

Functional assessment of endogenous NK₁ receptors using the CellKey™

Schizophrenia and Cognitive Disorders DPU

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Content

- Overview of the CellKey™ system
- Functional assessment of neurokinin 1 (NK₁) receptors endogenously expressed in U373-MG cells using the CellKey™
- CellKey™ utilization in primary cells
- Summary and conclusions

Label-free detection: interrogating native signalling

- Cellular label-free detection is a powerful technology for probing native cellular systems:
 - Changes in cell 'phenotypes' monitored via biosensors
 - Overall response
- Methodology is non-invasive, measures responses in living cells
 - Recombinantly and endogenously-expressed targets
- Readout is cumulative and independent of signalling pathways
 - Sensitive to distinct events e.g. functional selectivity elicited by a ligand
 - Sensitive to multiple events e.g. pleiotropic actions elicited by a ligand
 - Sensitive to alternative events not easily monitored e.g. $G\alpha_{12/13}$ signalling
- Response is resolved temporally and is mechanistically informative
 - Screening and detailed MoA studies possible

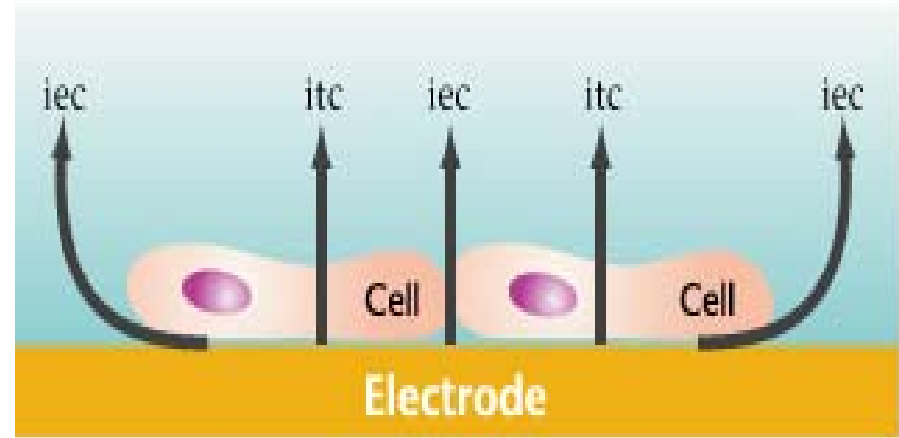
CellKey™ system: a cell-based assay platform (1)

- **Label-free technology:** measures impedance ($Z = V/I$, Ohm's law) using Cellular Dielectric Spectroscopy.
- **Real time, kinetic measurements** – two or ten seconds update rate.
- **Thermal control** – RT to 37°C.
- **Automated liquid handling** – simultaneous compound addition and read.
- **Software** package for data analysis of complex changes in impedance (dZ).



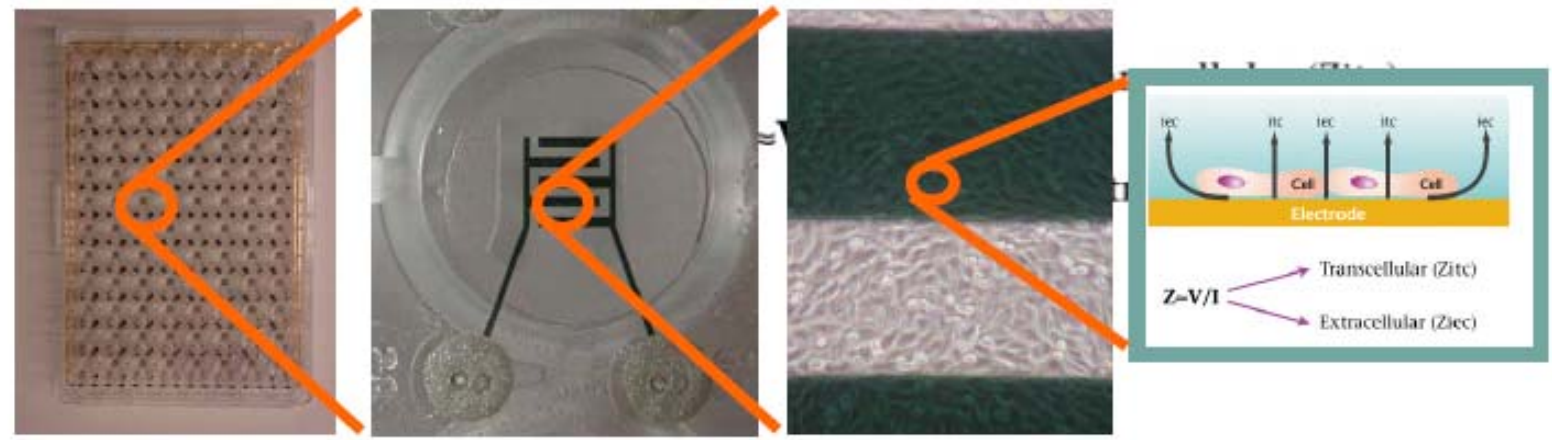
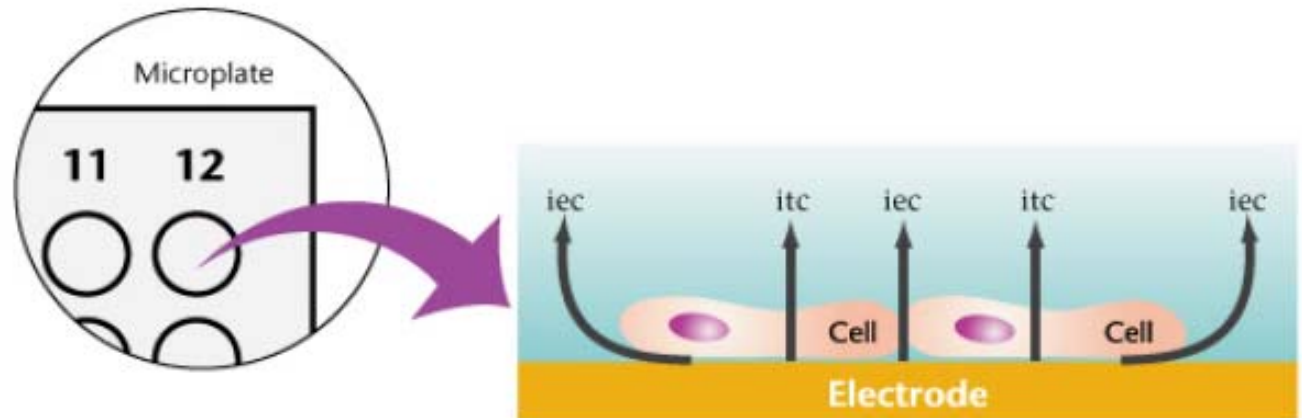
CellKey™ system: a cell-based assay platform (2)

- **Custom plates** contain electrodes in each well. The instrument applies voltages at different frequencies, inducing extra-cellular currents at low frequencies (iec) and trans-cellular currents at high frequencies (itc).
- **Universal platform:** Changes in impedance (dZ) represent a common endpoint measurement for G-protein coupled receptors, tyrosine kinase receptors and nuclear receptors.
- Can be used to measure **endogenous receptor** activity reliably.



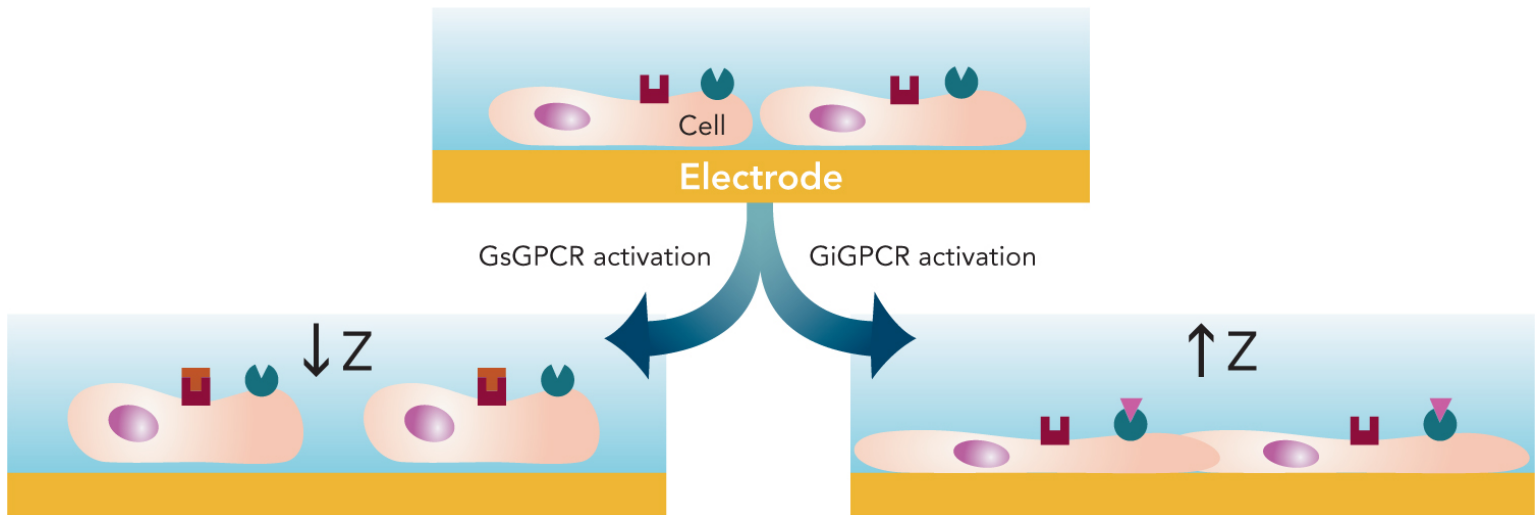
CellKey™ system: a cell-based assay platform (3)

CellKey™ system measures impedance (Z)

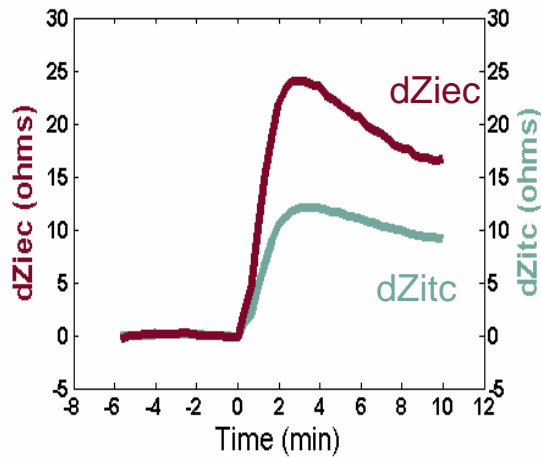
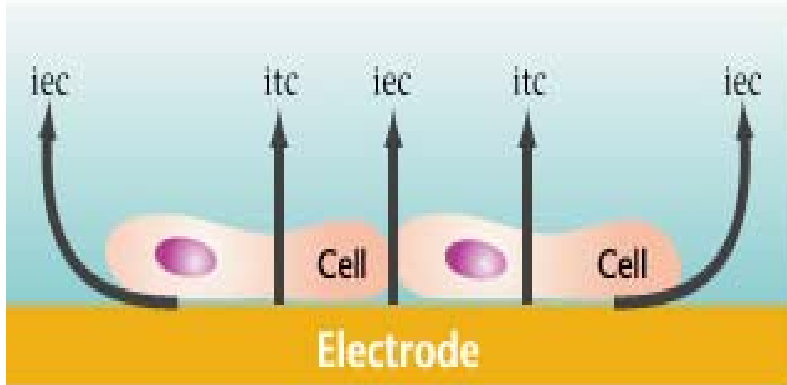


CellKey™ system measures impedance (Z)

- Contributors to changes in impedance are changes in cytoskeletal organization induced following receptor activation:
 - Cell morphology (volume, shape)
 - Cell adhesion to electrode
 - Cell-cell interaction
- These changes affect Z_{itc} and Z_{iec}



Assay data is quantitative and qualitative



Quantitative pharmacological analysis

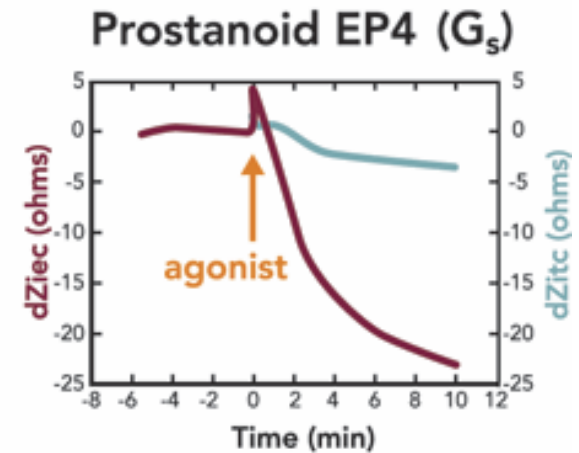
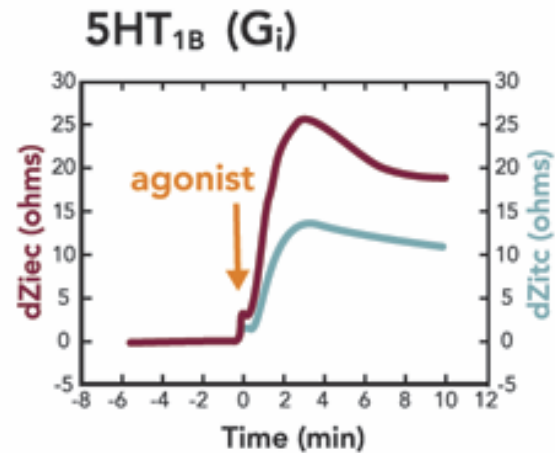
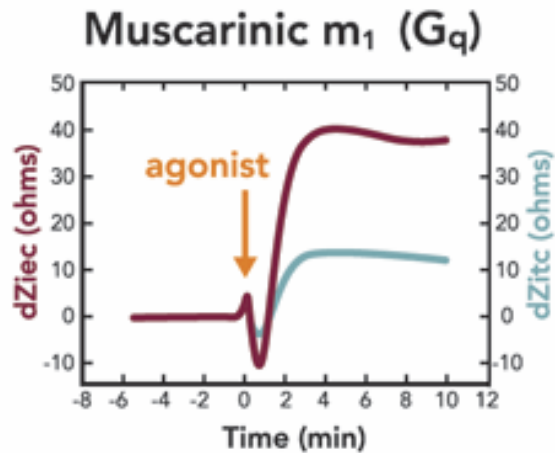
- Partial and inverse agonism
- Antagonists and modulators
- Schild analysis/Operational model

Qualitative analysis

- CellKey™ response profile is indicative of the signalling pathway being activated
- Insight into MoA

The CellKey™ system generates characteristic response profiles of G-protein signalling

- Activation of GPCRs leads to cytoskeletal changes measured by the CellKey™
- Signature is system-dependent (requires validation)



*CHO-M₁ cells

Potential applications of the CellKey™ system

- **Hit identification;**
- **Receptor panning:** identification of functional endogenous receptors expressed in cell lines commonly used in drug discovery;
- **Pharmacological characterization** of molecules (MoA, potency, intrinsic activity);
- **Signal transduction identification** and **Exploration of signalling pathway** through qualitative analysis of kinetic traces.

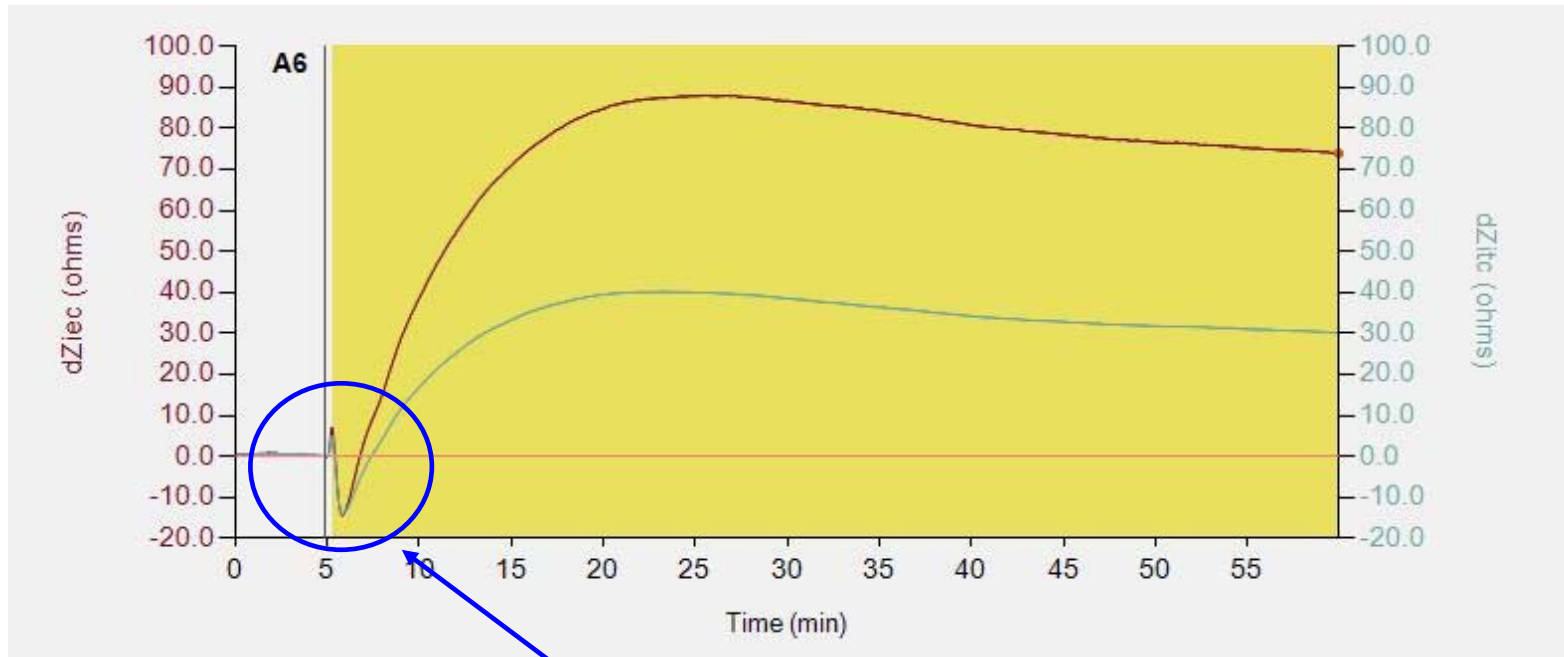
NK₁ receptor antagonism – U373-MG

- Neurokinin (NK) 1 receptors couple through a G_{q/11} α subunit to stimulate inositol phosphate (IP) production and raise intracellular calcium.
- The need for the functional characterization of compounds in a more native-like system arose when discrepancies were observed in some NK₁R antagonist potencies when tested in two different recombinant systems (HEK293-hNK₁ vs CHO-hNK₁ in [³H]-inositol phosphate accumulation assays).
- Some human astrocytoma cell lines express endogenous hNK₁ receptors, i.e. U373-MG (*Torrens et al., 2000*).
- U373-MG gives small, unworkable IP response; small though workable changes in intracellular calcium using FLIPR, which, however, is a non-equilibrium assay (see non-surmountable profile for slowly dissociating competitive antagonists)



U373-MG response to 1 nM substance P using the CellKey™

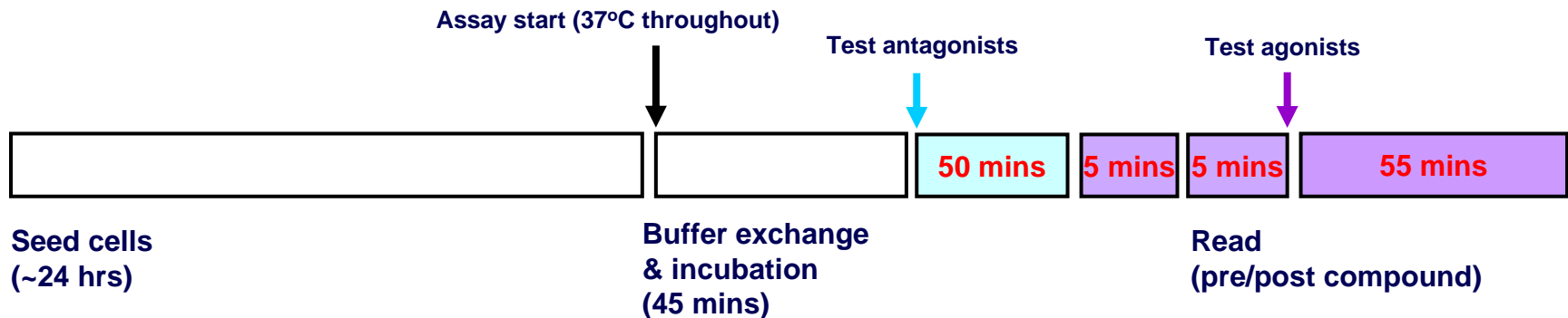
- Sensitivity of the CellKey™ allows monitoring of endogenous receptor activation;
- Measurements can be taken for up to 1 h allowing time for equilibration of the system.



SP-elicited response is G_q-like

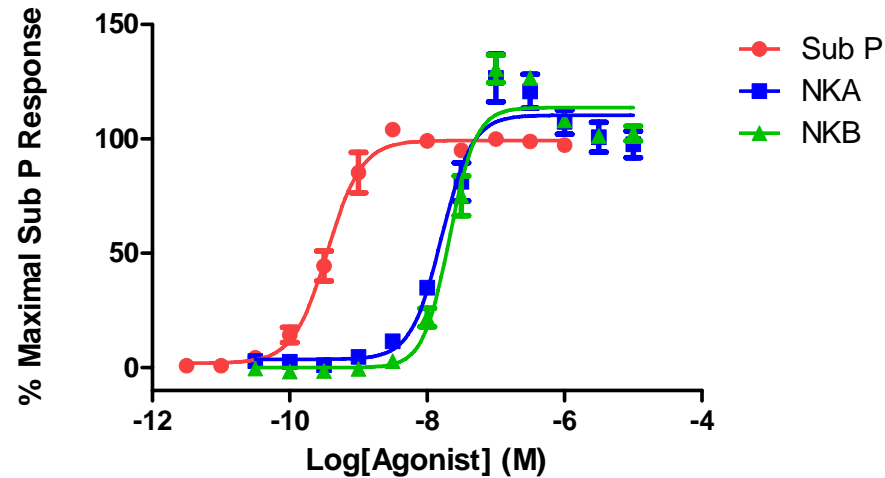
Optimised assay conditions

- Cell density at time of plating: 60K/well
- Assay temperature: 37 °C
- Tolerance to DMSO: 0.1 % 
- Pre-incubation with test antagonists for 60 minutes (where applicable)
- Responses monitored in real time following ligand addition



Neurokinin characterization in the U373-MG using the CellKey™

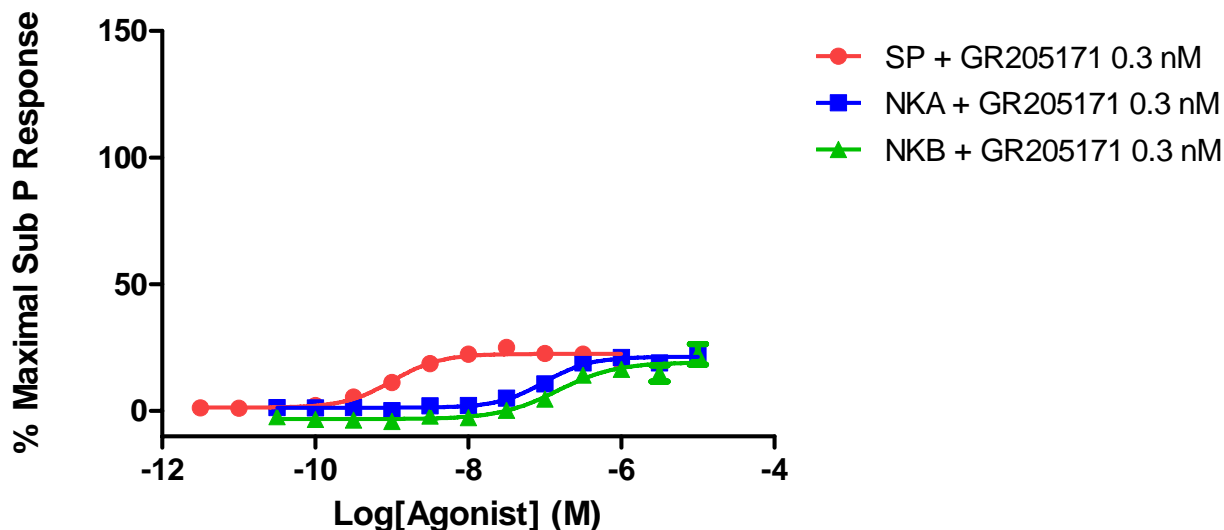
Extracellular impedance changes after 55 min incubation with substance P, or neurokinin A or B



	SP	NK _A	NK _B
pEC ₅₀ Mean ± SEM (n = 3)	9.4 ± 0.14	7.7 ± 0.07	7.6 ± 0.11

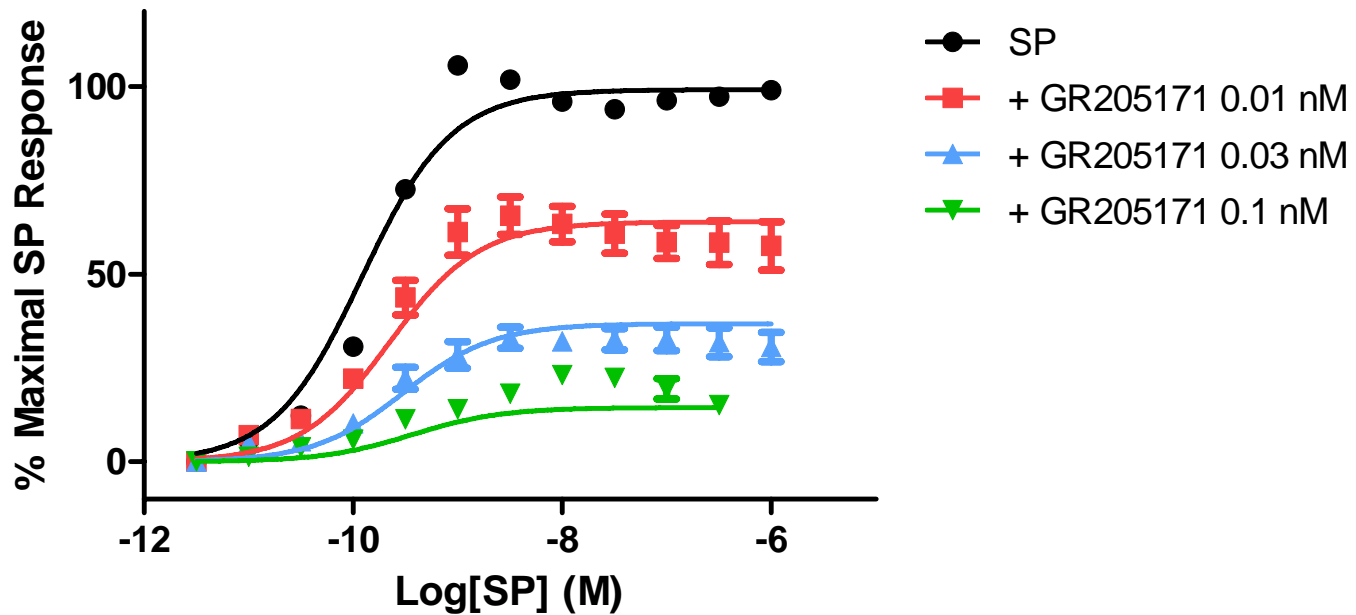
Effect of GR205171, a selective NK₁R antagonist

- GR205171 is a selective and potent NK₁ receptor antagonist (*Gardner et al., 1996*)
- Pre-incubation with GR205171 0.3 nM for 60 minutes inhibited the agonist-evoked response in a similar manner for the three neuropeptides, indicating that the response is NK₁ receptor-mediated.



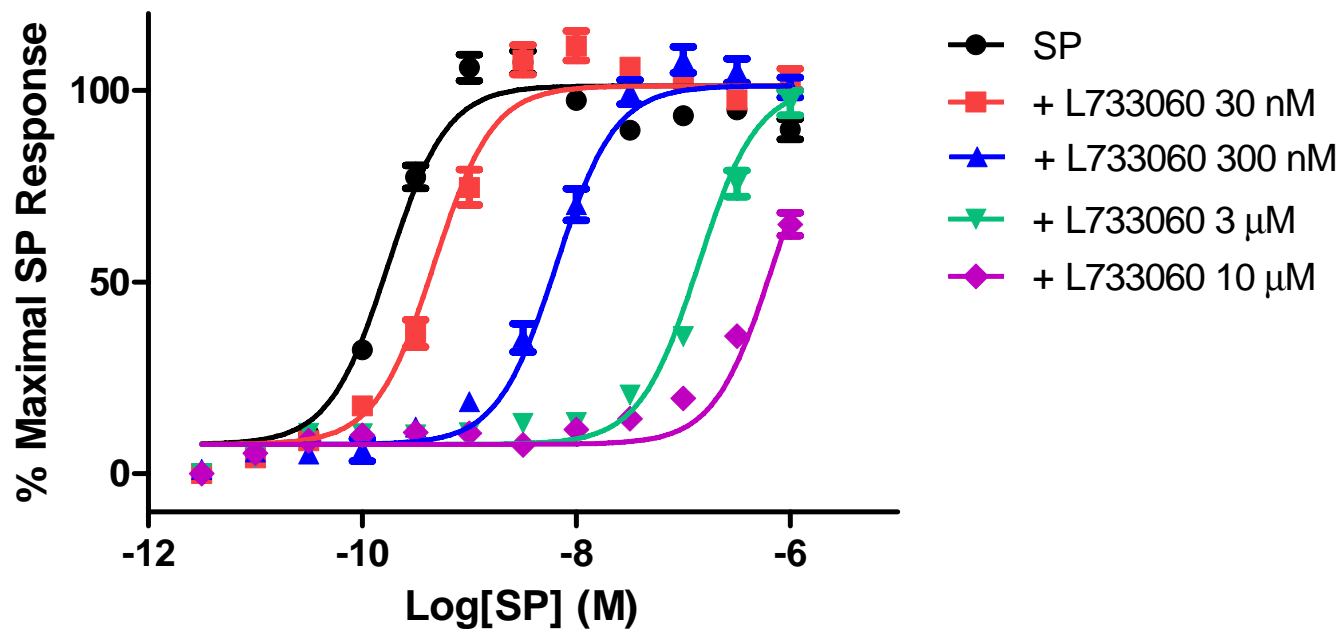
GR205171 behaves as an insurmountable NK₁R antagonist

Impedance changes at 55 min
Operational model for non-competitive antagonism



L-733060 behaves as a surmountable NK₁R antagonist

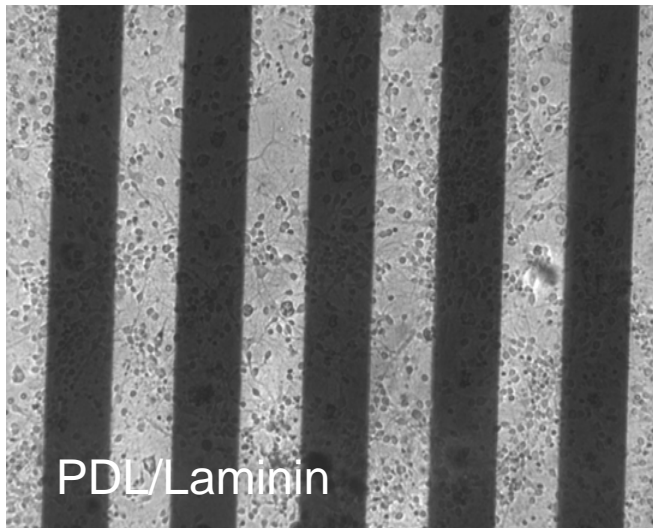
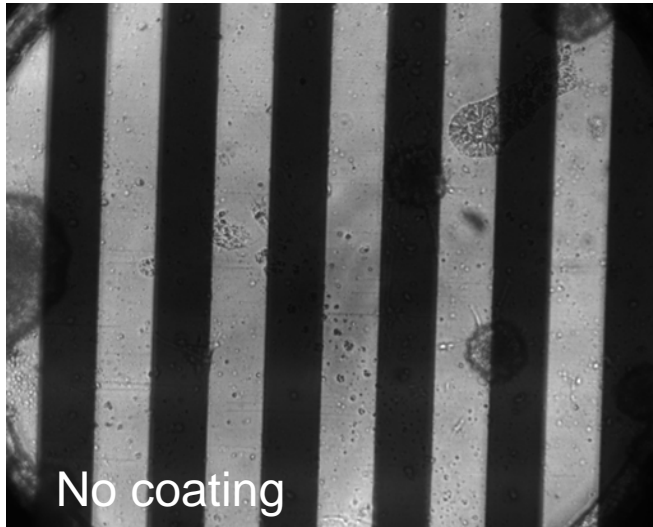
Impedance changes at 55 min
Gaddum/Schild EC₅₀ shift equation



Conclusions

- The CellKey™ system enabled the measurement of a robust and reliable endogenous NK₁ receptor-mediated response in the U373-MG cell line;
- The response profile indicated the activation of a G_{q/11} α subunit, consistent with neurokinin-mediated increase in IPs previously reported in this cell line (*Torrens et al., 2000; Lee et al., 1992*);
- The rank order of potency of endogenous neurokinins at hNK₁ receptors found using the CellKey™ system is in agreement with the literature (*Pennefather et al., 2004*);
- Using the CellKey™ system we demonstrated a functional MoA for GR205171 and L-733060 at hNK₁Rs which agreed with previous functional data (*Griffante et al., 2005; Seabrook et al., 1996*);
- These results suggest that the CellKey™ may provide an alternative functional assay technology for the pharmacological characterization of compounds acting at GPCRs, including endogenous NK₁ receptors.

Using the CellKey™ in primary neurons (1)



- Conditions: CellKey™ small sample 96-well plates, striatal neurons derived from E18 CD rats, 9 days *in vitro* (DIV)
- No coating:
 - Few cells attached, clump formation, abnormal morphology.
 - Cells unsuitable for assay.
- PDL/Laminin coating:
 - Good cell adherence
 - Correct morphology, cells extending neurites

Mary Matthews

Using the CellKey™ in primary neurons (2)

- Cells look morphologically healthy on PDL/Laminin coated plates;
- Purinergic stimulation as a positive control for cell health and stimulation with various standard agonists for expressed receptors reveal no impedance changes.
- Are there insufficient cells to elicit a measurable response?
 - Optimum cell density? – enough cells for cultures to develop but not too densely packed as to adversely effect culture quality
- Are cells immobilised?
 - Coating regime to facilitate cell adherence: balance between adherence and cytoskeletal motility
- Titration of coating and density optimisation ongoing – initial results show no adverse effects on cell morphology of titrating the coating to 1:15 of original concentrations (7 DIV) – signal testing ongoing.

Summary and conclusions

- CellKey™ is a cell-based label-free technology which measures cellular impedance
- It allows measurement of G-protein coupled receptor activation
- Assay setup and development are more rapid than for many traditional assay formats
- Data are quantitative and qualitative
- CellKey™ is particularly suitable for the study of endogenous receptors:
 - Low expression levels compensated by a high sensitivity
 - Physiologically relevant cellular background and coupling
- CellKey™ is independent of signalling pathway
- CellKey™ allows equilibrium measurements
 - Problematic with intracellular calcium measurements

Some literature references

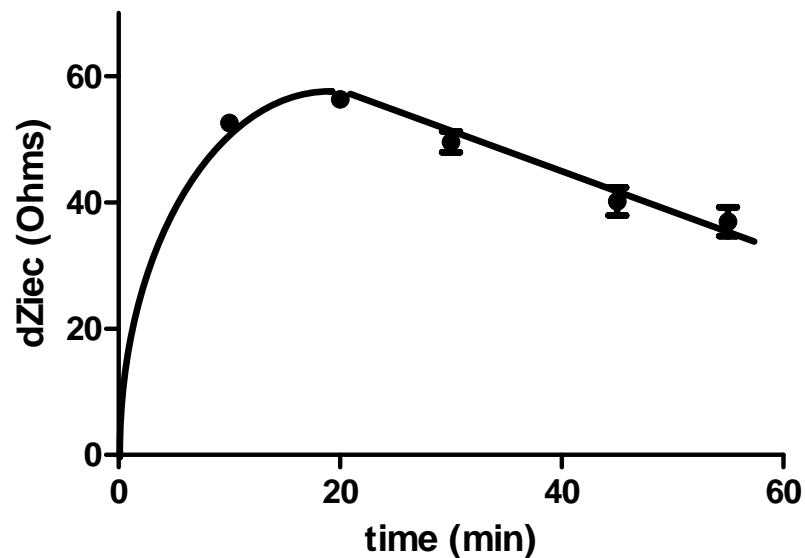
- *Cellular dielectric spectroscopy: a label-free technology for drug discovery.* Leung G. et al., Jala 2005; 10:258-69
- *Evaluating cellular impedance assays for detection of GPCR pleiotropic signaling and functional selectivity.* Peters M. F. and Scott C. W., Journal of Biomolecular screening 2009: 246-255)

Back-ups

Saturation binding values in U373-MG membranes

Radioligand	K_d (nM)	B_{max} (pmol/mg protein)	pK_d
[³ H]-GR205171	0.017 ± 0.009	0.365 ± 0.020	10.93 ± 0.28
[³ H]-SP	0.214 ± 0.128	0.190 ± 0.039	9.82 ± 0.25

Effect of Sub P in U373 MG in CellKey
Response at 1uM Sub P vs Time



About data analysis

Response Settings

Select the data and methods to use when reducing the impedance data to a single extracted value for each well. All response settings apply to all wells in the current plate.

Analysis Time Period

Start Time (hh:mm:ss) 00:05:18 Stop Time (hh:mm:ss) 00:59:58

Analysis Method

Kinetic Response
dZiec

Extracted Value Calculation
Specific Time
Max - Min
Max Change
Specific Time

Well Example

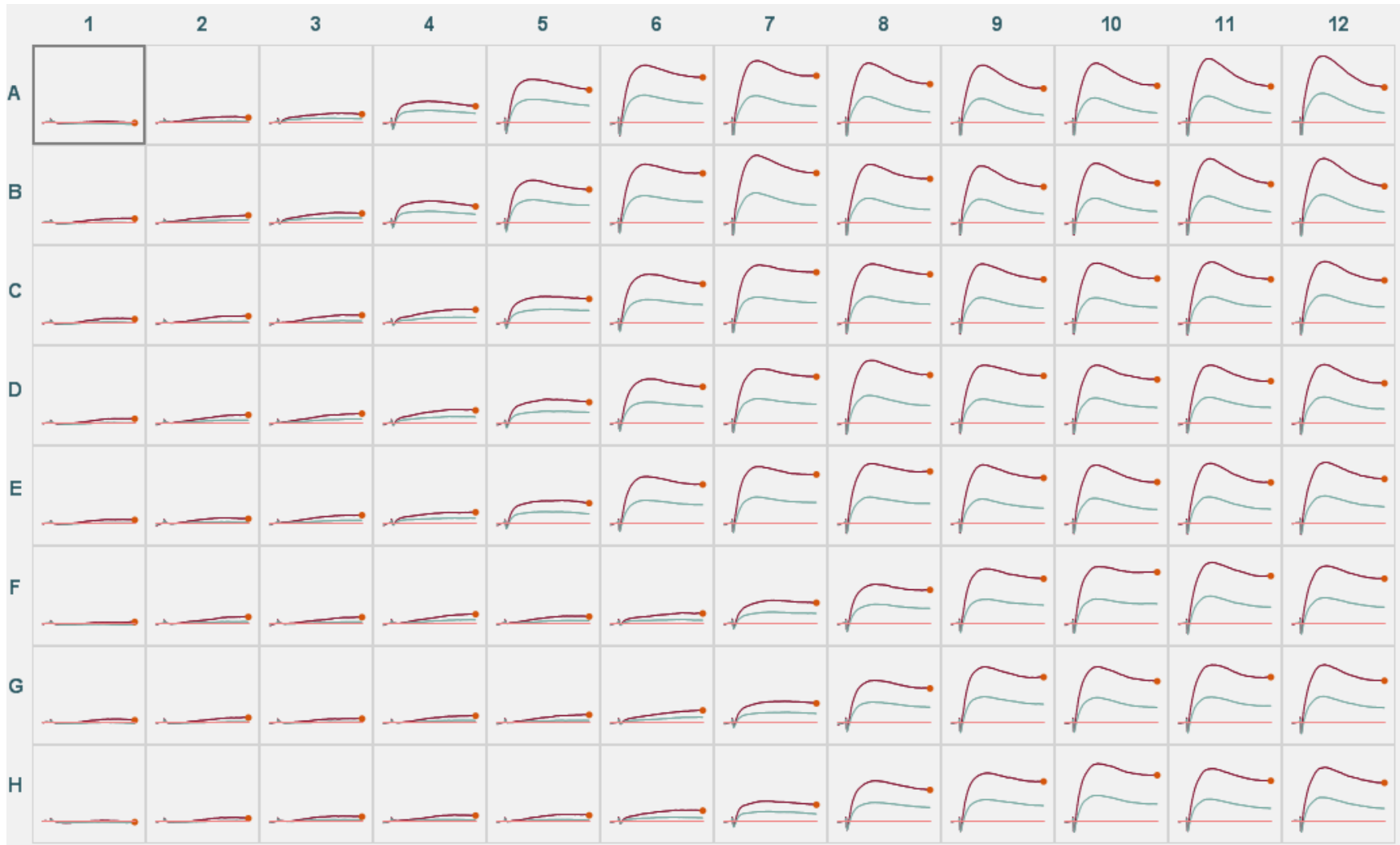
Well: A6 Sample 6

Kinetic Response

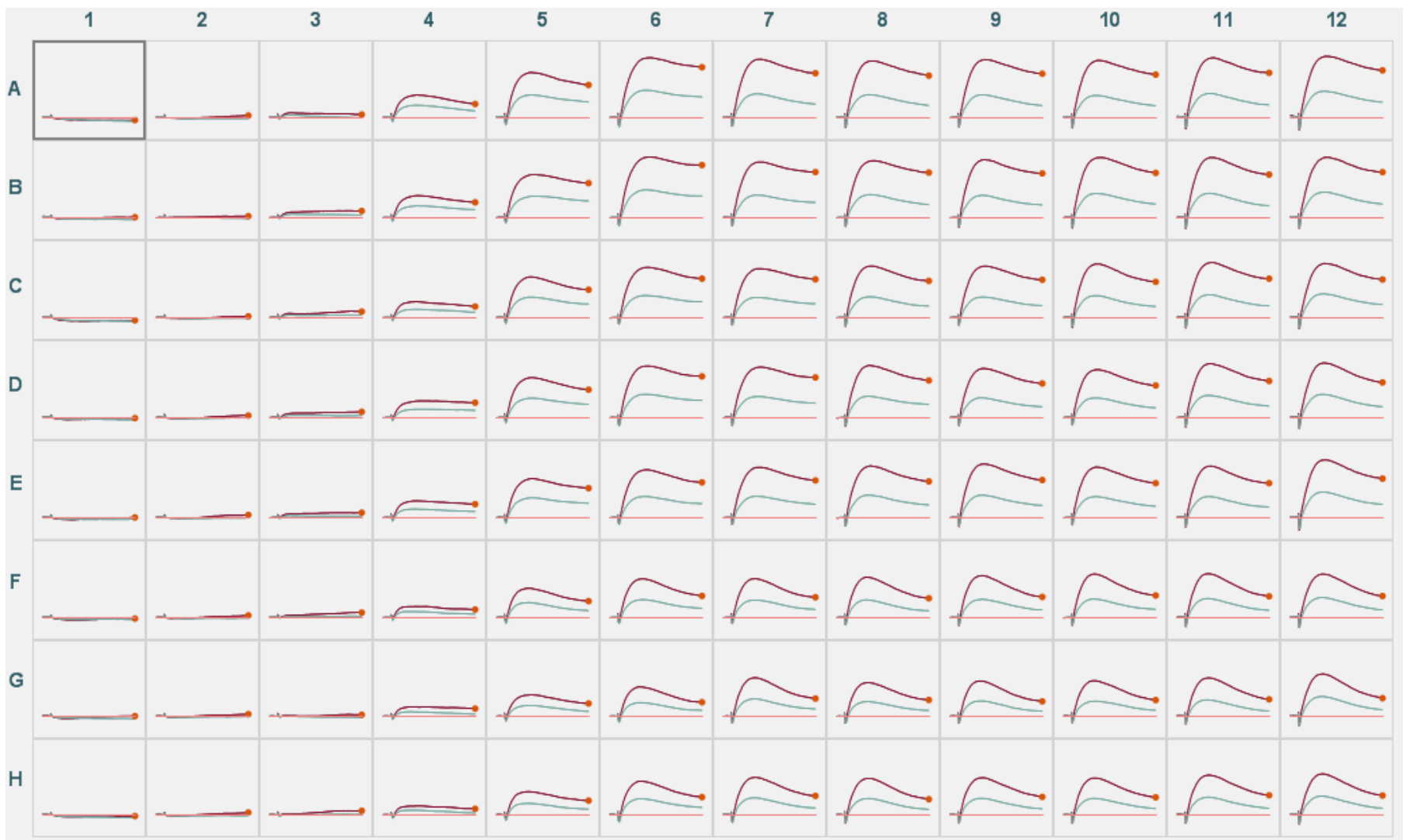
Extracted Value (ohms) 65.8

Buttons: Restore Factory Settings, Set As Default, OK, Cancel

Kinetic traces for L-733060

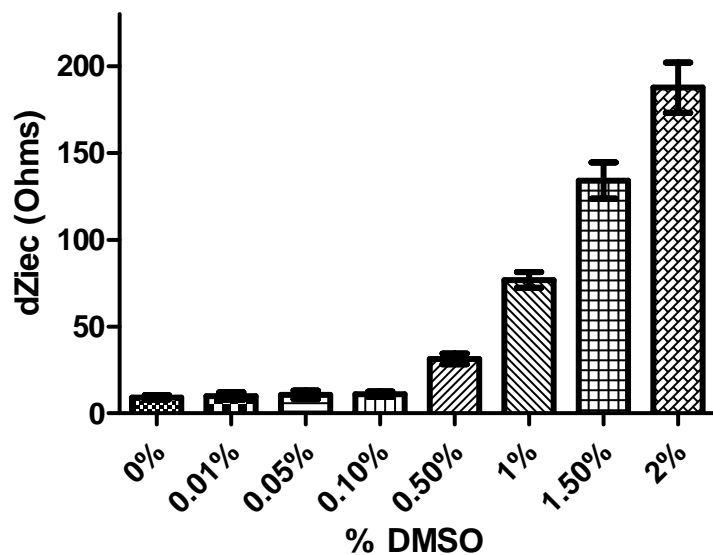


Kinetic traces for GR205171

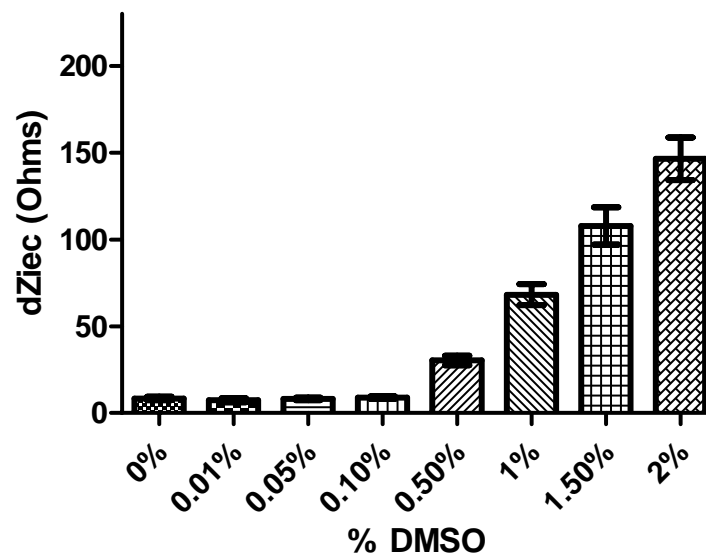


Effect of DMSO – max impedance changes (2 minutes)

No pre-incubation with DMSO

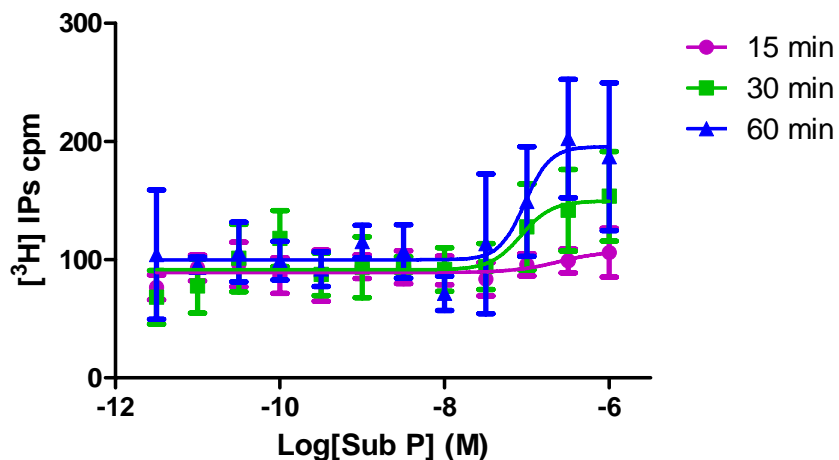


1 h pre-incubation with DMSO

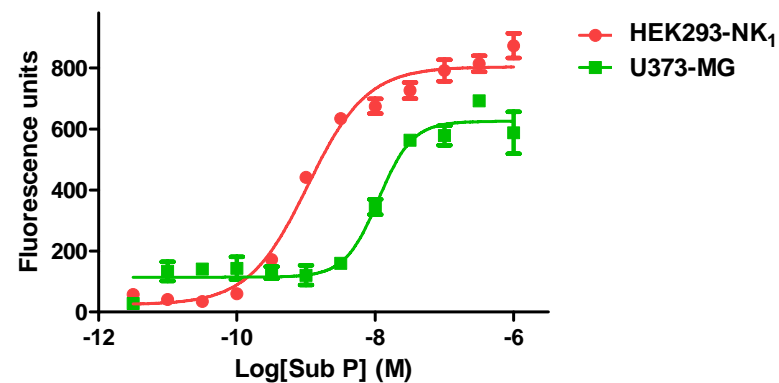


Response of U373-MG cells to SP in PI and FLIPR assays

U373-MG - [³H]-inositol phosphate accumulation



Intracellular Ca²⁺ mobilisation (FLIPR)



	HEK 293 NK1	U373 MG
log(agonist) vs. response -- Variable slope		
Best-fit values		
Bottom	24.11	114.3
Top	803.8	625.3
LogEC50	-8.976	-7.952
HillSlope	1.017	1.784

