and 10 treated cells). Afterwards, cells were washed once with TBS and lysed directly in 25 cm2 plates with ice cold cell lysis buffer (0.1% Titon X-100, 10% Glycerol and 50 mM HEPES pH 7.4; ) containing a cocktail of protease inhibitors (Sigma-Aldrich, Buchs, Switzerland) and incubated on ice for 20 min. Lysates were centrifuged at 10,000 g for 15 min and the protein concentration in the supernatants (whole cell extracts) was determined with the Quant-iTM assay kit and read with the Qubit-TM fluorometer (Invitrogen®, Basel, Switzerland). To eliminate free phosphate (Pi) from samples, whole cell extracts and buffers were treated with PiBind™ resin (Innova Biosciences, Cambridge, UK) according to the manufacturer’s instructions. The PiBind™ resin provides a quick way to remove contaminating Pi from samples. Afterwards the release of Pi (from whole cell extracts) was measured in the direction of ATP hydrolysis using a high-sensitivity colorimetric ATPase Assay kit (Innova Biosciences, Cambridge, UK) as per manufacturer's recommendations. The kit employs a 96-well plate format with a PiColorLock™ Gold reagent (an improved malachite green formulation). Briefly, 100 µl of diluted whole cell extracts were incubated with 100 µl substrate/buffer mix (0.5 M Tris pH 7.5, 0.1 M MgCl2, 10 mM specially purified ATP) in a 96 well plate for 30 min at 30°C. Afterwards 50 µl of Gold mix (by adding Accelerator to PiColorLockTM Gold) was added to the samples to stop the reactions, and 2 min later 20 µl of stabilizer was added. After 30 min, the absorbance was measured at 650 nm using a VERSAmax microplate reader (Molecular Devices, Orleans Drive Sunnyvale, CA, USA). Enzymatic activities (the amount of Pi released) of the samples were calculated using the Pi standards supplied by the company. The blanks were assayed under the same conditions, except stop mix was added to samples before the substrate solution. The activities of the samples were expressed as µmol Pi/min/mg cellular protein at 30°C.

**Measurement of cell-free in vitro fentanyl-stimulated permeability glycoprotein (Pgp) ATPase activity**

Cell-free in vitro fentanyl-stimulated Pgp ATPase activity was measured using Pgp-Glo™ Assay System (Promega, Madison, USA) according to the manufacturer’s instructions. The kit contains all the reagents required to run the assay: Pgp reaction buffer, MgATP, Verapamil, Na3VO4, a lyophilized ATP detection reagent and its reconstitution buffer and recombinant human Pgp membranes. The assay is based on the ATP dependence of the light-generating reaction of firefly luciferase. Briefly, 25 µg of recombinant human Pgp membrane fractions were incubated in a 96-well plate with fentanyl at 2, 10 and 40 ng/ml (n=4 each, according to the manufacturer’s instructions). Sodium orthovanadate (Na3VO4)-treated control samples (0.25 mM) (n=4) and verapamil-treated samples (positive control) (0.5 mM) (n=4) were included in addition to untreated control samples. Na3VO4 is a selective inhibitor of Pgp, and samples treated with Na3VO4 have no Pgp ATPase activity. In the absence of Na3VO4, basal and drug-stimulated Pgp ATPase activities could be detected. ATP consumption in the presence of Na3VO4 is attributed to minor non-Pgp ATPase activities present in the membrane preparation. Verapamil is a substrate for transport by Pgp that stimulates Pgp ATPase activity. The samples were incubated in the Pgp reaction buffer with a non-limiting concentration of ATP (5mM) for 40 minutes at 37°C. Afterwards, ATP standards (3.0, 1.5, 0.75 and 0.375 mM) were added to the plate and luminescence was read on a plate-reading luminometer (Tecan Infinite M1000 plate reader, Tecan, Männedorf, Switzerland). The ATP concentrations of the Pgp reactions were determined by comparing relative light units (RLU) values from samples to ATP standards. Stimulation of Pgp ATPase activity would induce a reduction in ATP levels. The Pgp ATPase was calculated activity as a specific activity (i.e., in terms of ATP consumed):

Basal recombinant human Pgp activity:

([ATP Na3VO4] – [ATP Untreated sample]) ÷ (25 μg human Pgp × 40 min) = nmol ATP consumed/μg Pgp/min

Test compound (Fentanyl- or Verapamil [positive control]-stimulated) recombinant human Pgp activity:

([ATP Na3VO4] – [ATP Test compound]) ÷ (25μg human Pgp × 40 min) = nmol ATP consumed/μg Pgp/min

Fold stimulation by a test compound:

Test compound stimulated activity ÷ Basal activity