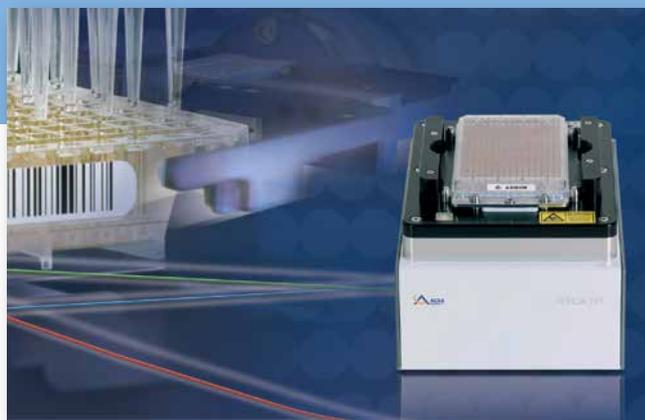# xCELLigence System RTCA HT Instrument

High-Throughput GPCR Screening

Introduction

- **Accurate determination of cell response kinetics.**
- **Analysis of disease-relevant primary cells and endogenous receptors.**
- **Modular integration with liquid handling automation.**

The xCELLigence System allows for sensitive and robust assessment of cellular behavior in real time. A number of research applications, including cell proliferation, cytotoxicity, cell adhesion, and receptor responses have been proven using this system. Cells are seeded onto plates with microelectrodes, allowing for precise measurement of changes in electrical impedance, to measure cell number, cell morphology, and substrate attachment (1). Subtle changes in cytoskeletal structure and cellular contraction, such as those induced by activation of many types of receptors, can be sensitively detected (2). Label-free real-time technology used by the xCELLigence System offers several advantages for assaying receptor responses, including accurate determination of response kinetics, the ability to use native receptors (especially in the context of disease-relevant cells), and the opportunity to capture biological responses involving multiple second messenger pathways.

For life science research only.
Not for use in diagnostic procedures.

Introduction

To bring xCELLigence System technology to the forefront of the drug discovery and drug development process, ACEA Biosciences, Inc. developed the RTCA HT Instrument. This platform offers all the advantages and features of label-free and real-time monitoring of cellular behavior in a 384-well format. The RTCA HT Instrument is designed for high-throughput screening, allowing for integration into a user's existing liquid handling and lab automation platform (see Figure 1).

Up to four RTCA HT Stations, each having a small footprint (L: 240 mm × W: 165 mm × H: 135 mm), may be integrated onto the automation platform deck at a time. Each station accommodates one Society for Biomolecular Sciences (SBS)-compatible 384-well plate (E-Plate 384), which is maintained at physiological temperature by an integrated heating system. The stations are connected to the RTCA HT Analyzer, which modulates the electric field required for impedance readout.

The system is controlled by the HT Control Unit, running the RTCA HT Software, which interfaces with the user's scheduling software during operation, and also allows analysis of results after screening. In addition, RTCA HT Software can output quantitative parameters that measure the kinetic responses obtained from each test well for entry into the user's data analysis pipeline.

Here, we present the experimental results of a proof-of-concept screen for small molecule inhibitors of the angiotensin I receptor using the RTCA HT Instrument. The angiotensin I receptor (AGTR1) is a Gq-coupled GPCR that plays a central role in the renin-angiotensin regulatory system for control of blood vessel constriction. Renin is released by the kidneys in response to low blood pressure, and converts the circulating peptide angiotensinogen to angiotensin I by proteolytic cleavage. Angiotensin I is converted to angiotensin II by angiotensin converting enzyme (ACE), the target of the common hypertension drugs known as ACE inhibitors. Angiotensin II (ATII) binds to AGTR1 in blood vessels, resulting in calcium mobilization via Gq-stimulation of phospholipase C, and ultimately vasoconstriction.

AGTR1 is a major target for treating hypertension, especially in cases where ACE inhibitors are contraindicated, and is also involved in congestive heart failure and diabetes-related kidney disease. Several antagonists of this receptor are currently used clinically for these indications, and several more are under development (3). The assay developed on the RTCA HT Instrument for AGTR1 antagonist screening in research is both rapid and robust, with a Z factor > 0.5 at three minutes after agonist addition.

The RTCA HT Instrument was used as both a primary and secondary screening platform to precisely identify all known angiotensin I receptor antagonists present in the test library. Subsequent testing revealed that rank order of potency as assayed on the RTCA HT Instrument matched well with the order obtained in an independent second messenger assay as well as when using disease-relevant primary cells. These experimental results demonstrate the broad utility of the RTCA HT Instrument for high-throughput small molecule screening early in the drug discovery process.

Methods

Assay development: Background readings were made with only 20 µl cell growth medium per well. CHO-K1 cells expressing the angiotensin I receptor were seeded at 12,000 per well in an E-Plate 384 in 30 µl growth medium per well, spun briefly in a centrifuge, incubated at room temperature for 30 minutes to allow cells to attach, and then grown overnight in a tissue culture incubator. In one experiment, growth medium was exchanged for assay buffer (HBSS containing 0.1% BSA and 20 mM HEPES), and incubated for 30 minutes at +37°C. In the other, the growth medium was left in place.

Plates were then placed on an RTCA HT Station set at +37°C and monitored for impedance signals at 30-second intervals for 5 minutes prior to agonist addition. Angiotensin II was added in a volume of 5.5 µl in assay buffer using a liquid handling instrument (Biomek FX Laboratory Automation Workstation from Beckman Coulter), while monitoring was ongoing. Plates were read every 15 seconds for an additional 10−20 minutes.

Primary screen: The above protocol was performed using the Biomek FX Laboratory Automation Workstation (Beckman-Coulter), as diagrammed in Figure 1, using the manufacturer's scheduling software. Spectrum library compounds were diluted to 100 µM in assay buffer prior to compound transfer, for a final screening concentration of 10 µM.

Secondary screening: CHO-K1 cells expressing angiotensin I or α−2A adrenergic receptor were screened by the protocol outlined above. The IP-One ELISA assay (Cisbio, Bedford, MA) was performed in 96-well plates, according to the manufacturer's recommendation, with the exception that fewer cells were used (30,000 per well) to more closely match the cell density to well surface area ratio used in the xCELLigence System RTCA assay. The VSMC (T/G HA-VSMC; ATCC, Manassas, VA) cells were assayed using the protocol described above, with the exception that 5,000 cells were seeded in E-Plates 96 and assayed on the xCELLigence System RTCA MP Instrument.
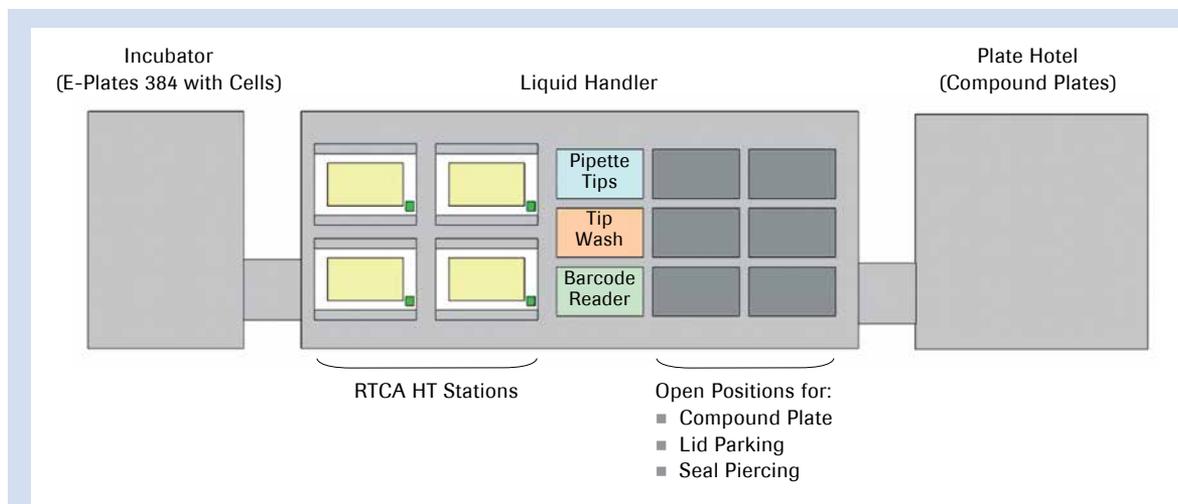
Figure 1: Schematic showing the RTCA HT Instrument together with the automation platform layout. Up to four RTCA HT Stations may be incorporated directly onto the deck of the automated liquid handling platform. These are connected by cables to the adjacent RTCA HT Analyzer (not shown). In a typical assay, E-Plates 384 containing growth medium (dispensed on- or off-line) are loaded into the incubator, and background impedance readings are made using the RTCA HT Station after barcode reading. Cells are then plated onto the E-Plates 384 (dispensed on- or off-line), which are returned to the incubator. Cells in E-Plates 384 are monitored for cell growth over time by cycling in and out of the RTCA HT Stations at regular intervals. Up to four plates can be read simultaneously for a user-defined number of independent impedance readings. For compound addition, the user's standard automation workflow can be used. Compounds may be added to the E-Plates 384 at an open position or directly on the RTCA Station for rapid detection of responses in GPCR assay.

Results

A CHO-K1 cell line over-expressing the angiotensin 1 receptor (CHO-AGTR1) was first evaluated for responsiveness to agonist stimulation using the xCELLigence System. In one experiment, the growth medium was exchanged for assay buffer, and in the other, the growth medium was left in place. The results of dose-response experiments are shown in Figure 2A. The xCELLigence System assay for changes in morphology in response to angiotesin II stimulation was very sensitive, with an EC_{50} value less than 1 nM in either assay buffer or growth media. The assay buffer condition produced a slightly greater maximal Cell Index (1.5 versus < 1.4 at 10 μM), and also appeared to be more sensitive to detection of angiotensin II response; this protocol was fixed for subsequent experiments.

Experimental results of the assay's ability to detect small molecule antagonists of the angiotensin I receptor are shown in Figure 2B. CHO-AGTR1 cells were assayed as described above, with the exception that antagonists or controls were included during the 30-minute incubation in assay buffer. Angiotensin II was then used to stimulate the cells at a final concentration of 10 μM to ensure saturation

of the cell morphological response. Saralasin, a potent but non-selective AGTR1 antagonist, blocked the CHO-AGTR1 response to angiotensin II in a dose-dependent manner, with an IC_{50} of 12.5 nM (see Figure 2B). The less potent, but AGTR1-selective inhibitor losartan exhibited an IC_{50} value of 863 nM, whereas the ATR2-selective inhibitor PD 123319 had no effect. Assay parameters described here were used for subsequent antagonist screening. Research results indicate that the RTCA HT Instrument can be used to assay selective antagonists of GPCR function, and antagonists of differing potency can be distinguished from each other.

To evaluate the robustness of the assay for angiotensin I receptor function, and to optimize the time point used for picking hits in the antagonist assay, the real-time kinetic readout feature of the RTCA HT Instrument was utilized. The response of the CHO-AGTR1 cells to a saturating concentration of agonist was measured over time, and plotted as a Normalized Cell Index (NCI) value (see Figure 3A). By this measure, changes in cell morphology rapidly produce a Cell Index value of 1.4 within the first 2 minutes, after which the value gradually increases to approximately 1.8 after 12 minutes. In this case,
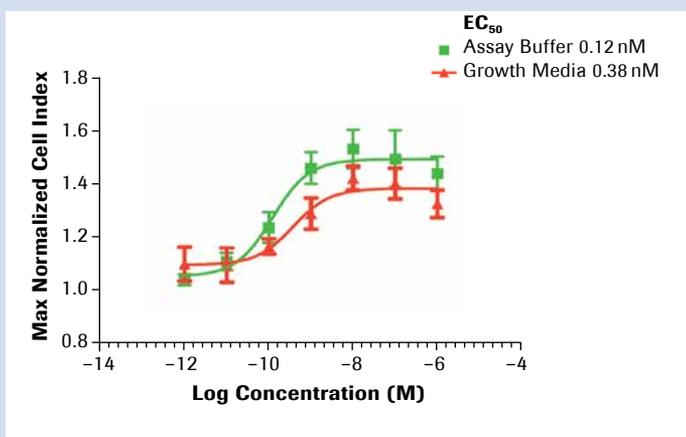

Figure 2: Assay development.
A. Dose–response curve for angiotensin II stimulation of the angiotensin I receptor on the RTCA HT Instrument. CHO-K1 AGTR1 cells plated in E-Plates 384 were grown overnight, then stimulated with the indicated concentrations of angiotensin, with or without a prior change of growth medium for assay buffer. The maximal Normalized Cell Index reached during the duration of the experiment is plotted as the mean of 10 replicates; error bars represent one standard deviation, and EC_{50} values are indicated.
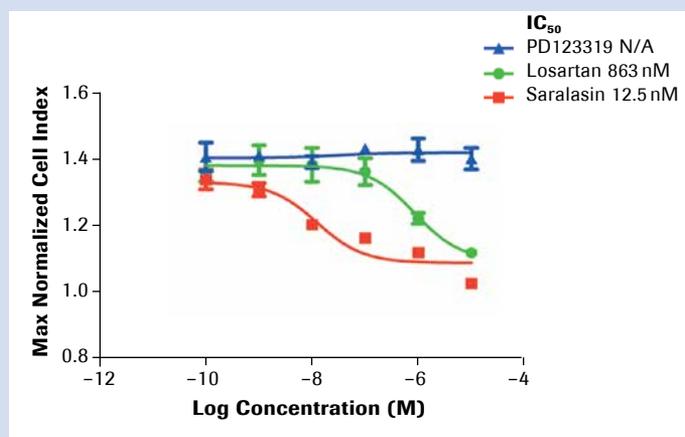
B. Dose–response curve for inhibitors of angiotensin II–stimulated angiotensin I receptor activation. CHO-K1 AGTR1 cells plated in E-Plates 384 were grown overnight and the growth medium was exchanged for assay buffer. The indicated concentrations of antagonists were added, and after 30 minutes, cells were stimulated with 10 nM of angiotensin II and monitored on the RTCA HT Instrument. The maximal Normalized Cell Index reached during the duration of the experiment is plotted as the mean of four replicates; error bars represent one standard deviation, and IC_{50} values are indicated.
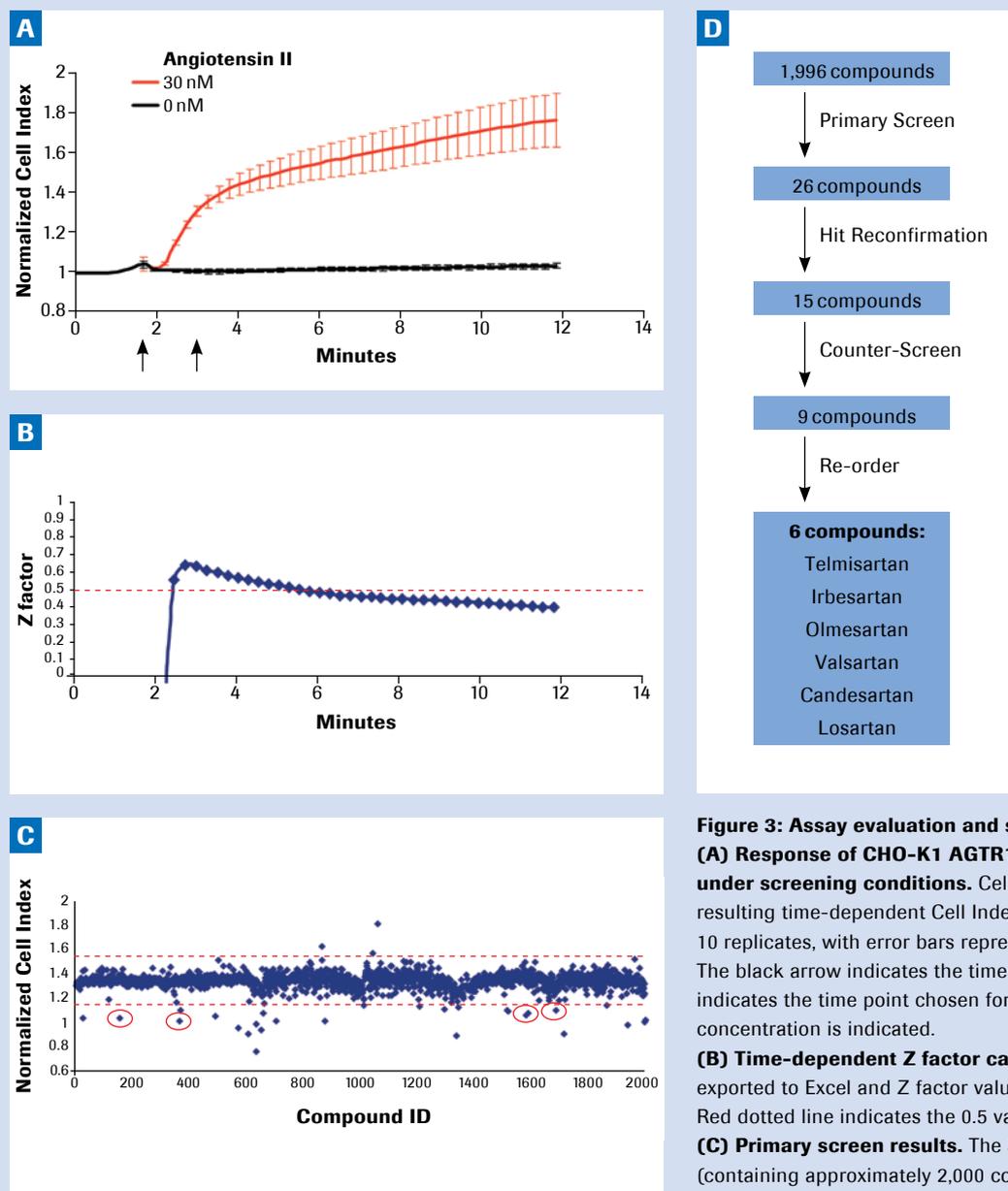
Figure 3: Assay evaluation and screening results.
(A) Response of CHO-K1 AGTR1 cells to angiotensin stimulation under screening conditions. Cells were assayed as in Figure 2A and the resulting time-dependent Cell Index values plotted as mean values of 10 replicates, with error bars representing one standard deviation. The black arrow indicates the time of compound addition; the red arrow indicates the time point chosen for hit selection and angiotensin II concentration is indicated.
(B) Time-dependent Z factor calculation. Results from (A) were exported to Excel and Z factor values calculated for each time point. Red dotted line indicates the 0.5 value cutoff for a highly robust assay.
(C) Primary screen results. The Spectrum small molecule library (containing approximately 2,000 compounds) was tested for antagonist activity on CHO-K1 AGTR1 cells using the assay described in Figure 2B. Normalized Cell Index values at the 3-minute time point after compound addition are plotted by compound identity number. Red lines indicate the value of three standard deviations from the mean; compounds below this threshold were considered candidate antagonists. Red circles indicate compounds that proved to be true selective antagonists after secondary screening.
(D) Schematic of overall primary and secondary screening results.

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well-to-well variability also increases significantly after the initial (approximately 2) minutes. The Z factor metric, which takes both assay window and well-to-well variation into account (4), was determined for the Cell Index value obtained at each time point for 10 minutes after agonist addition (see Figure 3A). A Z factor > 0.5, which is a very robust assay, was obtained 2 to 5 minutes after agonist addition, suggesting that this time frame is optimal for screening. These experimental results indicate the utility of the real-time kinetic readout of the xCELLigence System, which allows for precise determination of optimal assay conditions during assay development.

Using the experimental assay parameters developed above, a small-scale proof-of-concept screen was conducted for antagonists of angiotensin II-mediated activation of the AGTR1 receptor. The 2,000-compound Spectrum Collection (MicroSource Discovery Systems, Inc.; Gaylordsville, CT) was chosen. Approximately half of the library is composed of approved drugs, including six AGTR1 antagonists used to treat hypertension (telmisartan, irbesartan, olmesartan, valsartan, candesartan, and losartan). These compounds serve as internal positive controls for the screen. The remainder of the library is composed of natural products, known toxins, and other bioactive compounds.

The CHO-AGTR1 cells were seeded in E-Plates 384 as described above, and on the following day treated with Spectrum Collection compounds for 30 minutes prior to addition of 30 nM angiotensin II using the assay buffer protocol described above. Normalized Cell Index values at the 3-minute time point obtained for each compound are shown in Figure 3 (C). The majority of compounds produced Cell Index values within the range of three standard deviations from the mean for all compounds (red lines in Figure 2C), but 26 (1.3 %) were below the cutoff of three standard deviations, identifying them as potential AGTR1 antagonist candidates.

These 26 hits from the initial creen were found to fall into three classes. In the first class, false positives due to random statistical variation can be expected when screening compound libraries, especially when the sample size is small (in this case, 2,000 compounds). Such compounds were eliminated by repeating the assay under identical experimental conditions using the RTCA HT Instrument. This hit-reconfirmation step resulted in fifteen positive compounds (see Figure 3D).

A second class of false positive compounds could suppress the morphological response to angiotensin II independently of AGTR1 signaling. Indeed, some compounds in the Spectrum Collection are known toxins, or have other bioactive properties which could independently alter cell responsiveness. To eliminate those compounds, a counter-screen was run on the RTCA HT Instrument using the same experimental assay conditions and a different receptor, the α–2A adrenergic receptor, expressed in the same parental cell line.

Compounds that antagonize α–2A adrenergic receptor function were considered nonspecific. This resulted in nine compounds with selective AGTR1 antagonist activity (see Figure 3D). Stocks of these compounds were re-ordered and again tested on the CHO-AGTR1 cell line using the xCELLigence System. Six of these were found to be active, corresponding exactly to the six known antagonists in the Spectrum Collection. The three false positive compounds were most likely due to compound management or handling errors.

These results demonstrate the utility of the xCELLigence System for primary screening, as well as for hit reconfirmation and counter-screening, allowing for identification of compounds with true antagonist activity from a compound library.

A

Cho-AGTR1

B

CHO-AGTR1

C

T/G HA-VSMC
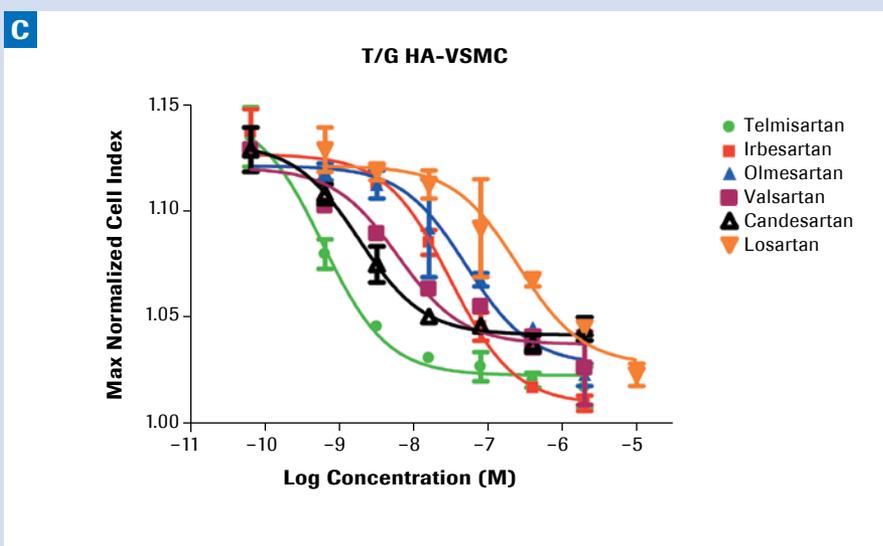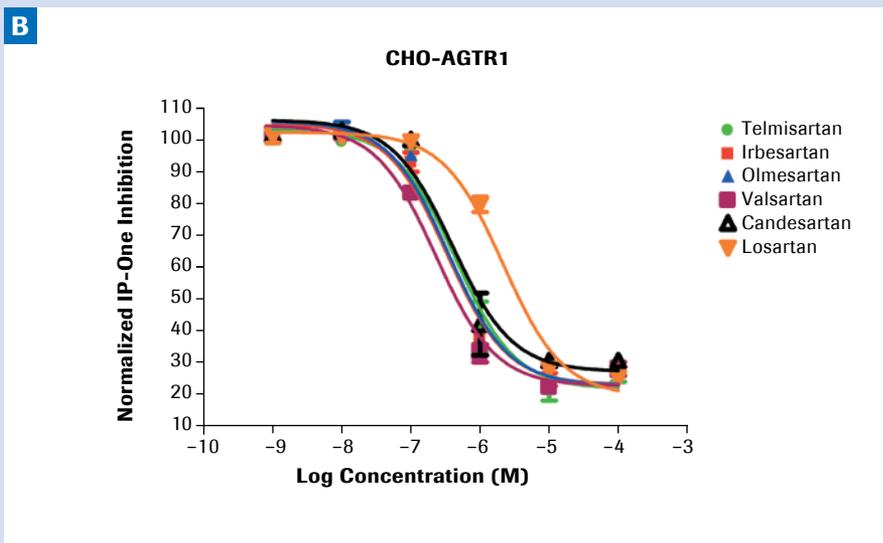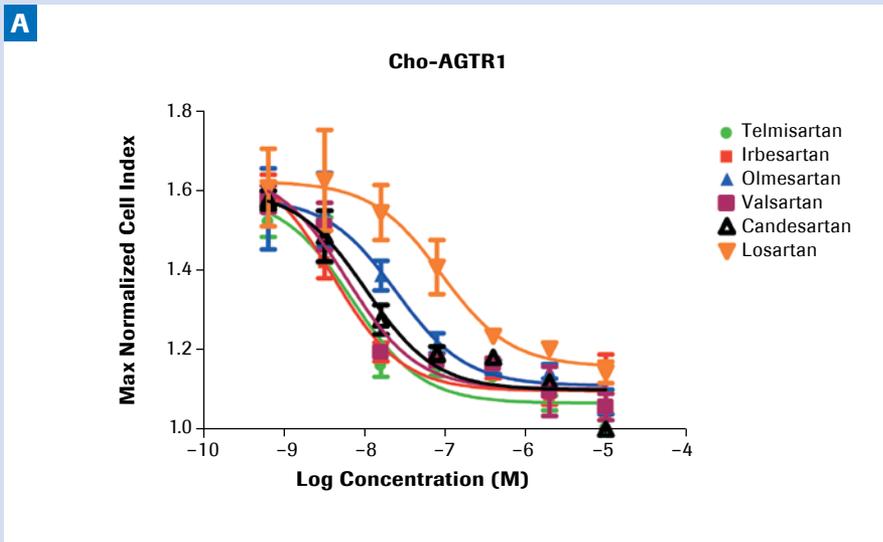
Figure 4: Characterization of screening hits.
This experiment shows dose-response curves for angiotensin I receptor antagonists.
A: xCELLigence System assay on CHO-K1 AGTR1 cells.
B: IP-One ELISA assay on CHO-K1 AGTR1 cells.
C: xCELLigence System assay on T/G HA-VSMCs.

We next tested the potency of these six antagonists using the RTCA HT Instrument. Dose-response curves were obtained from CHO-AGTR1 cells using the maximal Normalized Cell Index value during a 10-minute window after angiotensin II (ATII) addition for each dose of each compound (see Figure 4A). Five of the antagonists showed similar potencies for inhibition of the ATII-stimulated morphology change. All effects were found to be in the 10 nM range (telmisartan, irbesartan, olmesartan, valsartan, and candesartan), while the sixth, losartan, was significantly right-shifted (to >100 nM). These potencies are in good agreement with the expected values.

To evaluate whether the relative potencies measured by the RTCA HT Instrument are comparable to those obtained in a second messenger endpoint assay, an inositol phosphate-1 (IP-1) accumulation assay was performed. The phospholipase C activation resulting from Gq-coupled GPCR stimulation leads to calcium mobilization via inositol tris-phosphate (IP-3). IP-3 in turn is degraded to IP-1, which can accumulate in the cell and be detected by an ELISA assay (IP-One ELISA; Cisbio, Bedford, MA). Dose-response curves for these compounds acting on the CHO-AGTR1 cells in the IP-One assay are shown in Figure 4B. A comparison to those obtained using the RTCA HT Instrument reveal close agreement between the two assays in terms of the rank order.

A major advantage of the xCELLigence System for assaying GPCR function is the label-free nature of the readout, allowing for direct detection of receptor function in a biologically meaningful context (5). This in turn enables the use of disease-relevant cells, including primary cells, in screens and follow-up assays. We therefore tested the AGTR1 antagonists on a human vascular smooth muscle cell (VSMC) line. These cells should more closely reflect the *in vivo* biology of AGTR1 function than the recombinant CHO-K1 cell line in terms of receptor expression levels and G-protein or other regulatory partner coupling.

In this experiment the dose-response curves for the AGTR1 inhibitors in VSMCs showed a similar relationship to the CHO-K1 AGTR1 cells (see Figure 4C). Thus, the xCELLigence System can also be used for secondary assays that bridge the divide between artificial, receptor over-expressing cell line screens and more disease-relevant models.

Discussion

This study demonstrates that the RTCA HT Instrument is a powerful platform for conducting robust high-throughput screening using intrinsic cell biological changes as a readout. The proof-of-concept screen presented here shows that the RTCA HT Instrument can be used to identify true modulators of a GPCR response in an antagonist screening mode. A CHO-K1 cell line over-expressing the human angiotensin I receptor, a key regulator of vascular biology and a major target for treating hypertension, was screened using a library containing well-characterized small molecules including known antagonists. The RTCA HT Instrument was used in the primary screen, where an initial hit rate of 1.3% was achieved (see Figure 2), as well as for hit reconfirmation and a counter-screen to evaluate receptor specificity, resulting in a final specific hit rate of approximately 0.3%.

This approach identified all six of the known angiotensin I receptor antagonists present in the library, showing the utility of the cell morphology-based readout employed by the xCELLigence System for high-throughput screening. The relative potency of these hits was comparable to a second messenger endpoint assay (IP-One), confirming the relevance of the morphological changes detected by the RTCA HT Instrument to canonical GPCR signaling pathways.

The advantage of the xCELLigence System for assaying endogenously expressed receptors in biologically relevant cells showed the similarity of these hits for a human vascular smooth muscle cell line. This study shows that with the xCELLigence System, high-throughput screening can be conducted from the primary screening to hit reconfirmation and secondary screening, using biologically relevant cells to maximize hit quality and minimize subsequent attrition rates in drug discovery.

In summary:

- The RTCA HT Instrument is a flexible tool for automated, label-free small molecule screening.

- In a proof-of-concept screen, the RTCA HT Instrument was used to develop a rapid and robust assay for GPCR antagonists, correctly identifying all known inhibitors in the library.

- Screening hits were analyzed by secondary screening for specificity and for activity in a biologically relevant human cell line using the xCELLigence System.

- Compound effects measured by the xCELLigence System were highly correlated to those obtained using a second messenger endpoint assay.

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Ordering Information

Product	Cat. No.	Pack Size
RTCA HT System Bundle	**00380601070**	**1 Bundle**
RTCA HT Station	05919878001	1 Instrument
RTCA HT Analyzer	05919843001	1 Instrument
RTCA HT Control Unit	06200176001	1 Instrument
E-Plate 384	05867681001 05867673001	2 × 5 Plates 8 × 5 Plates

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