Novel Methodology to Identify TRPV1 Antagonists Independent of Capsaicin Activation

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Abstract

TRPV1 was originally characterized as an integrator of various noxious stimuli such as capsaicin, heat, and protons. TRPV1-null mice exhibit a deficiency in sensing noxious heat stimuli, suggesting that TRPV1 is one of the main heat sensors on nociceptive primary afferent neurons and a candidate target for heat hypersensitivity in chronic pain. Several different potent and selective TRPV1 antagonists have been developed by more than 50 companies since the characterization of the receptor in 1997. A consequence of this competitive interest is the crowding of patentable chemical space, because very similar in vitro screening assays are used. To circumvent this issue and to expand our understanding of TRPV1 biology, we sought to take advantage of recent advancements in automated patch-clamp technology to design a novel screening cascade. This SAR-driving assay identified novel modulators that blocked the depolarization-induced activation of outwardly-rectifying TRPV1 currents independent of agonist stimulation, and we correlated the pharmacology to three other innovative assays for higher-throughput screening. Ultimately, we have identified a screening paradigm that would have good predictive value for future TRPV1 drug discovery projects and novel chemical space with a higher probability of gaining intellectual property coverage.

Introduction

Capsaicin, the active ingredient in chili peppers, has long been known to cause nocifensive behaviors in both rodent and man and has been proposed as a target for the treatment of chronic inflammatory pain.[1,2] Its receptor, TRPV1, was first cloned by Caterina et al.[3] in 1997, initiating a new wave of pharmacologic research to harness the therapeutic potential from this nonselective cation channel. Both agonists to desensitize nociceptive small-diameter afferent neurons, and antagonists to block pain-evoked neuronal transmission, have been discovered for this channel.[4] However, more monetary and intellectual investment has been placed on the latter type of therapeutic, with more than 50 different pharmaceutical and biotechnology companies reporting research and development activities.[5] A consequence of this popularity within the pharmaceutical industry is the extensive amount of patentable space that is covered. At last count, 511 patents that cite the discovery of small- and large-molecule TRPV1 modulators have been filed (Scifinder, American Chemistry Society and Voight

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and Kort[6]). Most of these compounds are extremely lipophilic, like capsaicin itself, and have very poor aqueous solubility that has been problematic for the development of these compounds in clinical trials. For the most part, the discovery of small-molecule TRPV1 antagonists has relied on high-throughput screening (HTS) technology that measures differences in intracellular calcium concentrations. Many of the early lead compounds, including BCTC,[7] SB-366791,[8] and AMG9810,[9] as well as clinical candidates, such as ABT-102,[10] AMG-517,[11] and SB-705498,[12] were identified using fluorescent imaging plate reader (FLIPR) Ca^{2+} influx or $^{45}Ca^{2+}$-uptake assays. These assay methods measure the ability of chemical entities to block an agonist-induced Ca^{2+} flux through the TRPV1 channel, with either capsaicin, protons, or heat (>42 °C) used to activate the channel.[3] In most of the reported and patented cases, however, capsaicin is used as the preferred ligand to probe TRPV1 SAR under HTS conditions.

When TRPV1 was first cloned, it was described not only as a ligand-gated channel that responded to capsaicin and protons but also as a weakly voltage-gated channel with outward rectification properties.[3] In fact, this is a trait shared by many other members of the TRP channel family.[13] The recent advancements in the area of automated patch-clamp technologies have opened the door to higher-throughput pharmacological screening for many voltage- and ligand-gated ion channels.[14–20] To explore the potential of a new chemical landscape for TRPV1, we took advantage of the voltage-gated properties of TRPV1 and developed a medium-throughput automated patch-clamp assay method using the IonWorks Quattro system. Although this method proved to be very effective in identifying novel chemical entities for TRPV1, its potential for use in an HTS screening paradigm was limited in terms of the cost per data point and throughput capabilities. Three other novel TRPV1 screening platforms were validated, namely, (1) the voltage-sensitive dye detection of membrane potential variation after proton-gated stimulation of TRPV1, (2) atomic absorption spectroscopic measurement of spontaneous rubidium-flux through TRPV1, and (3) the Ca^{2+}-induced activation and Ca^{2+}-influx of TRPV1. By correlating the quantity and quality of the hits generated from each of these three novel assays with the automated electrophysiological assay, we identified the atomic absorption method as an initial HTS prior to using a lower-throughput but data-rich electrophysiology method. The development and validation of these assays are described here, along with the correlations that enabled the implementation of an innovative screening cascade to discover new TRPV1 antagonists.

Methods

Stably Expressing Inducible TRPV1 Cell Lines

Human TRPV1 sequence (accession No. AX002940) was subcloned in a pcDNA4/TO inducible expression vector (Invitrogen, Carlsbad, CA) and then transfected into Chinese hamster ovary (CHO) or human embryonic kidney (HEK) cells stably expressing the T-REx repressor gene (Invitrogen) using an electroporation method as previously described.[21] A stable pool was generated by maintaining the cells in DMEM/F12 culture medium (Wisent, St-Bruno, QC, Canada) supplemented with 10% fetal bovine serum, 1% glutamine, and 350 µg/mL Zeocin and 5 µg/mL Blasticidin (Invitrogen). Colonies were selected and plated in a 96-well plate at a concentration of 1 cell/well and maintained in the presence of the selection antibiotics. Clones that survived the selection process were assessed for the functional expression of TRPV1 using the standard capsaicin Fluo-4 FLIPR experimental protocol described below. The highest expressing clones were further characterized for expression stability over a span of 3 mo. The highest stable clone was used for all subsequent assays.

Standard Capsaicin FLIPR Assay with Fluo-4 Ca^{2+} Detection

hTRPV1-HEK cells were plated in a black, clear-bottom 384-well plate coated with poly-D-lysine (Becton Dickinson Labware, Bedford, MA) at a concentration of 10 000 cells per well in 50 µL and incubated overnight at 37 °C and 5% CO_2 along with 0.1 µg/mL tetracycline to induce the expression of TRPV1. The following day, cells were washed in an assay buffer of 1xHBSS (without Ca^{2+} or $Mg^{2+;}$ Wisent) supplemented with 5 mM glucose and 1 mM $CaCl_2$ before being loaded with 2 µM Fluo-4 AM (Invitrogen) for 1 h. The cells were washed three times before adding 25 µL of assay buffer. The plates were read in a FLIPR-384 instrument during the application of 12.5 µL fourfold the final concentration of test compound prepared in a separate 384-well plate in serial threefold dilutions, and then the subsequent addition of 20 nM capsaicin (final, prepared as fourfold final concentration stock of 80 nM in a 384-well compound plate). Responses to both test compound and capsaicin additions were recorded at 1 Hz for 60 s to capture the acute effects and then at 0.1 Hz for an additional 280 s. Data were extracted and analyzed using FLIPR-384 software (Molecular Devices, Sunnyvale, CA) and Microsoft Excel.

IonWorks Quattro Voltage-Ramp Assay

hTRPV1-CHO cells were maintained as described above. On the day prior to the experiment, cells were induced with 0.1 µg/mL tetracycline. Cells were washed and prepared for the experiment as per the manufacturer's instructions. The final cell pellet was resuspended to a concentration of 1.2×10^6 cells/mL in Dulbecco's phosphate-buffered saline (D-PBS; with Ca^{2+} and Mg^{2+} (Wisent). For the experiment, D-PBS (in mM NaCl 136.9, KCl 2.7, KH_2PO_4 1.5,

Na_2HPO_4 8, $CaCl_2$-$2H_2O$ 0.9, and $MgCl_2$-$6H_2O$ 0.5; Wisent) with 10 mM glucose was used as the extracellular assay buffer. The intracellular solution was composed of the following (in mM): K-gluconate 100, KCl 40, $MgCl_2$ 3.2, HEPES 5, and EGTA 3, pH adjusted to 7.2 with KOH. An additional buffer with 0.1 mg/mL amphotericin B was prepared to gain access to the cells via the perforated patch method using the IonWorks instrument.

Cells were loaded onto the instrument, and once a seal was formed, perforated access was achieved with the amphotericin B solution. Cells were maintained at a holding potential of –60 mV, and in order to elicit TRPV1 voltage-gated currents, continuous voltage-ramp sweeps of 500 ms from –80 mV to +80 mV at 1 Hz were applied to the cells prior (at baseline) and after compound addition. The peak amplitude for the resulting outward current at +80 mV was used to compare compound effects on TRPV1.

FLIPR Assay with Voltage-Sensitive Reporting Dye and pH Stimulus

hTRPV1-HEK cells were prepared, plated, and induced in the same manner as described above for the standard FLIPR assay with capsaicin. The day of the experiment, culture media were removed by inversion and then the cells were loaded with the blue membrane potential dye (Molecular Devices) prepared in 1x-HBSS (with Ca^{2+} and Mg^{2+}, Wisent) for 1 h. After the incubation with the membrane potential dye, plates were placed directly in the FLIPR 384 instrument and read during the application of 12.5 μL of a pH 5.2 DPBS solution, and the subsequent second addition of a fourfold the final concentration of compound prepared in a separate 384-well plate in serial threefold dilutions. Responses to both test pH and compound additions were recorded at 1 Hz for 60 s to capture the acute effects and then at 0.1 Hz for an additional 280 s. Data were extracted and analyzed using FLIPR-384 software and Microsoft Excel.

Calcium-Induced Activation of TRPV1 Assay

hTRPV1-HEK cells were prepared, plated, and induced in the same manner as described above for the standard FLIPR assay with capsaicin. The day of the experiment, culture medium was removed from the cells by inversion, and the cells were loaded with a Fluo-4 no-wash dye (Fluo-4NW; Invitrogen). Briefly, one vial of the Fluo-4NW kit was diluted in 10 mL of an assay buffer with reduced calcium (in mM): KCl 5.3, KH_2PO_4 0.4, NaCl 137.9, Na_2HPO_4 0.34, EGTA 5, D-glucose 10, HEPES 10, $NaHCO_3$ 4.5, $CaCl_2$ 0.000016. The minimal amount of calcium is required so that the cells remain adherent to the plates. Cells were incubated with the dye for 1 h before reading in a FLIPR-384 instrument during the application of 12.5 μL

fourfold the final concentration of test compound prepared in a separate 384-well plate with serial threefold dilutions and then the subsequent addition of an assay buffer containing 4.8 mM $CaCl_2$. Responses to both test compound and high extracellular Ca^{2+} additions were recorded at 1 Hz for 60 s to capture the acute effects and then at 0.1 Hz for an additional 280 s. Data were extracted and analyzed using FLIPR-384 software and Microsoft Excel.

Atomic Absorption Spectroscopy-Based Rubidium Flux Assay

hTRPV1-CHO cells were maintained as described above. Fifty-six hours prior to the experiment, cells were detached and replated on a black poly-D-lysine–coated 384-well plate (Becton-Dickenson) at a concentration of 10 000 cells/well. Twenty hours prior to the experiment, the cells were induced with 0.1 μg/mL tetracycline. On the day of the experiment, compound plates were prepared at a starting concentration of 40 μM in threefold dilutions, representing four times the final concentration intended for the assay. The induction media were removed, and cells were washed three times with a buffer consisting of (in mM) NaCl 150, HEPES 25, $CaCl_2$ 2, $MgCl_2$ 1, and Na_2HPO_4 0.8. Of the wash buffer, 12.5 μL was left in each well after the final wash step, and then 12.5 μL of the fourfold final concentration of test compound or control solutions was added for 10 min at room temperature. Subsequently, 25 μL of 2× rubidium loading buffer (in mM: NaCl 130, HEPES 25, $CaCl_2$ 2, $MgCl_2$ 1, Na_2HPO_4 0.8, glucose 10, RbCl 11) was added for 3 h at 37 °C. After loading, the plates were washed eight times in a buffer consisting of (in mM) NaCl 150, KCl 5.4, HEPES 25, $CaCl_2$ 2, $MgCl_2$ 1, and Na_2HPO_4 0.8, leaving 12.5 μL per well after the final wash. Plates were frozen overnight and then thawed the next day, and pellets lysed with 80 μL of 1% Triton X-100 solution in water. The ICR8000 single-channel atomic absorption flame spectrometer (Aurora Biosciences, Vancouver, Canada) was used to determine the concentration of rubidium present in cells. The instrument was calibrated by generating a standard curve with known rubidium concentrations prior to each run. Fifty microliters of each well was injected, followed by 200 μL of ICR wash buffer, resulting in the concentration of rubidium as calculated from rubidium's absorbance of light at 780 nm.

Whole-Cell Patch-Clamp Recordings

hTRPV1-CHO cells were maintained as per the cell culture methods described above. On the day of the experiment, cells were treated with 2 mL Accutase for 2 min before washing with 1 mL 1x-D-PBS. The cells were tapped gently to dissociate them from the culture flask and then placed in an Eppendorf tube. A droplet of cells was transferred to

a recording chamber fixed to the stage of an inverted microscope (Olympus) and allowed to settle for 2 min prior to being constantly perfused (0.5 mL/min) with D-PBS, a standard extracellular bathing solution containing (in mM) 137 NaCl, 8.1 Na_2HPO_4•$7H_2O$, 2.68 KCl, 1.47 KH_2PO_4, 0.9 $CaCl_2$, 0.5 $MgCl_2$•$6H_2O$, 5.6 glucose, and 0.33 Na-pyruvate at pH 7 and 275 to 290 mOsm. Manual whole-cell patch-clamp recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Patch electrodes were pulled from borosilicate glass (World Precision Instruments Inc., Sarasota, FL) on a Flaming/Brown puller (Sutter Instrument Co, Novato, CA) to form a tip diameter of 1.5 to 2.5 mM, which translated to a 2 to 5 MΩ tip resistance. These electrodes are filled with intracellular pipette solution (pH 7.3, 290 mosM) containing the following (in mM): 8 NaCl, 20 KCl, 1 $MgCl_2$, 10 HEPES, 110 K-glutamate, 10 EGTA, and 4 ATP-Mg.

A small patch of the cell membrane underneath the tip of the pipette is aspirated to form a tight, high-resistance seal between the membrane and the pipette, known as a gigaseal. Negative pressure is then applied to rupture the membrane, thereby establishing a whole-cell configuration. The adjustment of capacitance compensation and series resistance compensation (<15 MΩ) is done prior to recording membrane currents. The currents are recorded at a holding potential of –60 mV. Data acquisition is done with Clampex 9.2 software and stored for offline analysis.

High temperature to activate hTRPV1 is delivered to the individual cells via a DAD-VC Voltage Command and Valve Control System (ALA Scientific Instruments, Farmingdale, NY) coupled to a TC2BIP 2 channel bipolar temperature controller and MPRE8 direct perfusion preheater (Cell MicroControls, Norfolk, VA), which use a computer-controlled micromanifold to quickly release and heat a sample of extracellular solution via an electronic valve. Heated solution is applied for a short 1 s burst, and activation of the hTRPV1 channel is observed as a fast-activating and desensitizing inward current (mostly Ca^{2+} and Na^+ ions). Capsaicin (1 μM) or low-pH 5.2 solutions were delivered to the cells with the same DAD-VC valve control system in 1 s bursts.

Compound Sets

Two sets of compounds were used for comparisons. A small set of 96 compounds was used to first test on the IonWorks voltage ramp assay and determine if it would be sensitive enough for further pharmacologic studies. To compare the higher-throughput capabilities and results of other novel screening methods, a diversity set of 7509 compounds was tested on the voltage-sensitive dye, calcium add-back, capsaicin, and atomic absorption assays. The physical chemical properties of some of the more novel hits identified during this preliminary screening process are presented in

the results section in **Table 1**. All compounds were synthesized by AstraZeneca plc.

Data Analysis and Statistics

Concentration-response curves were generated by fitting data to a four-parameter logistic equation using Prism 4.0 software (GraphPad, San Diego, CA). The percentage effect data were calculated using Microsoft Excel, and visualizations were created using TIBCO Spotfire (TIBCO Software Inc., Palo Alto, CA). For the larger set of compounds, the individual percentage effect in the traditional capsaicin-based FLIPR assay and the various novel assays is plotted against each other to provide a qualitative assessment of the novelty of the hits that were identified. Correlations made between the novel FLIPR assays and the IonWorks-based voltage-ramp assay were calculated using the Spotfire simple linear regression tool. Here, the pIC_{50} values for the assay in question were plotted against the pIC_{50} values for the same compounds tested on the IonWorks platform, and the correlation coefficients were calculated by a least-squares method to give an indication of how closely the two data sets compared with one another.

Results

Gating Properties of TRPV1 Channels

The TRPV1 ligand-gated ion channel is a nonselective cation channel that can be activated by capsaicin, the pungent substance found in chili peppers, as well as low pH and heat >42 °C. Representative whole-cell patch-clamp recordings from the hTRPV1-CHO/Trex stable cell line used for all subsequent experiments are shown in **Supplemental Figures 1a–c**. As first described by Caterina et al.[3] when the molecular identity of the capsaicin receptor was discovered, TRPV1 is also weakly voltage gated, and its current-voltage relationship is characterized by a distinct outward rectification and a reversal potential around 0 mV. These same characteristics were observed in the inducible hTRPV1-CHO/Trex (**Supplemental Fig. 1d**) and –HEK/Trex cell lines (data not shown). The expression level of TRPV1 was such that even without the addition of agonists such as capsaicin or resiniferatoxin, currents elicited by the voltage-ramp protocol described in **Supplemental Figure 1d** were large enough to offer a window for the pharmacologic study of TRPV1 antagonists.

Voltage-Gated TRPV1 Pharmacology

The pharmacology of commonly used TRPV1 antagonists and agonists was studied on the voltage-gated currents elicited by a voltage-ramp protocol that depolarized the cell

Table 1. Characteristics of TRPV1 Antagonists Identified by Novel Screening Paradigms.

Compound No.	Search	MW	ClogP	Number H-Bond Donors	Number H-Bond Acceptors	Molecule PSA ($Å^2$)	No. of Atoms	Solubility (mM)	Capsaicin pIC_{50}	IonWorks pIC_{50}
AZ7457	C	377.4	4.97	1	4	53	27	1.7	5.04	6.45
AZ7387	A, C, V	355.4	3.3	1	8	95	26	6.2	5.12	6.85
AZ7194	A, C, V	373.3	3.95	1	5	61	27	5.4	5.23	6.22
AZ7117	A, C	385.5	3.68	2	5	66	29	3.4	5.64	6.05
AZ7452	C	321.4	4.2	1	4	44	24	16.5	5.91	6.65
AZ7466	A, C	348.2	4.02	1	4	44	23	6.2	5.93	6.52
AZ7118	A, C	365.5	3.92	2	5	66	27	7.1	5.94	6.43
AZ7448	C, V	400.2	3.52	2	6	85	27	16.7	6.10	6.56
AZ7514	V	351.5	3.17	2	5	66	26	29.5	6.24	6.27
AZ7449	A	405.4	3.18	1	6	70	29	15.0	6.44	6.05
AZ7090	A, C, V	419.3	3.47	1	6	55	29	7.1	6.68	6.93
AZ7400	C, V	402.2	4.18	2	6	85	27	20.6	6.71	7.08
AZ7463	V	402.2	4.18	2	6	85	27	89.2	6.91	7.41
AZ7503	V	416.9	3.85	1	5	62	29	33.6	6.97	6.07
AZ7513	V	398.9	3.71	1	5	62	28	30.7	6.97	6.03
AZ7501	V	394.4	3.26	1	5	62	29	26.9	7.09	7.00
AZ7095	A, V	310.3	3.52	3	4	70	23	78.7	7.23	7.34
AZ7455	C, V	403.2	3.29	1	7	88	25	2.8	7.51	7.05
AZ7473	V	371.4	4.76	1	4	44	27	5.9	7.76	6.68
AZ7499	V	345.3	4.16	2	4	57	25	3.2	8.39	7.93
SB-705498	*	429.2	3.63	2	5	58	26	0.4	6.63	6.73
AMG-517	*	430.4	4.63	1	6	73	30	<0.03	7.93	6.58

C = Ca^{2+} add-back; A = atomic absorption spectroscopy; V = voltage-sensitive dye.

*SB-705498 and AMG-517 are clinical candidates previously described by GSK and Amgen, respectively, and included in this table for comparison.

from −80 mV to +80 mV in 500 ms using an IonWorks automated patch-clamp instrument. The protocol was identical to that used in the preceding manual patch-clamp experiments, except that the stimulation protocol was repeated every second for the duration of the experiment. After seals were made on the instrument and a perforated whole-cell access was achieved, each well on the IonWorks PatchPlate received a single concentration of a given compound, and responses were measured for 30 s. An example of the response to 1.25 μM capsaicin is shown in **Figure 1a**. The current measured at +80 mV was significantly greater in the presence of capsaicin as compared with the basal level, and the EC_{50} value for capsaicin was calculated to be ~1 μM (**Fig. 1b**).

Antagonists, in the absence of any exogenous ligand to stimulate TRPV1 channels, were also capable of blocking the outward current measured at +80 mV. Capsazepine, the prototypical TRPV1 antagonist, was able to block the outward current elicited by the voltage-ramp protocol (**Fig. 1c**). Although the effect was dose dependent, the highest concentration of capsazepine was not capable of completely blocking the voltage-gated TRPV1 current at +80 mV, with a maximal percentage inhibition of ~35% (**Fig. 1d**). Another reference TRPV1 antagonist, ruthenium red, was capable of completely blocking the voltage-gated currents with a calculated IC_{50} of ~13.6 μM (**Fig. 1e**). One of the few TRPV1 antagonists previously described to have differential pharmacology depending on the activation stimulus and species, JYL-1421,[22–24] was also tested on the voltage-gated currents. **Figure 1f** shows that JYL-1421 was not capable of blocking the responses to the voltage-ramp protocol.

Development of Higher-Throughput Assays to Correlate to Voltage-Ramp Pharmacology

Automated patch-clamp instruments generate a wealth of data that are comparable with that of the gold-standard patch-clamp technique in a medium-throughput setting; however, they are still not capable of testing a very large set of compounds in a time- and cost-efficient manner. To circumvent this problem, three novel TRPV1 assays were developed and tested for (1) their potential to identify novel chemical entities compared with what more traditional capsaicin-based Ca^{2+} flux assays are capable of identifying and (2) their correlation with the voltage-gated pharmacology generated on the IonWorks assay. To develop these
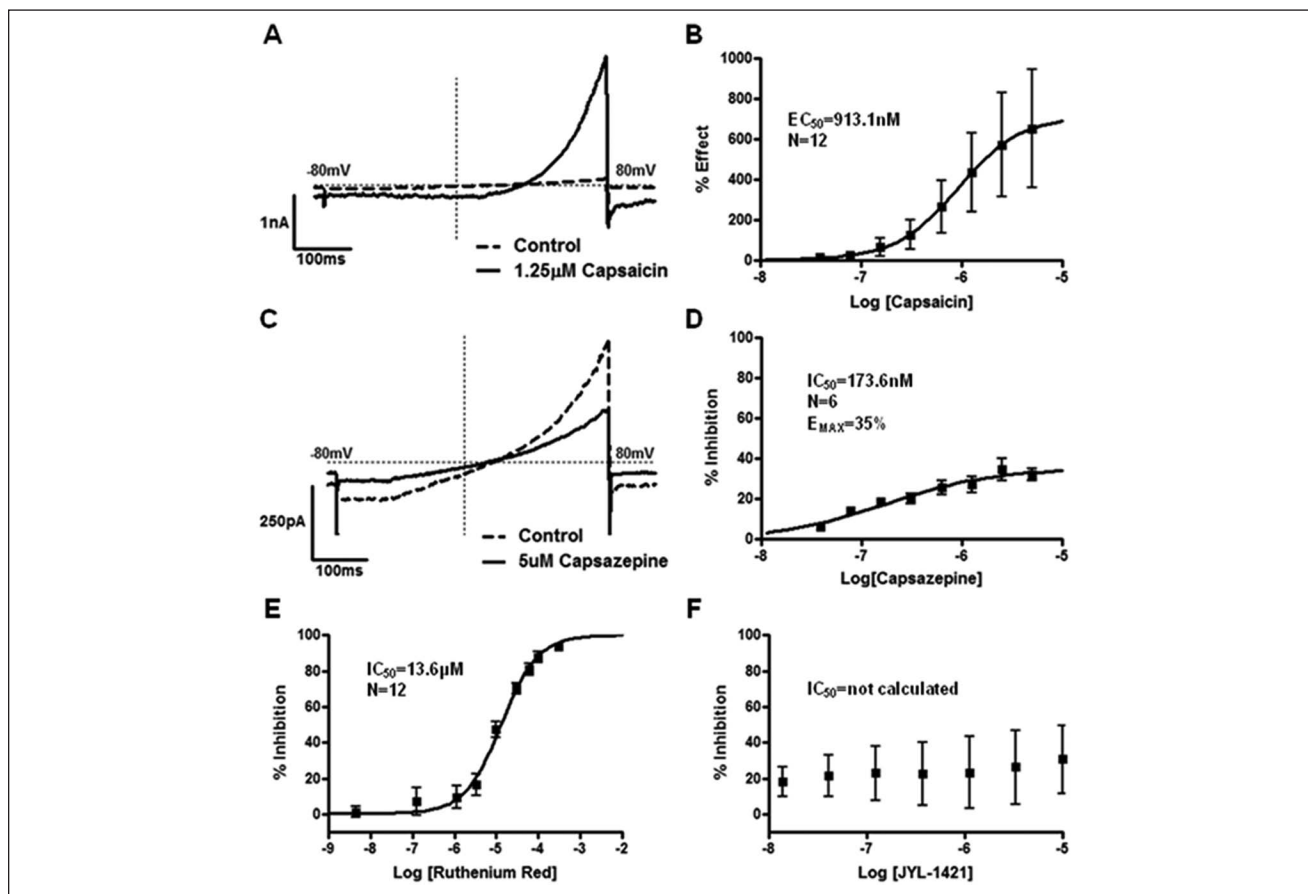
Figure 1. (**A**) Current response to voltage-ramp stimulus on hTRPV1-CHO/Trex cell in the absence (dotted line) and presence of 1.25 µM capsaicin (solid line). (**B**) Dose-response relationship of capsaicin applied to hTRPV1-expressing cells using a voltage-ramp stimulus to activate the channels. The peak current amplitude at +80 mV was used to measure capsaicin potency. (**C**) Basal response (dotted line) and in the presence of 5 µM capsazepine (solid line) for voltage-ramp stimulus on hTRPV1-expressing cell. (**D**) Dose-response relationship for capsazepine using the current amplitude at +80 mV as a measure of efficacy. Capsazepine was able to completely inhibit the voltage-evoked TRPV1 currents. Dose-response relationships for ruthenium red (**E**) and JYL-1421 (**F**) using the voltage-ramp protocol. Ruthenium red completely inhibited voltage-gated TRPV1 currents, whereas JYL-1421 had no effect.

comparisons, two sets of compounds were tested on the novel assays. Set A contained 7509 compounds that represented ~75% of the diversity found within the AstraZeneca HTS library and adhered to Lipinski's rules for compounds with druglike properties.[25] Set B contained 96 compounds that were known TRPV1 modulators, along with the compounds identified from each of the Set A screens with a percentage inhibition >50% from the novel assays.

A voltage-sensitive dye-based assay was developed using pH 5.2 as a stimulus. The voltage-sensitive dye captures any changes to the resting membrane potential, and TRPV1 channels are activated prior to compound addition, representing a more physiological scenario when channels would be activated prior to compound being available. **Figure 2a** shows a typical example of the responses generated on the FLIPR 384-well instrument. The results from screening Set A with the voltage-sensitive dye assay are

presented in **Figure 2b**. Of the 7509 compounds screened at a single concentration of 10 µM, 86 were found to have an inhibition >50%. Only two of these compounds were also found to have an inhibition >50% on the more traditional capsaicin-induced Ca^{2+}-flux assay, indicating that the voltage-sensitive dye assay was capable of identifying TRPV1 antagonists with novel pharmacology. To assess whether the voltage-sensitive dye assay would be an appropriate HTS assay to reliably predict potency values on the voltage-gated IonWorks assay, the 86 novel compounds were included as part of the set B compounds that were tested in a full dose response in both assays. The correlation of pIC_{50} values obtained with the two methods is plotted in **Figure 2c**. The two assays had a significant correlation coefficient of $r^2 = 0.423$; however, there were several compounds in the lower quadrants (red, VSD $pIC_{50} < 5$) that could potentially represent false-negatives. These compounds were not active
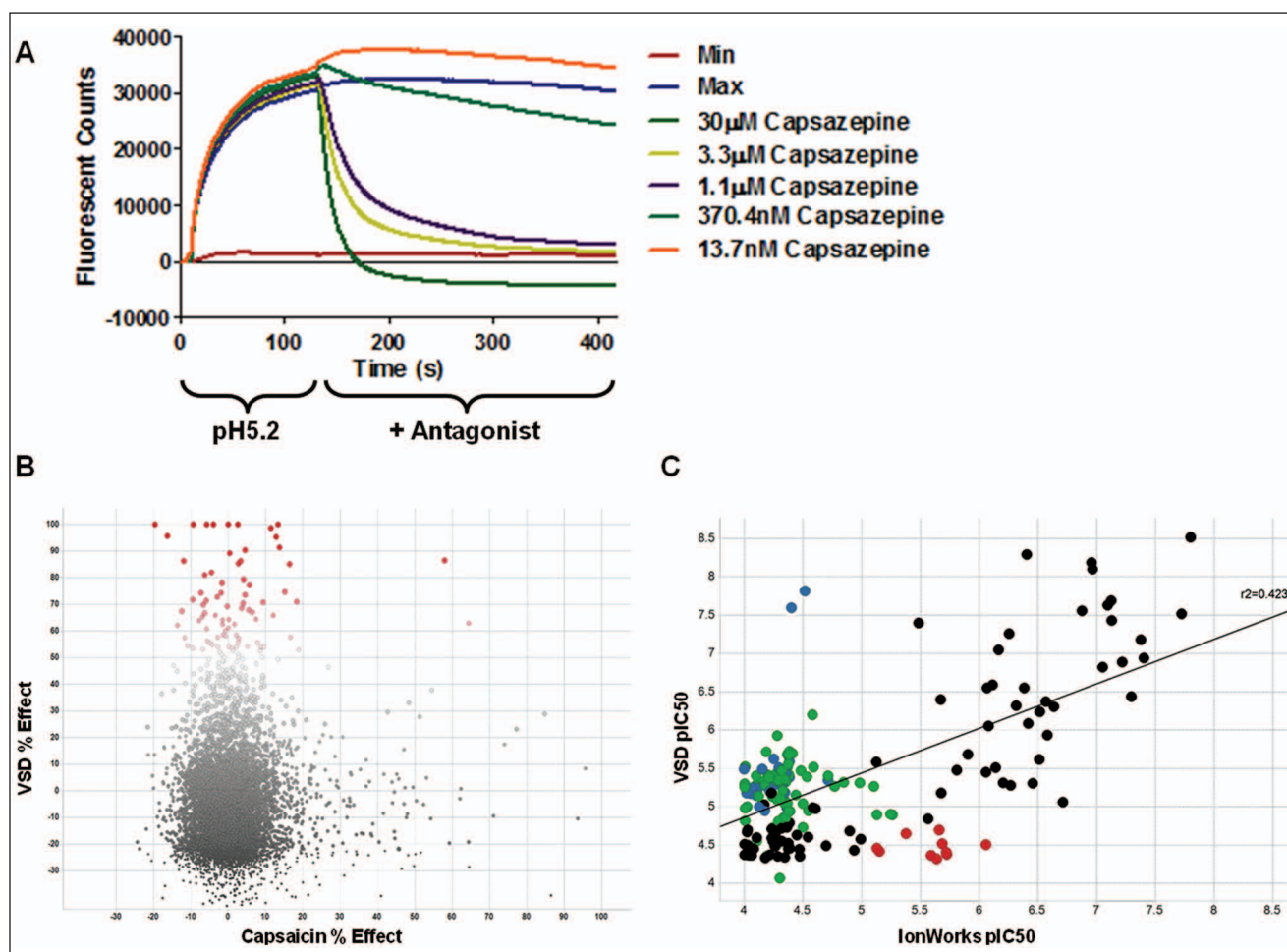
Figure 2. (**A**) Typical response of hTRPV1-HEK/Trex cells to the application of a pH 5.2 solution measured with a voltage-sensing fluorophore on a fluorescent imaging plate reader instrument. Various concentrations of the TRPV1 antagonist capsazepine were applied after channel stimulation and fluorescence decreases indicating an inhibition of channel activity. (**B**) Comparison of the percentage inhibition on the pH 5.2–elicited responses measured using a voltage-sensitive dye with the percentage inhibition on 100 nM capsaicin-elicited responses measured with a Fluo-4 Ca^{2+}-sensing fluorophore. A single concentration of 10 μM was used for all of the compounds tested. (**C**) Correlation between the pIC_{50} values for inhibiting the pH 5.2–elicited responses measured with a voltage-sensitive dye and the agonist-independent voltage-gated responses measured with an IonWorks automated patch-clamp system. The compounds tested were a mix of known TRPV1 modulators and compounds identified in **B** with a >50% inhibition on the voltage-sensitive dye–based assay. Compounds highlighted in red are considered false-negatives, blue are false-positives, and green are the novel compounds never identified by a previous capsaicin-based assay.

in the voltage-sensitive dye assay but were considered active on the IonWorks assay. Conversely, there were also several compounds in the left-hand quadrants (blue, IonWorks $pIC_{50} < 5$) that were considered false-positives.

TRPV1 is sensitive to intracellular calcium concentrations,[26] and binding of Ca^{2+}/calmodulin to the TRPV1 receptor plays an important role in channel desensitization after activation by capsaicin. Another TRP family member, TRPA1, has also been described to be sensitive to extracellular Ca^{2+} concentrations, where higher levels are capable of increasing channel gating.[27] We sought to determine if elevated extracellular Ca^{2+} concentrations could modulate TRPV1 in a similar

fashion to TRPA1. **Figure 3a** shows the response of TRPV1-expressing cells to an elevated 4.8 mM Ca^{2+} concentration after being incubated in the presence of low 0.016 μM extracellular Ca^{2+}. This response was not identified in nontransfected HEK293s cells nor in the noninduced hTRPV1-HEK/Trex cell line used for these experiments (data not shown). This calcium add-back assay was used in a similar screening paradigm as the voltage-sensitive dye method. Ten compounds were found to inhibit the Ca^{2+}-induced responses >50% (**Fig. 3b**). Of these, five were previously identified using the traditional capsaicin-based FLIPR screen. Correlation of the data generated by the calcium add-back method to the
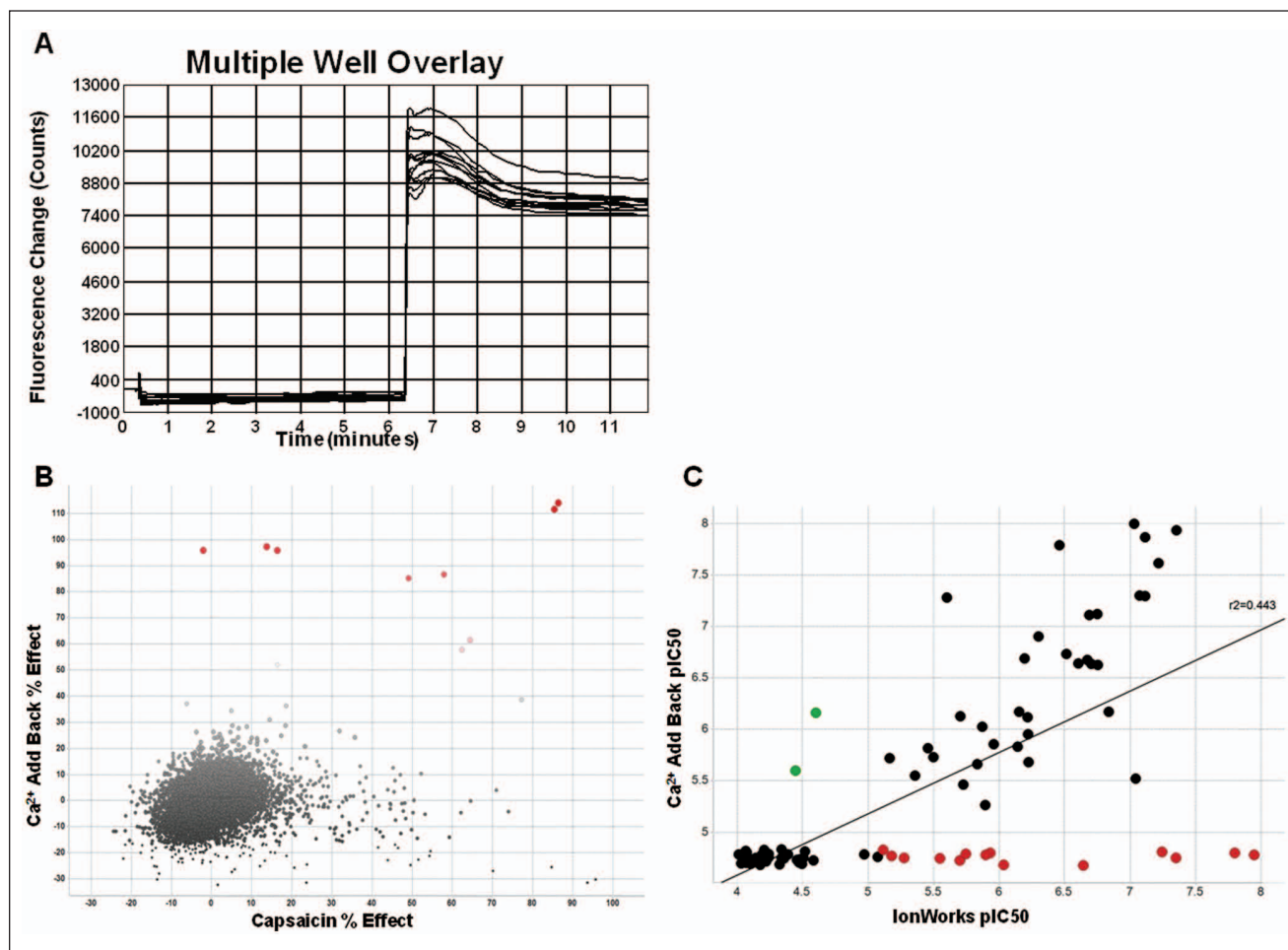
Figure 3. (**A**) Example of responses elicited by the addition of 4.8 mM CaCl to hTRPV1-HEK/T-rex–expressing cells that were loaded with a Fluo-4 Ca^{2+}-sensing dye in the virtual absence of Ca^{2+}. (**B**) Comparison of the percentage inhibition of responses elicited when a high concentration of CaCl (4.8 mM) is applied to hTRPV1-HEK/Trex–expressing cells with the percentage inhibition on 100 nM capsaicin-elicited responses. Both are measured with a Fluo-4 Ca^{2+}-sensing fluorophore. A single concentration of 10 µM was used for all of the compounds tested. (**C**) Correlation between the pIC_{50} values for inhibiting the calcium add-back responses and the agonist-independent voltage-gated responses measured with an IonWorks automated patch-clamp system. The compounds tested were a mix of known TRPV1 modulators and compounds identified in **A** with a >50% inhibition on the calcium add-back assay. Compounds highlighted in red are considered false-negatives, blue are false-positives, and green are the novel compounds never identified by a previous capsaicin-based assay.

voltage-gated IonWorks method using the set B compounds found a significant correlation coefficient of $r^2 = 0.443$ (**Fig. 3c**). However, several compounds were found in the lower two quadrants of the correlation (red, CAB $pIC_{50} < 5$), including some with very high potency on the IonWorks assay ($pIC_{50} > 7$), indicating that the calcium add-back method might have the risk of generating too much false-negative data.

A third assay to screen for novel TRPV1 antagonists was developed using an atomic absorption spectroscopy-based rubidium flux method. Initially, the assay was developed to mimic the responses of TRPV1 channels to the voltage-ramp stimulus by adding a high concentration of KCl to depolarize the cells after loading with Rb^+. Because cells normally treat Rb^+ in a similar fashion to K^+ ions, it was anticipated that

measuring the effluxed Rb^+ in the assay media after depolarization would give an indication of TRPV1 channel activation. Contrary to this expected response, no increase in Rb^+ was detected in the extracellular assay media. While investigating the cause for this discrepancy, it was determined that TRPV1 antagonists such as ruthenium red and capsazepine were capable of inhibiting the uptake of Rb^+ in TRPV1-expressing cells in a dose-dependent fashion. Although Rb^+ is readily absorbed by nontransfected HEK cells in the absence of TRPV1, it appears as if Rb^+ preferentially conducts through the TRPV1 channel when it is expressed, even in the absence of any other agonist to activate the channel. This unexpected response of TRPV1 channels offered an opportunity to assess novel TRPV1 pharmacology by
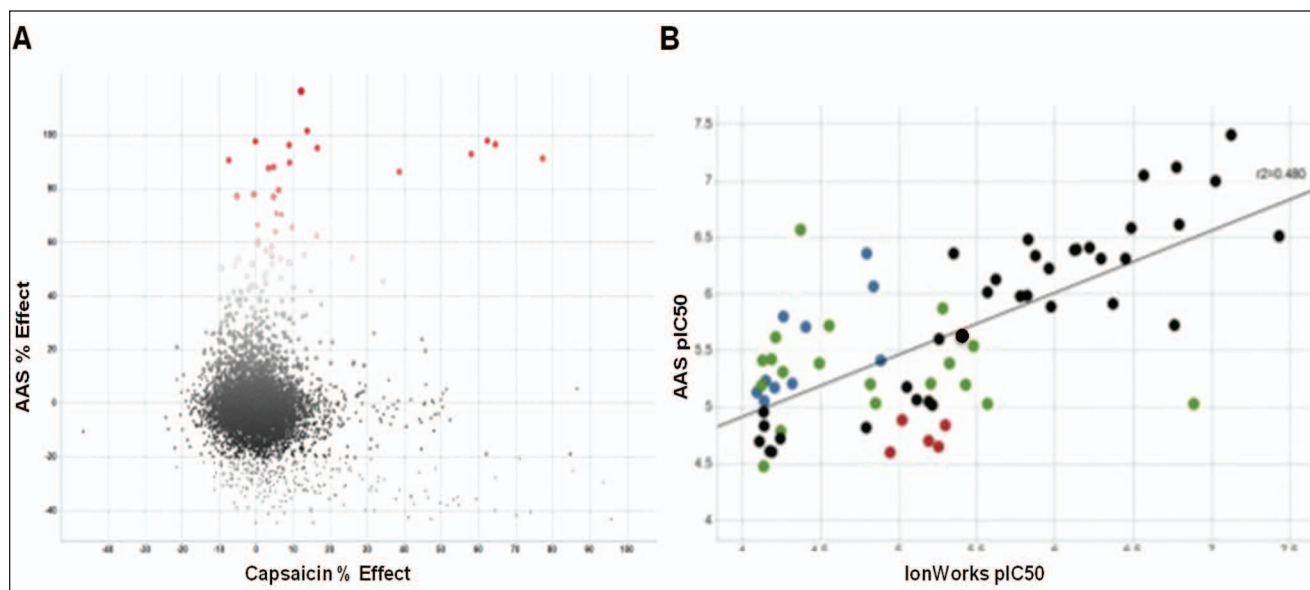
Figure 4. (**A**) Comparison of the percentage inhibition of spontaneous rubidium influx into hTRPV1-CHO/Trex–expressing cells measured by atomic absorption spectroscopy with the percentage inhibition of 100 nM capsaicin-elicited responses measured with a Fluo-4 Ca^{2+}-sensing fluorophore. A single concentration of 10 μM was used for all the compounds tested. (**B**) Correlation between the pIC_{50} values for inhibiting the atomic absorption spectroscopy responses and the agonist-independent voltage-gated responses measured with an IonWorks automated patch-clamp system. The compounds tested were a mix of known TRPV1 modulators and compounds identified in **A** with a >50% inhibition on the atomic absorption spectroscopy assay. Compounds highlighted in red are considered false-negatives, blue are false-positives, and green are the novel compounds never identified by a previous capsaicin-based assay.

measuring the amount of Rb^+ capable of entering the cell after an antagonist preincubation. When screening the Set A collection of compounds, 39 compounds were found to inhibit Rb^+ influx by >50% (**Fig. 4a**). Of these, only four were previously identified by traditional capsaicin-based screening methods. Comparing results from screening the set B compounds on both the atomic absorption method and the voltage-ramp method resulted in a correlation coefficient of $r^2 = 0.48$ (**Fig. 4b**). Although several compounds were found in the lower-left quadrant that represent false-negative values (red, atomic absorption spectroscopy $pIC_{50} < 5$), these represented only ~27% of the total number of compounds, compared with ~55% in the voltage-sensitive dye method (**Fig. 2c**).

TRPV1 Antagonists Identified by Novel Screening Paradigm Independent of Capsaicin

Examples of the chemical properties for some compounds identified through the novel screening paradigms described above are listed in **Table 1**. Several of these compounds were not previously identified with the more traditional capsaicin-induced Ca^{2+}-flux assays and were some of the more potent molecules found with the novel assays. All

compounds adhere to the Lipinski Rule of 5 in terms of druglike properties.[25] Two of the TRPV1 antagonists tested recently in clinical trials, AMG-517[11] and SB-705498,[12] are included in the table for comparison. In general, solubility is improved for the novel compounds compared with these two clinical candidates. The pIC_{50} values from the traditional capsaicin-based FLIPR assay and the IonWorks voltage-gated assay can be found in the last two columns of **Table 1**. It is interesting to note that several compounds exhibit a very different pharmacologic profile depending on the stimulus used to activate TRPV1 channels, and therefore the ability of a compound to inhibit the channel voltage-gated opening independent of any other agonist stimulation or shift in voltage dependence of activation should be added to the profiles of TRPV1 antagonists described previously.

Discussion

We have described the development of a novel assay to screen for TRPV1 antagonists using the previously reported voltage dependence for activation of the channel as a pharmacodynamic endpoint. Studying the pharmacology of known modulators on TRPV1 voltage-gated currents indicated that another dimension could be added to the previously described pharmacologic profiles for antagonists.[28]

Although compounds have been differentiated before in terms of their ability to modulate capsaicin, low pH, and heat stimuli,[28] we have demonstrated that TRPV1 antagonists also possess differential effects on antagonizing agonist-independent voltage-gated responses of the channel. Some compounds such as ruthenium red are capable of fully antagonizing the voltage-evoked responses, whereas others such as capsazepine were very weak and partial antagonists.

Realizing this might offer new opportunities from a drug-discovery perspective, we sought to determine whether the voltage-gated activation of TRPV1 could be used to identify undiscovered chemical space in the otherwise relatively crowded patent literature[6] and improve on some of the chemical liabilities related to pharmacokinetic profiles and solubility that the majority of known TRPV1 antagonists possess. Although the pharmacodynamic effect on the voltage dependence of TRPV1 activation can be studied using automated patch-clamp instruments such as the IonWorks platform, this type of assay is not well suited for true high-throughput screening because its lower throughput and cost constraints. We therefore developed three novel assays capable of screening for TRPV1 antagonists independently of using capsaicin as an agonist to activate the channel. A medium-sized compound library was used to test each assay for its ability to identify new chemical starting points that had not been identified by more traditional capsaicin-based screening platforms.

Each of the three assays proved capable of finding such compounds, with the assays based on the voltage-sensitive dye detection of pH elicited responses and the spectroscopic atomic absorption of Rb^+ retained in cells after spontaneous flux through TRPV1 giving the greatest number of new potential inhibitors (86 and 39, respectively). The ultimate goal was to return to the voltage-gated assays and compare these novel assays to determine which one would best predict the pharmacology on the automated patch-clamp assay. All three novel assays had similar correlation coefficients ($r^2 = 0.423$ to 0.480). However, when looking at the distribution of the hits using TIBCO Spotfire visualization software, there was a very high incidence of false-negatives in the Ca^{2+} add-back assay. Although the goal of an HTS campaign is to trim the number of compounds down to a manageable set that can be profiled in greater depth, having the potential for too many false-negatives also runs the risk of not being able to identify the most ideal structures for future research. Furthermore, by screening the set of ~96 compounds, 11 of 26 compounds that had a $pIC_{50} > 6$ on the voltage-gated assay were not identified using the Ca^{2+} add-back HTS method, indicating that some highly potent antagonists could also be missed. In contrast, the voltage-sensitive dye method tended to identify several false-positive hits, with several compounds being inactive in the
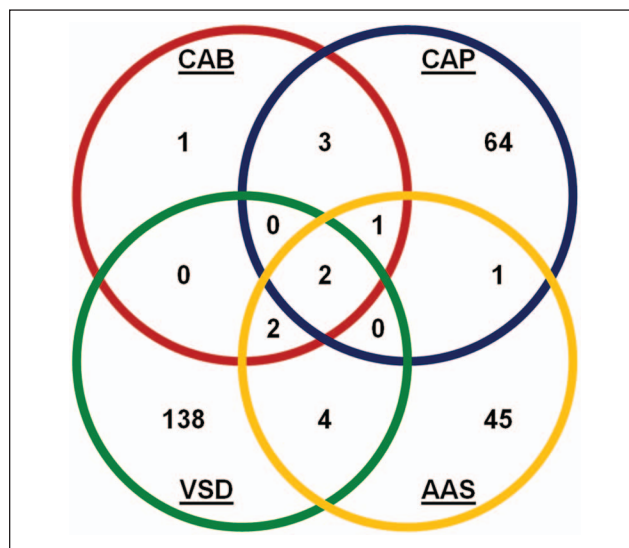

Figure 5. Venn diagram to highlight the differences and similarities in the compounds identified by the three novel assays and the traditional capsaicin-based assay. CAPS = capsaicin-based assay; VSD = voltage-sensitive dye–based assay; CAB = Ca^{2+} add-back assay; atomic absorption spectroscopy = the atomic absorption spectroscopy–based Rb^+ flux assay.

automated patch-clamp assay (**Fig. 2b**). The atomic absorption spectroscopy–based assays did not have the same issue of generating false-negative data compared with the Ca^{2+} add-back assay, had fewer than the voltage-sensitive dye assay, and had the best correlation with the automated patch-clamp assay. The atomic absorption spectroscopy–based assay was able to positively identify 11 of 12 compounds that also had a $pIC_{50} > 6$ on the voltage-gated assay compared with 17 of 26 with the voltage-sensitive dye method.

Several compounds were identified by these novel assays that had previously not been identified in capsaicin-based HTSs. Therefore, after their identification, they were further characterized using the more traditional capsaicin-based FLIPR assay to verify their potency. Data in **Table 1** indicate that although some of the compounds were first identified by the novel assays described here, they still maintained a good potency in the capsaicin-based assay. However, several compounds were characterized by a significant difference in terms of the potency to inhibit the voltage-gated activation of TRPV1 compared with the capsaicin activation. These represent a novel profile of TRPV1 antagonists that should be added to those described previously.[28] The differences and relationship of the novel pharmacology identified using the three novel screening assays can be visualized in the Venn diagram presented in **Figure 5**. Each assay was capable of identifying supposed inhibitors of TRPV1 using their respective endpoint measures; however, there is very little overlap between these novel pharmacologies.

The voltage-gated activation of TRPV1 is not a novel concept. In fact, it has been well documented that while capsaicin and resiniferatoxin reveal a voltage-independent gating for TRPV1, the more natural ligands such as protons (low pH) and heat shift the voltage dependence of gating to more physiological negative potentials.[13,29] An allosteric model to describe the synergy between voltage gating and other independent stimuli to regulate channel gating has already been described.[30] Several molecular determinants responsible for the proton- and heat-dependent shifts in the voltage dependence of activation have been described, including F660[31] in the S6 transmembrane domain and I696, W697, and Q700 in the TRP box.[32] It would be interesting to assess the types of molecular interactions the compounds identified here have with TRPV1 and determine if they have a better profile than some of the previous TRPV1 antagonists, particularly with respect to causing hyperthermia in vivo.[33] In addition, because TRPV1 is hypothesized to be a target for the treatment of chronic inflammatory pain, the analgesic potential of these compounds should be investigated and will be the subject of future research.

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Declaration of Conflicting Interests

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