Supplemental material to this article can be found at: https://doi.org/10.1124/molpharm.121.000271.

High-Throughput Screening of TRPV1 Ligands in the Light of the Bioluminescence Resonance Energy Transfer Technique

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Received March 5, 2021; accepted May 17, 2021

ABSTRACT

Ion channels are attractive drug targets for many therapeutic applications. However, high-throughput screening (HTS) of drug candidates is difficult and remains very expensive. We thus assessed the suitability of the bioluminescence resonance energy transfer (BRET) technique as a new HTS method for ion-channel studies by taking advantage of our recently characterized intra- and intermolecular BRET probes targeting the transient receptor potential vanilloid type 1 (TRPV1) ion channel. These BRET probes monitor conformational changes during TRPV1 gating and subsequent coupling with calmodulin, two molecular events that are intractable using reference techniques such as automated calcium assay (ACA) and automated patch-clamp (APC). We screened the small-sized Prestwick chemical library, encompassing 1200 compounds with high structural diversity, using either intra- and intermolecular BRET probes or ACA. Secondary screening of the detected hits was done using APC. Multiparametric analysis of our results shed light on the capability of calmodulin inhibitors included in the Prestwick library to inhibit TRPV1 activation by capsaicin. BRET was the lead technique for this identification process. Finally, we present data exemplifying the use of intramolecular BRET probes to study other transient receptor potential (TRP) channels and non-TRPs ion channels. Knowing the ease of use of BRET biosensors and the low cost of the BRET technique, these assays may advantageously be included for extending ion-channel drug screening.

SIGNIFICANCE STATEMENT

This study screened a chemical library against TRPV1 ion channel using bioluminescence resonance energy transfer (BRET) molecular probes and compared the results with the ones obtained using reference techniques such as automated calcium assay and automated patch-clamp. Multiparametric analysis of our results shed light on the capability of calmodulin antagonists to inhibit chemical activation of TRPV1 and indicates that BRET probes may advantageously be included in ion channel drug screening campaigns.

Introduction

Ion channels are pore-forming membrane proteins allowing ions to flow across membranes. Widely regarded as attractive drug targets for many therapeutic applications, ion channels are the second largest class of membrane proteins for drug discovery behind G protein–coupled receptors (GPCRs). They account for worldwide sales above $18 billion, which highlights their tractable nature (Wickenden et al., 2012; Global data, 2020). Nonetheless, despite its commercial potential and academic relevance, in vitro pharmacological profiling of most ion channels remains unaddressed (Bagal et al., 2015).

Automated patch-clamp (APC) is rapidly emerging and provides increased throughput screening of ion channel targets (Obergrussberger et al., 2018), but it remains expensive

Reference:

https://doi.org/10.1124/molpharm.121.000271

[This article has supplemental material available at molpharm.aspetjournals.org.]
and requires expert handling (Terstappen et al., 2010; Yu et al., 2016). As a consequence, indirect readout technologies are often used for initial screening to be later confirmed by APC. Generally, these techniques take advantage of fluorescent probes to monitor changes in membrane potential or concentration of cytoplasmic ions such as calcium (Terstappen et al., 2010; Yu et al., 2016; McGivern and Ding, 2020). Such assays give only an indirect readout of channel activity, since they monitor molecular mechanisms that are spatially or temporally distant from the studied channel with the risk that the tested compound could up- or downmodulate nonspecific targets. They are therefore prone to a high yield of false positives (Clare, 2010). This drawback can be bypassed by measuring events proximal to the studied ion channel once activated.

For the last twenty years, resonance energy transfer–based techniques have revolutionized molecular pharmacology and biochemistry, allowing measurement of protein-protein interaction and protein conformational changes in real-time in live cells (Miyawaki and Niino, 2015). These techniques are based on the nonradiative intra- or intermolecular transfer of energy between an energy donor and a compatible fluorescent energy acceptor. Such quantum mechanism strictly relies on molecular proximity (around 100 Å) and orientation between donor and acceptor molecules for energy transfer, making it ideal for probing either protein conformational changes or the dynamic of protein-protein interactions. Independence from an external energy source for donor excitation gives bioluminescence resonance energy transfer (BRET) some advantages over related methods, such as fluorescence resonance energy transfer, by avoiding cells photodamage, fluorophore photo-bleaching, background autofluorescence, or direct acceptor excitation (Pfleger et al., 2006). Thanks to these advantages, BRET assays have been widely implemented for GPCR and kinases drug screening (Bacart et al., 2008; Kocan and Pfleger, 2011; Schann et al., 2013; Ayoub, 2016).

Ironically, although ion channels have been perceived as the next GPCR for the last 15 years, according to their importance as a drug target (Kaczorowski et al., 2008), they only recently benefited from BRET technology (Robertson et al., 2016; Ruigrok et al., 2017). Such BRET probes monitor molecular events related to ion-channel activation (conformational changes during gating and protein-protein interactions dynamics) that are of utmost importance for ion channel pharmacology while being intractable using either one of the aforementioned reference techniques for ion-channel HTS. They, therefore, opened up new prospects for improving the effectiveness of ion-channel drug screening. Nonetheless, acceptance of intra- and intermolecular BRET assays as novel tools for ion-channel drug screening relies on their efficiency with regards to conventional methods and need therefore a solid proof-of-concept of their operability and effectiveness under real drug screening conditions.

Here, we assessed the suitability of the BRET technique as a new HTS method for ion channels by taking advantage of our recently characterized intra- and intermolecular BRET probes targeting TRPV1 conformational changes during gating and subsequent coupling with calmodulin (CaM), two events leading to TRPV1 activation and regulation (Ruigrok et al., 2017). We then screened the small-sized Prestwick chemical library, encompassing 1200 FDA- and EMA-approved compounds with high structural diversity using either automated calcium assays (ACAs) or our intra- and intermolecular BRET probes. We next performed a secondary screen of the detected hits with an APC. Multiparametric analysis of our results put into light the power of the BRET technique to unravel hits compounds that would not have been detected with conventional methods such as ACA and APC. Finally, we present data exemplifying the use of intra-molecular BRET probes for the study of other TRPs and non-TRPs ion channels.

Materials and Methods

Plasmids. The mammalian expression vector encoding sYFP2-TRPV1-Luc2, TRPV1-Luc2, and sYFP2-CaM were described in (Ruigrok et al., 2017). To generate the other BRET constructs, mNeonGreen and nanoluciferase were used to improve the brightness of the assay in some experiments, and are referred as mNeonG and nLuc for short in the rest of this paper. Mammalian expression vectors for the expression of the nLuc-ion channel-mNeonG or mNeonG-ion channel-nLuc fusion proteins were constructed using cDNA bricks obtained by gene synthesis (Genescript, Leiden, Netherlands) that encode, respectively, nLuc or mNeonGreen (brick 1), any ion channel described in the text (brick 2), and mNeonGreen or nLuc (brick 3). Using the BsmBI type IIS enzyme and T4 DNA ligase, the cDNA Bricks were assembled in frame and in the right order into pcdNA 3.1(+)-Lac Z vector, which allowed direct visualization of the assembly efficiency using a colorimetric test based on alpha complementation (Supplemental Table 1). Briefly, a ligation mix containing 2 μl of T4 DNA ligase buffer 10X, 1 U of BsmBI enzyme, 1 U of T4 DNA ligase, 0.4 mM ATP, 4 mM dithiothreitol, or direct acceptor excitation (Pfleger et al., 2006). Thanks to these advantages, BRET assays have been widely implemented for GPCR and kinases drug screening (Bacart et al., 2008; Kocan and Pfleger, 2011; Schann et al., 2013; Ayoub, 2016).

Ironically, although ion channels have been perceived as the next GPCR for the last 15 years, according to their importance as a drug target (Kaczorowski et al., 2008), they only recently benefited from BRET technology (Robertson et al., 2016; Ruigrok et al., 2017). Such BRET probes monitor molecular events related to ion-channel activation (conformational changes during gating and protein-protein interactions dynamics) that are of utmost importance for ion channel pharmacology while being intractable using either one of the aforementioned reference techniques for ion-channel HTS. They, therefore, opened up new prospects for improving the effectiveness of ion-channel drug screening. Nonetheless, acceptance of intra- and intermolecular BRET assays as novel tools for ion-channel drug screening relies on their efficiency with regards to conventional methods and need therefore a solid proof-of-concept of their operability and effectiveness under real drug screening conditions.

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Reagents. Ovotan, OLDA, resinfetoxatin (RTX), AMG517, AMG9810, BotC, JNJ-17290212, and AMG21629 were all from Tocris (Bristol, UK). Capsaicin (CAPS) and capsazepine (CPZ) were from Sigma (Lyons, France). Coelenterazine H (NanoLight, Technology, Pineton, AZ) was added to a final concentration of 5 μM. The Prestwick chemical library, a collection of off-patent drugs with high chemical and pharmacological diversity, has been obtained from Prestwick Chemical Inc (Ilkirch-Graffenstaden, France; https://www.prestwickchemical.com/screening-libraries/prestwick-chemical-library/). Of note, we used the previous version of that library with 1200 compounds. Indeed, this library is regularly updated by adding new compounds of interest and removing others.

Cell Culture and Transfections for BRET Assays. HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium high glucose (catalog number D6429; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and streptomycin. HuH7 cells were maintained in Dulbecco’s modified Eagle’s medium low glucose (catalog number 3185502; Thermo Fisher Scientific, Waltham, MA) supplemented with 1% glutamax (catalog number 35050-068; Thermo Fisher Scientific), 10% fetal bovine serum, 100 units/mL penicillin, and streptomycin.

For BRET assays performed in 96-well plates, cells were seeded at a density of 500,000 cells in 6-well dishes, 24 hours before transfection. Transient transfections were performed using polyethylenimine
(PEI, linear, Mr 25,000; catalog number 23966 Polysciences, War-RING et al., 2009). For intramolecular BRET assays, HEK293T cells were transfected with 0.1 μg of sYFP2-TRPV1-rLuc2 and 1.9 μg of empty pcDNA3.1(−) vector, whereas HEK293T cells were transfected with 0.1 μg of TRPV1-rLuc2 expression vector and 1.9 μg of sYFP2-CaM expression vectors for intermolecular BRET assays. After overnight incubation, transfected cells were detached and resuspended in DMEM without red phenol (catalog number 21063-029; ThermoFisher Scientific) containing 10% fetal bovine serum and 100 units mL⁻¹ penicillin and streptomycin before being seeded at 10⁵ cells per well in 96-well white plates (ref 655093, Greiner Bio One, Courtabeuf, France). Cells were left in culture for an additional 24 hours before being processed for BRET assay.

BRET assays in high-throughput conditions were performed in 384-well plates using either HEK293T cells transfected with TRPV1-rLuc2 and sYFP2-CaM or HuH7 cells transfected with sYFP2-TRPV1-rLuc2. Briefly, on the day of the transfection, HEK293T and HuH7 cells were rinsed, detached, and resuspended at a density of 350,000–375,000 cells/mL in DMEM without phenol red (catalog number 11880028; ThermoFisher Scientific) supplemented with 1% glutamax, 5% fetal bovine serum, 100 units mL⁻¹ penicillin, and streptomycin. Two DNA mix containing either 50 ng/mL of the hTRPV1-rLuc2 expression vector, 200 ng/mL of the sYFP2-Calmodul ine expression vector, and 750 ng/mL of noncoding salmon sperm DNA for the intramolecular BRET assay, or 800 ng/mL of YFP-hTRPV1-Luc expression vector and 200 ng/mL of salmon sperm DNA for the intramolecular BRET assay, were prepared in 150 mM NaCl and mixed with an equal volume of PEI, 3 times more concentrated than total DNA [i.e., 3:1 (w/w) PEI:DNA ratio]. The DNA/PEI mix was then incubated for 15 minutes at room temperature before being added to the corresponding cell suspension at a ratio of 1:10 (v/v). HuH7 cells were then seeded directly into white opaque 384-well microplates (catalog number 781080; Greiner Bio One SAS, Les Ulis, France) at a rate of 20 μL per well, i.e., 7,500 cells per well, and were left in culture for an additional 24 hours before being processed for BRET assay. HEK293T cells were seeded into a 75-cm² flask (catalog number 15632011; Invitrogen) and were left in culture for an additional 24 hours before being detached and resuspended in equilibration buffer (NaCl 145 mM, KCl 5 mM, KH₂PO₄ 4 mM, CaCl₂ 1 mM, MgSO₄ 1 mM, glucose 10 mM, pH 7.5) at a density of 750,000 cells mL⁻¹. A 384-well plate was then filled with 20 μL (15,000 cells) of the cell suspension per well and left for equilibration for 1 hour at 22° in the dark before being processed for BRET assay.

**BRET Measurement in 96-Well Plates.** After the addition of Coelenterazine H into the red-phenol-free cell culture medium at a final concentration of 5 μM, BRET signals were measured using a multidector TriStar2 LB942 microplate reader (Beberthold Technologies, Bad Wildbad, Germany) and emission filters centered at 540 ± 20 nm for YFP and 480 ± 20 nm for Luc, or 515 ± 40 nm for mNeon-Green and 460 ± 20 nm for nLuc.

The BRET signal was determined by calculating the ratio of the emission intensity measured in the acceptor window (I_acceptor) over the emission intensity measured in the donor window (I_donor), according to eq. 1:

\[
BRET = \frac{I_{acceptor}}{I_{donor}} \quad (1)
\]

Because of the overlapping emission spectra of Luc and YFP, a fraction of the light detected in the YFP filter originates from the Luc emission, resulting in a contaminating signal [Hamdan et al., 2006]. In that configuration, the net BRET was therefore defined as the BRET ratio of cells coexpressing Luc and YFP constructs minus the BRET ratio of cells expressing only the Luc construct in the same experiment. To assess the functionality of the ion channel BRET-based probes, Coelenterazine H was added to the cell culture medium 5 minutes before the injection of agonists and antagonists and subsequent BRET readings. In these experiments, the maximal quantity of DMSO was 0.3%. All experiments were performed at 37° C and pH 7.4 unless otherwise indicated.

**Concentration-Response and Drug Screening Using Intramolecular and Intermolecular BRET Assays in 384-Well Plates.** Screening of the Prestwick chemical library was performed at 22° C (intramolecular test) or 37° C (intramolecular test) using a 2-step injection protocol. One minute after the injection of Coelenterazine H in each well (10 μL, 5 μM final) to initiate the bioluminescent reaction catalyzed by the luciferase enzyme, 10 μL of the tested compounds (15 μM final) or vehicle alone was injected to assess each compound ability to activate TRPV1 (hereafter designated as activation mode). BRET measurements were performed 5 minutes (intermolecular BRET assay) or 15 minutes (intramolecular BRET assay) after compound injection using an EnVision Multimode Plate Reader (Perkin Elmer, Villebon-sur-Yvette, France) with emission filters centered at 535 ± 15 nm for YFP (I_acceptor) and 480 ± 15 nm for Luc (I_donor). Immediately after this first BRET measurement, 20 μL of the prototypic agonist CAPS (final concentration: 500 nM) was injected in each well to assess the ability of each compound to inhibit chemical activation of TRPV1 (hereafter designed as “inhibition mode”). A second BRET measurement was then performed after CAPS injection. In the inhibition mode, the final concentration of the tested compound was 10 μM.

The data in the activation mode are expressed according to eq. 2, and data in the inhibition mode are expressed according to eq. 3.

\[
\text{% of CAPS effect} = 100 \times \frac{BRET_{\text{com}p} - BRET_{\text{base}}}{BRET_{\text{max} \text{-} BRET_{\text{base}}}} \quad (2)
\]

\[
\text{% of inhibition} = \frac{BRET_{\text{com}p} - BRET_{\text{CAPS}}}{BRET_{\text{base}} - BRET_{\text{CAPS}}} \times 100 \quad (3)
\]

where BRET_comp is the net BRET in the presence of the compound, BRET_base is the basal BRET before injection of the compounds, BRET_CAPS is the net BRET measured in presence of 500 nM and 15 μM capsaicin, respectively. Two independent runs (n1 and n2) were performed, and an arbitrary percent activation or inhibition cutoff of 30% was chosen to select hit compounds. A counter screen was performed using HEK293T cells transfected with TRPV1-rLuc2 alone. The compound effects on TRPV1 intramolecular and intermolecular BRET probes were validated only if the basal BRET of TRPV1-rLuc2 remained unaffected during the counter screen step.

In all experiments performed in 384-well plates, the final quantity of DMSO was 1% in the activation mode and 0.87% in the inhibition mode. Reference compounds effects were assessed both in the activation and inhibition modes using identical protocols, except that concentration-response curves were performed instead of a single concentration measurement. All injection steps were done using a TECAN EVO Freedom 150 Platform (TECAN, Mannendorf, Switzerland).

**Automated Calcium Assay.** Automated calcium assays were outsourced to Eurofins Pharma Discovery Services (St. Charles, MO). Briefly, HEK293 cell line stably expressing human TRPV1 (Eurofins catalog number Cyl3068) were plated in 384-well plates in maintaining medium and incubated at 37° C and 5% CO₂. After 24 hours, the medium was aspirated from the 384 wells and 40 μL of dye loading buffer (Hanks’ balanced salt solution supplemented with 20 mM HEPES pH 7.4, 2.5 mM probenecid, and 5 μg/mL Fluo-8 Ca²⁺ dye) was added to the cells in each well. The assay plate was incubated at 30° C and 5% CO₂ in a humidified chamber for at least 80 minutes prior to washing and addition of the Assay buffer (Hanks’ balanced salt solution supplemented with 20 mM HEPES, pH 7.4).
The calcium-flux assays were performed on a Molecular Devices’ FLIPR<sup>extra</sup> plate reader (San Jose, CA) using an excitation filter centered at 482.5 ± 12.5 nm and an emission filter centered at 545 ± 30 nm. Concentration-response curves were obtained in duplicate by either injecting increasing concentrations of the indicated reference agonist compounds or by injecting increasing concentration of the indicated reference antagonist compound followed, 3 minutes later, by an injection of 0.1 μM CAPS. Single-point screening of the Prestwick chemical library was performed using an initial injection of the tested compounds at a final concentration of 15 μM to assess each compound’s ability to activate TRPV1 for 180 seconds, followed by a second injection of 0.1 μM CAPS to assess each compound’s ability to inhibit CAPS-activated TRPV1 for another 180 seconds. In the inhibition mode, the final concentration of the tested compound was 10 μM. The compound wells, reference agonist, reference antagonist, and background vehicle controls were prepared in DMSO at 0.44% final in the activation assay and 0.33% final in the inhibition assay. Two independent experiments were performed on all duplicate tests. All plates were subjected to appropriate baseline corrections. Once baseline corrections were processed, maximum fluorescence values were exported to calculate the normalized Ca<sup>2+</sup> flux relatively to CAPS activation according to eq. 4:

\[
\text{normalized Ca}^{2+}\text{ flux} = \frac{\text{RFU}_{\text{max}} - \text{Baseline}_{\text{avg}}}{\text{RFU}_{\text{CAPS}} - \text{Baseline}_{\text{avg}}} \tag{4}
\]

where RFU<sub>max</sub> and Baseline<sub>avg</sub> are the maximal fluorescence signal and the baseline signal measured during the recording session of the tested compound respectively, and RFU<sub>CAPS</sub> is the maximal fluorescence signal measured with either 4 μM CAPS (activation mode) or 0.1 μM CAPS (inhibition mode). In the activation mode, the results are expressed as % of CAPS effect according to eq. 5:

\[
\% \text{ of CAPS effect} = \frac{\text{normalized Ca}^{2+}\text{ flux} \times 100}{C_{0}} \tag{5}
\]

In the inhibition mode, the % of inhibition induced by a compound is given by eq. 6:

\[
\% \text{ of inhibition} = 100 - \left( \frac{\text{normalized Ca}^{2+}\text{ flux} \times 100}{C_{0}} \right) \tag{6}
\]

**Manual Patch-Clamp.** For whole-cell electrophysiological study, transiently transfected HEK293T cells were bathed in an extracellular medium containing 135 mM NaCl, 5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10 mM glucose, and 10 mM HEPES. The osmolarity (measured with a cryo-osmometer type 15 from Gonotec, Jolla, CA) was used for plotting concentration-response curves. Statistical analyses were performed using GraphPad Prism v6.00 for Windows (GraphPad Software, La Jolla, CA) was used for plotting concentration-response curves. The size of the error bars indicates the S.D. within the data set.

In both activation and inhibition mode, the current was monitored using a ramp protocol from −100 mV to +100 mV over 300 milliseconds, from a holding potential of −60 mV, which was repeated every 20 seconds. The maximum outward current at +100 mV was used for analysis. In each condition, the maximum DMSO concentration at the end of the run was 0.3%.

**Data Preparation, Normalization, Analysis, and Statistics.** GraphPad Prism v6.00 for Windows (GraphPad Software, La Jolla, CA) was used for plotting concentration-response curves. The size of the error bars indicates the S.D. within the data set. Potencies of chemicals to activate or inhibit TRPV1 are expressed as pEC<sub>50</sub> 5 ± S.E.

In activation mode, the results are expressed as % of CAPS effect or to inhibits CAPS-activated TRPV1 (inhibition mode).

Activation Mode: The protocol consisted of two applications (control period) of an external solution containing 140 mM NaCl, 4 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, and 5 mM glucose, at pH 7.4, followed by the addition of 10 μM of the test compound (1 to 2 minutes). Then, a maximum concentration of agonist (3−10 μM capsaicin) was added to confirm the presence of the TRPV1 channel, and lastly, addition of the full block with 10 μM capsaicin (1 minute) was done. Data points that did not fulfill these controls were discarded. Data were normalized according to eq. 7:

\[
\% \text{ of CAPS effect} = 100 \times \frac{I_{\text{comp}} - I_{\text{base}}}{I_{\text{max}} - I_{\text{base}}} \tag{7}
\]

where \(I_{\text{comp}}\) is the current in the presence of the compound, \(I_{\text{base}}\) is the baseline current, and \(I_{\text{max}}\) is the max current in the presence of the maximum concentration of compound or capsaicin. The concentration-response curves are constrained between 0 (no activation) and 100 (maximum activation).

Inhibition Mode: The protocol consisted of two applications of external solution (control period), one application of the agonist capsaicin EC<sub>50</sub> (50 nM) for 1 to 2 minutes, followed by addition of 10 μM of the test compound (1 to 2 minutes) in the presence of capsaicin EC<sub>50</sub>, and lastly, addition of 10 μM capsaicin (1 minute) to control for full inhibition. Data were expressed as % of inhibition according to eq. 8:

\[
\text{normalized Current} = 100 \times \frac{I_{\text{comp}} - I_{\text{base}}}{I_{\text{ref}} - I_{\text{base}}} \times 100 \tag{8}
\]

where \(I_{\text{comp}}\) is the current in the presence of the compound, \(I_{\text{base}}\) is the baseline current, and \(I_{\text{ref}}\) is the current in the presence of the capsaicin EC<sub>50</sub>. The concentration-response curves are constrained between 0 (no inhibition) and 100 (maximum inhibition).

In both activation and inhibition mode, the current was monitored using a ramp protocol from −100 mV to +100 mV over 300 milliseconds, from a holding potential of −60 mV, which was repeated every 20 seconds. The maximum outward current at +100 mV was used for analysis. In each condition, the maximum DMSO concentration at the end of the run was 0.3%.
Hierarchical cluster analysis was performed using R and the dplyr, ggplot2, factoextra, and NbClust libraries (Charrad et al., 2014; Wickham, 2016; Kassambara and Mundt, 2020; R Core Team, 2020; Wickham et al., 2020).

Hierarchical agglomerative clustering is a multivariate statistical classification method of cluster analysis with the aim to build a hierarchy of clusters according to the similarity or the dissimilarity of their characteristics. It is an exploratory approach, and its interpretation depends on the experimental context. Here, the clustering was performed on 54 of the 59 identified hits compounds (see results) and applied to the characteristics listed in Table 1.

The values were first normalized (mean-centered and scaled), and an optimal number of clusters was automatically assessed using the NbClust algorithm, giving an optimal number of 7 clusters. To compute the NbClust algorithm, we used maximum distance and complete-linkage method which are often preferred and tend to produce more compact clusters.

Hierarchical cluster analysis was applied using the complete-linkage method. Three analyses were performed: one relying on the 12 parameters (Table 1), one without the intra- and intermolecular BRET measures, and one without the fluorescent probe-based calcium measurements. The clustering results were then visualized with dendrograms.

Results

Comparison of the capability of both intra- and intermolecular BRET probes, automated calcium assay, and automated patch-clamp to measure the potency and efficacy of reference compounds. We first addressed whether both intra- and intermolecular BRET biosensors discriminate between known TRPV1 agonists and antagonists that are expected to display different potencies. In these experiments, HEK293T cells transiently expressing either the intramolecular BRET probe sYFP2-TRPV1-rLuc2 (Fig. 1A) or the BRET pair TRPV1-rLuc2/sYFP2-CaM (Fig. 1B; intermolecular BRET assay), and plated in 96-well plate, were first challenged with increasing quantities of four known TRPV1 agonists. As expected, both CAPS, RTX, OLDA, and Olvanil induced a concentration-dependent increase of intra- (Fig. 1C) and intermolecular (Fig. 1D) basal BRET. CAPS, Olvanil, and RTX maximally increased the TRPV1 intramolecular BRET ratio by 50% from 0.5 to 0.75 (Fig. 1C), and the TRPV1 intermolecular BRET ratio by 700% from 0.05 to 0.35 (Fig. 1D). Although the absolute variation of the BRET ratio was similar and highly significant for both assays (0.25 for the intramolecular BRET assay and 0.3 for the intermolecular assay), the relative increase was lower when considering the TRPV1 intramolecular BRET probe. This is easily explained by a higher basal BRET ratio for the intramolecular BRET probe, which is expected given the proximity of N- and C-terminal extremities in the tetrameric quaternary structure of TRPV1 ion channels (De-la-Rosa et al., 2013). In sharp contrast, since CaM is only weakly coupled to TRPV1 in the resting state (Hasan et al., 2017; Ruigrok et al., 2017), the intermolecular basal BRET ratio is very low, leading to bigger relative changes after activation. In agreement with others, we found that OLDA maximal efficacy was lower than CAPS to activate human TRPV1 in transfected HEK293 cells (Bianchi et al., 2006). The rank order of EC50 values for each agonist was conserved for both BRET biosensors and is in full agreement with the literature (Winter et al., 1990; Ralevic et al., 2001; Bianchi et al., 2006) with RTX > CAPS > Olvanil > OLDA (Fig. 1 and Table 2).

We, therefore, assessed the efficacy and potency of various TRPV1 antagonists using our intra- and intermolecular BRET probes. As shown in Fig. 1E and Fig. 1F, using both intra- and intermolecular TRPV1 BRET probes, we confirmed that CFZ, AMG519, AMG9810, BCTC, NJU-17203212, and AMG21629 fully antagonized TRPV1 activation by CAPS [hereafter noted as TRPV1(CAPS)] in agreement with the literature (Gavva et al., 2005; Swanson et al., 2005; Bianchi et al., 2006; Gavva et al., 2007a; Gavva et al., 2007b; Papakosta et al., 2011). However, using both BRET assays, the antagonist SB366791 was found to only partially antagonize TRPV1(CAPS), which is in contradiction with the initial characterization of this compound as a full antagonist (Gunthorpe et al., 2004). We, however, confirmed that SB366791 is a weak antagonist (Table 2). As expected, RN1734, which is known to be a TRPV4-specific antagonist, failed to inhibit TRPV1(CAPS). These results indicate that both TRPV1 intra- and intermolecular BRET assays are fully functional to assess the agonist and antagonist behavior of chemical compounds. This statement is reinforced by the fact that both the shape of the current-voltage (IV) curve and the magnitude of the outward current flowing through both untagged TRPV1 and TRPV1 intramolecular BRET probe are similar in transiently transfected HEK293T cells challenged with CAPS (Supplemental Fig. 1). This further supports our previous observations that N- and C-terminal addition of either the YFP and/or Luc groups does not hinder TRPV1 activity (Ruigrok et al., 2017).

The acceptance of BRET probes as a novel tool for ion-channel drug screening relies on their operability and effectiveness with regards to conventional methods. We, therefore, performed concentration-response curves of the aforementioned TRPV1 agonists and antagonists using HTS platforms for both APC and fluorescent probe-based calcium measurement. The resulting potency of these chemicals to modulate TRPV1 activity were compared with the ones measured using our intra- and intermolecular BRET assays in the 384 well plate format. As shown in Fig. 2 and Table 2, the potency measured using each technique was in a similar range for the four agonists tested. Considering the data obtained with the antagonist compounds, we found that the pIC50 measured with both intramolecular and intermolecular BRET probes were again close to the ones measured with the ACA. APC yielded, however, significantly better pIC50 than the ones measured with either BRET probes or ACA for five antagonists out of the seven tested. Knowing that the

<table>
<thead>
<tr>
<th>Activation Mode (% of CAPS Effect)</th>
<th>Inhibition Mode (% of Inhibition)</th>
</tr>
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<tbody>
<tr>
<td>BRET intra</td>
<td>BRET inter</td>
</tr>
<tr>
<td>BRET intra</td>
<td>BRET inter</td>
</tr>
</tbody>
</table>

TABLE 1

List of characteristics used for data-driven hierarchical clustering
concentration of compounds tested in a primary high-throughput screening usually lies between 1 and 10 μM, our results nonetheless indicate that both TRPV1-based BRET assays are as fit to perform high-throughput screening as the conventional APC and automated Ca^{2+}-flux methods.

Assessment of Technical and Biological Reproducibility for Both Intra- and Intermolecular BRET Assays. We next assessed the suitability of our BRET assays for HTS purposes using transfected cells seeded in 384-well plates. We first assessed the technical and biological reproducibility of both intra- and intermolecular BRET assays by comparing the results of two independent experiments (done on two different days) where CAPS concentration-response curves were obtained on four consecutive 384-well plate assays (Fig. 3). All concentration-response curves fitting into the range of three S.D.s, our results indicated that both TRPV1 intra- and intermolecular assays offered good biological and technical reproducibility (Fig. 3, A and B). To determine the Z’-factor of the assay (Zhang et al., 1999), we measured the efficacy of 500 nM CAPS to trigger TRPV1 conformational change and calmodulin coupling over five independent experiments performed over three different days with 16–24 wells measured per plate (Fig. 3, C and D). All calculated Z’-factor were above or close to 0.5, indicating that both intra- and intermolecular assays for TRPV1 were of high quality and suitable for HTS [average Z’-factor were 0.58 ± 0.04 (average ± S.E.) and 0.54 ± 0.04 for intra- and intermolecular BRET assays, respectively].

The Primary Screen of the Prestwick Chemical Library for TRPV1 Activation and Inhibition. Based on this conclusion, we used HTS experimental conditions with ACA and both intra- and intermolecular BRET probes to screen the Prestwick chemical library for both activation (Fig. 4) and inhibition (Fig. 5) of TRPV1. The final drug concentration was 15 μM during the measurement of the compound efficacy to activate TRPV1 and was 10 μM during the measurement of the compound efficacy to inhibit TRPV1 activation after the injection of 500 nM CAPS (which is close to CAPS EC_{80} in our experimental condition, e.g., the concentration of CAPS inducing 80% of TRPV1 maximal activation). Two independent runs (n1 and n2) were performed, and an arbitrary percent activation or inhibition cut-off of 30% was chosen to select hit compounds. As expected, most compounds exhibited little to no effect whatever the assay considered, whereas a small percentage of compounds demonstrated positive or negative modulation of TRPV1 activity in either
pEC$_{50}$/pIC$_{50}$ values derived from curve fitting of TRPV1 agonists/antagonists dose-response measured using YFP-TRPV1-Luc intramolecular BRET probe, TRPV1-Luc/YFP-CaM intermolecular BRET probe, ACA, and APCBRET assays were measured both in 96-well plate and 384 well plate's formats. Values represent the mean ± S.E. of 3–9 independent experiments performed in duplicate. Values found in the literature are also indicated along with the method used, cellular model, and reference.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Intramolecular BRET Probe</th>
<th>Intermolecular BRET Probe</th>
<th>ACA</th>
<th>APC</th>
<th>Potency Found in the Literature</th>
<th>Method</th>
<th>Cellular Model</th>
<th>Reference</th>
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<tr>
<td></td>
<td>96 wells</td>
<td>384 wells</td>
<td>96  wells</td>
<td>384 wells</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsaicin</td>
<td>6.96 ± 0.07</td>
<td>6.83 ± 0.05</td>
<td>6.73 ± 0.10</td>
<td>7.11 ± 0.21</td>
<td>8.05 ± 0.08</td>
<td>7.45 ± 0.03</td>
<td>7.1 - 7.3</td>
<td>Ca$^{2+}$ (Fluo3/4)</td>
</tr>
<tr>
<td>Olvanil</td>
<td>5.89 ± 0.10</td>
<td>6.90 ± 0.05</td>
<td>6.42 ± 0.11</td>
<td>8.42 ± 0.16</td>
<td>7.93 ± 0.13</td>
<td>7.59 ± 0.04</td>
<td>6.36 - 7.73</td>
<td>Ca$^{2+}$ (Fluo3/4)</td>
</tr>
<tr>
<td>OLDA</td>
<td>6.96 ± 0.74</td>
<td>4.94 ± 0.11</td>
<td>4.90 ± 0.31</td>
<td>5.99 ± 0.30</td>
<td>6.20 ± 0.10</td>
<td>5.64 ± 0.05</td>
<td>5.49 ± 0.06</td>
<td>Ca$^{2+}$ (Fluo4)</td>
</tr>
<tr>
<td>RTX</td>
<td>8.02 ± 0.08</td>
<td>8.34 ± 0.06</td>
<td>7.59 ± 0.08</td>
<td>8.12 ± 0.18</td>
<td>8.19 ± 0.08</td>
<td>8.24 ± 0.04</td>
<td>7.92 - 8.69</td>
<td>$^4$Ca uptake Ca$^{2+}$ (Fluo4)</td>
</tr>
<tr>
<td>Antagonists</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>AMG 517</td>
<td>7.12 ± 0.10</td>
<td>7.95 ± 0.14</td>
<td>6.97 ± 0.07</td>
<td>7.87 ± 0.05</td>
<td>8.12 ± 0.09</td>
<td>8.40 ± 0.05</td>
<td>9.19 ± 0.25</td>
<td>$^4$Ca uptake</td>
</tr>
<tr>
<td>JNJ17203212</td>
<td>6.12 ± 0.11</td>
<td>6.91 ± 0.07</td>
<td>6.34 ± 0.06</td>
<td>6.47 ± 0.09</td>
<td>6.96 ± 0.02</td>
<td>8.24 ± 0.06</td>
<td>7.20 ± 0.11</td>
<td>Ca$^{2+}$ (Fluo3)</td>
</tr>
<tr>
<td>BCTC</td>
<td>8.05 ± 0.14</td>
<td>7.55 ± 0.05</td>
<td>7.54 ± 0.10</td>
<td>7.30 ± 0.02</td>
<td>8.02 ± 0.01</td>
<td>9.06 ± 0.02</td>
<td>8.77 ± 0.01</td>
<td>Calcium4</td>
</tr>
<tr>
<td>SB 366791</td>
<td>6.15 ± 0.35</td>
<td>6.47 ± 0.14</td>
<td>5.83 ± 0.21</td>
<td>6.45 ± 0.20</td>
<td>5.58 ± 0.08</td>
<td>7.84 ± 0.06</td>
<td>6.89 ± 0.22</td>
<td>$^4$Ca uptake</td>
</tr>
<tr>
<td>AMG 9810</td>
<td>6.12 ± 0.10</td>
<td>6.51 ± 0.32</td>
<td>6.55 ± 0.06</td>
<td>6.45 ± 0.09</td>
<td>6.31 ± 0.05</td>
<td>8.12 ± 0.04</td>
<td>7.72 ± 0.33</td>
<td>$^4$Ca uptake</td>
</tr>
<tr>
<td>AMG 21629</td>
<td>7.8 ± 0.18</td>
<td>8.64 ± 0.06</td>
<td>7.84 ± 0.09</td>
<td>8.35 ± 0.06</td>
<td>8.42 ± 0.04</td>
<td>8.64 ± 0.08</td>
<td>9.28 ± 0.23</td>
<td>$^4$Ca uptake</td>
</tr>
<tr>
<td>Capsazepine</td>
<td>6.31 ± 0.08</td>
<td>n.d.</td>
<td>6.19 ± 0.14</td>
<td>n.d.</td>
<td>6.32 ± 0.04</td>
<td>8.40 ± 0.06</td>
<td>7.90 - 8.27</td>
<td>$^4$Ca uptake Ca$^{2+}$ (Fluo4)</td>
</tr>
</tbody>
</table>
activation or inhibition modes (Fig. 4, A–C, Fig. 5, A–C, and Supplemental Table 2). Interestingly, when plotting the compounds’ percent distribution histograms, data from BRET experiments exhibited distribution profiles different than data issued from the Ca^{2+} flux method. The latter displayed an asymmetric profile with a significantly broader basis, especially in the inhibition mode (Fig. 4, D–F and Fig. 5, D–F). Reproducibility between the results obtained during the two independent runs was derived from scatter plots analysis (Fig. 4, G–H and Fig. 5, G–H) using different statistical methods. Firstly, the median distance between each experimental dot and a theoretical perfect duplicate assay was computed and compared between the three methods used (intramolecular BRET probe, intermolecular BRET probe, and ACA) for both activation and inhibition modes (Supplemental Fig. 2). We found that data dispersion was significantly lower for the intermolecular BRET probe than for the two other techniques in the activation mode and significantly lower for both intra- and intermolecular BRET assays in comparison with the results obtained with ACA in the inhibition mode. Secondly, the global dispersion of the pooled data (n1 and n2) was estimated using 4 classic dispersion metrics: the median absolute deviation, the difference between the largest and smallest values (range), the quartile coefficient of dispersion, and the interquartile range. For all these four metrics, the radar chart area is proportional to the data dispersion. As shown in Supplemental Fig. 3, the calcium-activated method exhibited a much larger area compared with values obtained from the two BRET assays, pointing toward a higher overall signal values dispersion of ACA. The overall conclusion is that TRPV1 BRET probes provide a statistically better signal reproducibility than ACA in high-throughput screening conditions. Accordingly, we found a significantly higher percentage of confirmed hits between both replicate assays in the activation mode when using intra- or intermolecular BRET probes (100% and 87.5% of confirmed hits, respectively) in comparison with the calcium assay, for which we found 18.1% of confirmed hits (Table 3). Although the percentage of confirmed hits was lower in the inhibition mode than in the activation mode for all three assays, the percentage of confirmed hits for both intra- and intermolecular BRET probes (42.6% and 58.8%, respectively) was still higher than the one found for the calcium assay (31.2%). This confirms that both BRET assays are sufficiently fit for reliable hit identification.

**Hit Confirmation with APC.** The Venn diagrams in Fig. 6 show the total number of unique hits detected by each assay in the activation mode (Fig. 6A) and the inhibition mode (Fig. 6B). A total of 22 compounds were shown to reproducibly trigger TRPV1 in both replicate screens, whereas 47 compounds were shown to reproducibly inhibit TRPV1(CAPS). Remarkably, only three hits were common to the three methods when regarding the inhibition mode (thioridazine hydrochloride, perphenazine, and benzethonium chloride), whereas no hits were common to the three methods when regarding the activation mode. Two hits were detected by both intramolecular and calcium assay to activate TRPV1. Considering the inhibition mode, one hit was common to both calcium assay and intermolecular BRET probe, two hits were common to both intermolecular BRET probe and calcium assay, and four hits were common to both intra- and intermolecular BRET probes. Since 10 compounds were identified in at least two different tests, all assays combined, this primary screen, therefore, identified a total of 59 hits (4.9% of the bank).

We then reassessed the efficacy of each of these 59 compounds to activate TRPV1 or inhibit TRPV1(CAPS) using APC. In a preliminary step, we first confirmed that TRPV1 behaved as an outwardly rectifying channel when stably expressed in HEK293T cells, as already described by others in several primary cells and cell lines (Caterina et al., 1997; Tominaga et al., 1998; Premkumar et al., 2002) (Fig. 7A). Knowing the outward rectifying properties of TRPV1, it is important to emphasize that most electrophysiologists assess TRPV1 activity by measuring the outward potassium current.
flowing through the TRPV1 ion channel at high positive membrane potential (e.g., between +60 and +100 mV). The reason is that, although this outward current measured at high positive membrane potential is less physiologically relevant, it is of much greater amplitude than the inward current measured at the negative resting membrane potential of cells (Priest et al., 2007). During a drug screening, the implicit assumption for such practice is that any hit displays an equal ability to activate or inhibit TRPV1 gating irrespective of the membrane potential. We, therefore, compared CAPS and CPZ potency to activate or inhibit TRPV1 ion channel when cell membrane potential was clamped at +100 mV, −25 mV (which is the resting membrane potential of HEK293T cells (Kirkton and Bursac, 2011)) and −100 mV. As shown in Fig. 7B, CAPS potency was right-shifted when the membrane potential was shifted from +100 mV to −25 mV and remained identical between −25 mV and −100 mV. CPZ potency to antagonize TRPV1 gating by CAPS was similar between +100 mV and −25 mV but was right-shifted at −100 mV (Fig. 7B, right panel). These results indicated that the membrane potential impacts CAPS and CPZ potency to activate or inhibit TRPV1. By the way, they (i) provided a potential explanation for the difference in the apparent potency of some TRPV1 agonists and antagonists measured with either APC, BRET probes, or ACA (Figs. 1 and 2), and (ii) called for an in-depth analysis of the efficacy of the 59 identified compounds to activate or inhibit TRPV1 gating as a function of the applied membrane potential during APC experiments.

Among the 59 compounds tested, 5 compounds known to be detergent molecules induced a high nonspecific current in untransfected HEK293T cells [chlorhexidine (#10), methyl benzethonium chloride (#38), benzethonium chloride (#39; which one was initially identified by all methods), alexidine dihydrochloride (#42), thonzonium bromide (#49)] and were discarded from the rest of the study (Supplemental Table 3).

Assessment of the ability of the 54 remaining drugs to either activate TRPV1 or inhibit TRPV1(CAPS) indicated a strong disparity between the results measured at −100 mV, −25 mV, and +100 mV (Fig. 7, C and D). Only six compounds were shown to be confirmed as TRPV1 activator by APC at
Among these six compounds, only two (compounds 40 and 47) were detected whatever the voltage used. Interestingly, compound 27 was found to activate TRPV1 very efficiently at +100 and −100 mV but not at −25 mV. Also, compounds 29 and 58 did not activate TRPV1 at +100 and −25 mV, whereas they did it at −100 mV. Eleven compounds inhibited CAPS-induced TRPV1 activation at +100 mV, but only three of them inhibited TRPV1 activation by CAPS whatever the voltage used (compounds 20, 32, and 58). Compounds 22, 30, and 48 partially blocked TRPV1 activation by CAPS at +100 mV, but displayed no inhibitory activity at either −25 mV or −100 mV. Compound 19 efficiently inhibited CAPS-induced TRPV1 activation at +100 mV but potentiated CAPS activation at −25 mV and had no effect at −100 mV. Compound 60 was as efficient as CPZ to antagonize CAPS activation of TRPV1 at both +100 and −25 mV, but potentiated CAPS activation at −100 mV. Compound 55 behaved as a partial antagonist of TRPV1 activation by CAPS at −25 and −100 mV, but had no effect at +100 mV. Compound 40 (not represented on Fig. 7C for simplification) strongly potentiated CAPS activation whatever the voltage considered (% of CPZ effect was −343.2, −52.4, and −95.75 respectively for +100 mV, −25 mV, and −100 mV). Altogether, these results indicate that, when using APC, the behavior of a given compound to modulate an ion channel can vary drastically depending on the plasma membrane potential.

The Correlation between Chemical Structure-Driven and Data-Driven Clustering indicates that Calmodulin Antagonists Act as TRPV1 Inhibitors.

Since no consensus emerged from the comparison of the results obtained with each assay taken independently, we next performed a structure-driven and a data-driven clustering of these 54 drugs to unravel a potential structure-function relation that might, in the light of the bibliography, provide insights into the ability of each drug to either activate or inhibit TRPV1.

We found that all 54 identified drugs could be sorted in only 11 different clusters (Supplemental Table 3). Cluster A contains drugs harboring a trifluoromethyl benzene group. Cluster C contains drugs harboring a bicycle in their structure. Cluster D is composed of detergent molecules. Cluster E contains drugs with only two aromatic rings joined by a linker. Cluster G contains tricyclic compounds such as phenothiazines. Clusters H, I, J, and S contain macrocyclic lactones, statins, dihydropyridines, and sterols-derived...
compounds respectively. Finally, 5 compounds did not fit in any of the aforementioned clusters and belong therefore to a miscellaneous cluster.

We next performed hierarchical clustering of the data acquired with these 54 hits, taking into account the 12 parameters integrating the results measured with both intra- and intermolecular BRET probes, ACA, and APC at $100 \, \text{mV}$, $25 \, \text{mV}$, and $100 \, \text{mV}$ in both activation and inhibition modes. The resulting dendrogram (Fig. 8) indicates the correspondence with the aforementioned structure-driven clustering and the measured activity of the drug as assessed with each technique in both activation and inhibition mode.

The most important correlation arising from this multi-parametric analysis points to a significant enrichment of drugs belonging to clusters E, G, and J into group 7 of the dendrogram (Supplemental Fig. 4). This prompted us to perform an in-depth analysis of the literature focusing on these clusters. Strikingly, we found that 12 of the 24 hits belonging to cluster E, G, and J are well known calmodulin antagonists [(trifluromazine (#01), chlorpromazine (#03), thioridazine (#04); cyproheptadine (#05); perphenazine (#06), loperamide (#11), clotrimazole (#18), perhexiline maleate (#20), quinacrine (#22), fluphenazine (#23), felodipine (#28), prenylamine lactate (#32)] (Rochette-Egly et al., 1982; Johnson and Wittnauer, 1983; Hatano et al. 2013; Montero et al., 1991; Cadrilola et al., 1992; Hegemann et al., 1993; Xin and Zhang, 1993; Olah et al., 2007; Lübker and Seifert, 2015).

Cluster J contains 3 compounds [felodipine (#28), lacidipine (#33), and cilnidipine (#56)] that are all derived from 3,5-diester-4-aryldihydropyridin and differ by structural variations on ester functions and aryl ring. All these three compounds were shown by the intramolecular BRET probe to antagonize the conformational changes occurring in TRPV1 after CAPS activation. No effects were detected using any other technique, except for APC at $25 \, \text{mV}$ that also

<table>
<thead>
<tr>
<th>% of confirmed hit between replicates assays</th>
<th>Intramolecular BRET Probe</th>
<th>Intermolecular BRET Probe</th>
<th>Calcium Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonist mode</td>
<td>100% (3)</td>
<td>87.5% (7)</td>
<td>18.1% (16)</td>
</tr>
<tr>
<td>Antagonist mode</td>
<td>42.6% (23)</td>
<td>55.8% (10)</td>
<td>31.2% (28)</td>
</tr>
</tbody>
</table>
measured an antagonist effect of lacidipine (Fig. 8 and Supplemental Table 3). To the best of our knowledge, among these three compounds, only felodipine has been reported to inhibit CaM (Johnson and Wittenauer, 1983). However, because of their similar chemical structure, it is highly possible that both lacidipine and cilnidipine, two Ca²⁺ channel blockers (Micheli et al., 1990; Chandra and Ramesh, 2013), also act as CaM antagonists.

Nine out of the ten compounds belonging to cluster G are antipsychotic drugs derived from phenothiazine [triflupromazine (#01), chlorpromazine (#03), thioridazine (#04), perphenazine (#06), quinacrine (#22), fluphenazine (#23), metixene (#30), methotrimeprazine (#44), and thieptylperazine maleate (#54)]. Phenothiazines are among the most potent calmodulin inhibitors, especially when the phenothiazine derivative is substituted by a halogen (Rochette-Egly et al., 1982; Caldirola

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Fig. 6. Venn diagram of the hits detected with the TRPV1 intramolecular BRET probe, TRPV1 intermolecular BRET assay, and ACA in the activation mode (A) and in the inhibition mode (B).

Fig. 7. Influence of the imposed membrane potential on the ability of hit compounds to activate TRPV1 or inhibit TRPV1 activation by CAPS. (A) I–V curves of vehicle (blue curve) or CAPS (1 μM, red curve)-evoked currents in HEK cells stably expressing hTRPV1. (B) The concentration-response curve of CAPS (left panel, n = 44) or CPZ (right panel, n = 21)-evoked current measured at +100 mV, −25 mV, and −100 mV in HEK cells stably expressing hTRPV1. CPZ concentration-response curves were measured 2 to 3 minutes after the addition of 50 nM CAPS in the assay buffer. (C) Scatter plot of the ability of hit compounds to activate TRPV1 when the membrane potential is clamped to −25 mV versus +100 mV (left panel) or −100 mV versus +100 mV (right panel). (D) Scatter plot of the ability of hit compounds to inhibit CAPS (50 nM)-activated TRPV1 when the membrane potential is clamped to −25 mV versus +100 mV (left panel) or −100 mV versus +100 mV (right panel).
Fig. 8. Data-driven hierarchical clustering of the effect of the hit compounds. The NbClust R package automatically sorted the 54 analyzed hits in 7 different groups. A complete linkage method (default method based on farthest neighbors' distance) was then applied to hierarchical clustering of the hits into each group using a maximum (Chebychev) distance metric (Abello et al., 2002). Correspondence with the structure-driven clustering and the measured activity of the drug as assessed with each technique in both activation and inhibition mode is also indicated. "X" indicates that the indicated drug efficacy was equal or above 30% of CAPS efficacy to trigger TRPV1 activation (activation mode) or inhibited TRPV1 activation by CAPS by at least 30% (see material and methods for details). "X*" indicates that the indicated drug did not inhibit but potentiated TRPV1 activation by CAPS (see Supplemental Table 3 for quantitative analysis). Whether each compound is identified as CaM inhibitor (Cam_Inh) or putative CaM inhibitor (p_Cam_Inh) is also indicated (see results for details).
et al., 1992). The tenth compound of the cluster G, cyproheptadine (#05), which is not a phenothiazine, has been described as a calmodulin inhibitor in one study (Xin and Zhang, 1993) and is structurally related to amitriptyline, a known calmodulin inhibitor (Reynolds and Claxton, 1982). To the best of our knowledge, among the ten compounds belonging to cluster G, the only compounds for which CaM antagonist activity has not been established are metizine (#30), methotrimeprazine (#32), and thiethylperazine maleate (#54) (Volpi et al., 1981; Prozialeck and Weiss, 1982; Rochette-Egly et al., 1982; Oláh et al., 2007; Lübbe and Seifert, 2015).

Nine of the twelve compounds belonging to cluster E are composed of poly-cycle rings-containing molecules. Interestingly, perhexilin maleate (#20) and prenylamine Llactate (#32) that had been detected as inhibitors by intramolecular BRET probe in the primary screen are both calmodulin antagonists (Caldirola et al., 1992), and have been confirmed as inhibitors of CAPS-induced TRPV1 activation by APC whatever the voltage used (Fig. 8 and Supplemental Table 3). Fluspirilen (#48), which triggered a conformational change in TRPV1 and was detected as an inhibitor by APC +100 mV, is known to bind CaM (Butts et al., 2013) and is structurally related to penfuridol, a first-generation neuroleptic shown to be a CaM antagonist (Lübbe and Seifert, 2015). Butocunazole (#25), which has been detected as an inhibitor of TRPV1(CAPS) by the intramolecular BRET probe is an imidazole-derived compound. Direct interaction of imidazole-derived compounds with calmodulin has been suggested as a possible mechanism for their antifungal activity (Hegemann et al., 1993; Breitholtz et al., 2020), further suggesting a functional link between CaM inhibition and TRPV1 activation. Sertindole (#35), which contains a 4-piperinyl moiety connected in position 3 of an indole ring, and astemizole (#08), which contains a 4-amino-piperinyl moiety connected in position 2 of a benzimidaole ring, share structural features with the calmodulin antagonist CGS 9343B (Norman et al., 1987). Loperamide (#11), a synthetic piperidine derivative, known to inhibit TRPV1 activation by ACA, is a recognized CaM antagonist (Merritt et al., 1982) and can prevent capsaicin-induced thermal allodynia in primates, in the absence of thermal antinociceptive effects (Butelman et al., 2004). Clotrimazole (#18) has a blurred profile since it was detected as an activator of TRPV1 by the intermolecular BRET probe and APC and inhibitor by intramolecular BRET probe and ACA. Nonetheless, clotrimazole (#18) is a potent calmodulin inhibitor (Montero et al., 1991; Hegemann et al., 1993) and has been detected as a TRPV1 activator (Meseguer et al., 2008). Of note, clotrimazole-derived compounds are weak competitive inhibitors of TRPV1(CAPS) (Oláh et al., 2007).

To the best of our knowledge, the only hit compound which has been shown to inhibit purified CaM in vitro (Schaeffer et al., 1987) and which does not belong to clusters E, G, or J is nicergoline (#12), a nitrogen polyheterocyclic compound filed in the miscellaneous cluster (Supplemental Table 3). In agreement with these considerations, most CaM inhibitors or putative CaM inhibitors belong to group 7 of the data-driven hierarchical clustering (Fig. 8). Interestingly, the cross-correlation between chemical structure-driven and data-driven clustering indicates that group 7 is mainly composed of drugs initially identified by intra- and/or intermolecular BRET probes (Fig. 8). Hierarchical clustering of the data acquired without ACA still yields to the formation of 7 groups with one of them being enriched with compounds belonging to clusters E, G, and J (Supplemental Fig. 4). In sharp contrast, hierarchical clustering of the data acquired without our BRET probes does not allow us to identify compounds belonging to clusters E, G, and J as part of a separate group (Supplemental Fig. 4). This observation points to a predominant detection of CaM inhibitors as inhibitors of TRPV1 activation by CAPS using our BRET probes.

**Analysis of Hit Compounds Not Displaying CaM Inhibitor Activities.** Bibliographic pieces of evidence also support an apparent CaM-independent modulation of TRPV ion channels, sometimes including TRPV1 itself, by several compounds identified as hits in our screens.

Four compounds, for which no CaM antagonist activity has been described, were identified by more than one technique to inhibit TRPV1(CAPS): [antimycin A (#17), lovastatin (#31), simvastatin (#47], and sertraline (#51)] (Supplemental Table 3 and Fig. 8). In the cluster F, sertraline (#51), a 1,2,3,4-tetrahydronaphthalen derivative, which is substituted by a methyamino at position 1 and a 3,4-dichlorophenyl at position 4 (the S, S diastereoisomer), has been detected as an inhibitor by the BRET intramolecular probe and APC at +100 mV and −100 mV. Interestingly, tetraline urea derivatives have been shown to display antagonistic properties against TRPV1 activation by CAPS (Meseguer et al., 2006; Jetter et al., 2007). Although sertraline is not a tetraline urea derivative, it would be interesting to assess whether substitution of the methyamino group of sertraline by an urea group improve sertraline potency and/or efficacy to inhibit CAPS-mediated TRPV1 activation. Lovastatin (#31) and simvastatin (#47) belong to cluster I, which also contains mevastatin (#59). All these three compounds displayed a blurred profile when comparing the results obtained using activation and inhibition modes with the different readout assays used. Cluster I is composed of statin molecules, also known as HMG-CoA reductase inhibitors, which are a class of lipid-lowering drugs reducing illness and mortality in persons who are at high risk of cardiovascular disease. They are the most common cholesterol-lowering drugs, and cholesterol binding has been shown to be of importance for TRPV1 gating (Saha et al., 2017), which may point toward an indirect action of these compounds on TRPV1 activity. In our study, simvastatin (#47) was detected as an activator by both the intermolecular BRET probe and APC, and as an inhibitor by calcium and intramolecular BRET probes. Lovastatin (#31) and mevastatin (#59) were both detected as an activator by APC but behaved both as TRPV1 activator and inhibitor using ACA. In the literature, lovastatin (#31) and simvastatin (#47) have been shown to trigger TRPV1-dependent Ca²⁺ influx in endothelial cells (Su et al., 2014; Negri et al., 2020). However, to the best of our knowledge, an effect of mevastatin on the activity of TRP ion channels has never been reported. Finally, two studies suggest that TRPV1 contributes to Ca²⁺ influx triggered in vagal nociceptive neurons by the well known antibiotic antimycin A (#17), which belongs to the miscellaneous cluster (Nesuashvili et al., 2013; Stanford et al., 2019).

In cluster E, raloxifen (#46), detected as a TRPV1 inhibitor only by the intramolecular BRET probe (Supplemental Table 3 and Fig. 8), has been shown to inhibit TRPV1 activation by
CAPS in the hippocampus and dorsal root ganglion of rats (Yazgan and Nazoroglu, 2017).

Finally, several compounds not described as CaM antagonists were shown to inhibit CAPS-induced TRPV1 activation by ACA, but not by either of the two BRET probes (Supplemental Table 3 and Fig. 8). Bibliographic evidence exists in support of the inhibitory efficacy against CAPS-induced TRPV1 activation of 6 of these compounds. In cluster A, flufenamic acid (#16), an anthranilic acid derivative carrying an N-(trifluoromethyl)phenyl substituent, has been shown to inhibit TRPV1 activation by CAPS (Hu et al., 2010; Guinamard et al., 2013). In cluster C, mefloquine (#7), which is a quinoline derivative and the antagonistic behavior of these compounds against TRPV1, has been recently discussed (Ambatkar and Khedekar, 2019). In cluster E, homochlorcyclizine (#19) shares structural determinants with dexbrompheniramine, which has been shown to inhibit TRPV1 in HEK293 cells (Sadofsky et al., 2008). In cluster F, rosiglitazone (#57), which belongs to the thiazolidinedione class, has been shown to inhibit TRP melastatin 3 ion channel while activating TRP canonical 5 ion channel (Majeed et al., 2011). Interestingly, during the screening of the Prestwick chemical library with the intermolecular BRET probe, the closely related compound Troglitazone enhanced TRPV1 activation by CAPS (Supplemental Table 2). Also, Troglitazone has been recently shown to directly activate TRPV1 (Krishnan et al., 2019). Still in cluster F, hexachlorophene (#58) is a polychloroaromatic compound shown to activate Potassium Voltage-Gated Channel Subfamily Q Member 1 (KCQ1) (Zheng et al., 2012), a molecular event known to inhibit TRPV1 (Ambrosino et al., 2019). Whether KCQ1 ion channels are expressed in HEK293T cells is not known, but outward potassium currents do exist in HEK293T cells (Ponce et al., 2018), leaving room for of an indirect effect of hexachlorophene on TRPV1. In cluster S, epiandrosterone (5α-androstan-3β-ol-17-one) (#26) is a dehydroepiandrosterone metabolite only differing by one π-bound from 5α-androsten-3β -ol-17-one, which one has been shown to antagonize CAPS-induced activation of TRPV1 (Chen et al., 2004). Auranoﬁn (#45), an oral chrysotherapeutic agent for the treatment of rheumatoid arthritis, which belongs to the miscellaneous cluster, has been shown to activate TRPA1 but not TRPV1 in transiently transfected HEK cells using calcium assay (Mannhold et al., 1987). Although we also found that no TRPV1 activation was detected using a calcium assay with this compound, Auranoﬁn, nonetheless, triggered a conformational change in TRPV1 that led to an increase of the BRET measured with the intramolecular BRET probes. Some compounds for which we found no bibliographic evidence linking them to the TRP ion channel, calcium, or calmodulin were also detected as hits by more than one technique and might be considered for further studies. Efavirenz (#27, cluster C), a noncompetitive inhibitor of HIV-1 reverse transcriptase, and beta-escin (#37, cluster S) has been detected as TRPV1 activator by both intermolecular BRET probes, calcium, and APC but, to the best of our knowledge, no evidence links these compounds to TRP ion channels. Importantly, beta-escin (#37) is known as a patch-clamp perforating agent but triggered only a very small current in nontransfected cells in our experimental conditions (Supplemental Table 3), thus validating it as a potential hit. Oxethazaine (#2, cluster F), a local anesthetic, has been shown to inhibit TRPV1 activation by CAPS using both ACA and intramolecular BRET probes. Finally, we found no bibliographic evidence linking several compounds detected as hits in our primary screen by only one technique. Among these compounds, diprydamole (#9, cluster C), nitrofurantoin (#14, cluster F), and repaglinide (#53, cluster F) were detected as
inhibitors or activators only by ACA. Of note, repaglinide has been shown to target neuronal calcium sensor proteins but not calmodulin (Okada et al., 2003), and its binding to TRP ion channel is not described. Pyrvinium pamoate (#52, cluster F) and ivermectin (#13, cluster H) were respectively detected as TRPV1 activator and inhibitor by the intramolecular BRET probe. Sulfameter (#41, cluster F) was detected as a TRPV1 activator using the intermolecular BRET probe. Altogether, the high structural diversity of these hits could be useful for structure-activated relationship studies (Tafesse et al., 2014).

Exemplification of the Concept of Intramolecular BRET Probe to Other ion Channels. The results obtained with intramolecular and intermolecular BRET probes prompted us to assess whether BRET-based biosensors could be derived for other ion channels. Since not all ion channels are in interaction with calmodulin, we focused on intramolecular BRET probes targeting ion channels having both N- and C-terminal extremities into the cytoplasm.

We first assessed whether the activity of two other TRPs ion channels, TRPV4 and TRPM8, could also be measured using intramolecular BRET probes. As shown in Fig. 9, the BRET signal measured on HEK293T cells transiently expressing mNeonG-hTRPV4-nLuc (Fig. 9A) and nLuc-hTRPM8-mNeonG (Fig. 9B) intramolecular BRET probes was concentration-dependently increased after addition of the specific agonists of TRPV4 and TRPM8 respectively (Bödding et al., 2014).
centration-dependent increase of the basal BRET ratio (Fig. 3). This produced a con-
trastation using patch-clamp or calcium-flux measurements on cells transiently expressing TRPV4 or TRPM8 (Bödding et al., 2007; Thorneloe et al., 2011). The pharmacological selectivity of the ligand-promoted BRET changes was further demonstrated by the competitive nature of the effects, as both HC060747 and MSB, two well known competitive antagonists of TRPV4 and TRPM8 respectively, right-shifted the corresponding agonist potency to higher values in both intramolec-
ular BRET tests. Altogether, these data strongly suggest that the agonist-promoted BRET changes in TRPV4 and TRPM8 intramolecular BRET probes correspond to activation of these two ion channels in live cells, as previously shown for TRPV1 (Ruigrok et al., 2017).

To go further in the exemplification of ion channel intramo-
lecular BRET probes, we constructed mNeon-KCa2.3-nLuc (Fig. 10A), mNeon-Kir6.1-nLuc (Fig. 10D), and mNeon-TREK1-nLuc BRET (Fig. 10G) intramolecular BRET probes targeting respectively 1) KCa2.3, a small conductance calcium-activated potassium channels sharing the same 6-trans-
membrane domains (TM) basic architecture with Shaker-like voltage-gated potassium channels and TRP ion channels, 2) Kir6.1, an ATP-sensitive inwardly-rectifying potassium channels, the structure of which contains two-TM domain per monomer, and 3) TREK1, a two-pore-domain background potassium channels containing two pairs of TMs per monomer, each flanking a pore domain. The functionality of KCa2.3 was assessed in two different ways. Firstly, we cotransfected mNeonG-KCa2.3-nLuc intramolecular BRET probe with TRPV1 and calmodulin in HEK293T cells, and triggered a Ca^{2+} influx into the cell through TRPV1 pore opening using a saturating concentration of CAPS (Fig. 10B). CAPS injection induced a rapid increase of the basal BRET signal until reaching a plateau. No such effect was detected when the solvent was injected alone (vehicle). Secondly, in HEK293T cells transiently expressing mNeonG-KCa2.3-nLuc intramolecular BRET probe alone, we triggered a rise in intracellular calcium by blocking calcium transport into the sarcoplasmic and endoplasmic reticula using an increasing dose of thapsigargin (Lu et al., 2014). This produced a concentra-
tion-dependent increase of the basal BRET ratio (Fig. 10C). The Kir6.1 intramolecular BRET probe was activated using cromakalim, a potent and selective ATP-sensitive potassium channel opener (Sanguinetti et al., 1988). As expected, cromakalim induced a rapid increase of the basal BRET signal until reaching a plateau while, again, no effect was detected when the solvent was injected alone (vehicle) (Fig. 10E). Importantly, the measured potency of cromakalim (Fig. 10F) fell in the range already described in the literature (Wilson et al., 1988). Also, repaglinide, a known inhibitor of KiR activation by cromakalim (Gasser et al., 2003), not only right-shifted cromakalim concentration-response curve but also decreased cromakalim efficacy and Kir6.1 basal BRET. These observations indicate that repaglinide is not a competitive antagonist of cromakalim as described by others (Gasser et al., 2003), but rather behaves as a noncompetitive unsurmountable antagonist of cromakalim by stabilizing Kir6.1 in a distinct conformational state. Finally, TREK1 was successfully activated using increasing quantities of the chemical activator BL1249 (Pope et al., 2018) (Fig. 10H). Altogether, these results confirm that intramolecular BRET biosensors can probe the conforma-
tional changes occurring during the gating of ion channels belonging to various ion channel families and not just the TRP ion channel family.

This led us to assess whether the intramolecular BRET sensor can also probe ligand-gated ion channels such as P2X purinergic receptors that have both extremities inside the cytoplasm. We therefore constructed the nLuc-P2X2-mNeonG intramolecular BRET probe (Fig. 11A) and transiently transfected it in HEK293T cells. The rat P2X2 ion channel was activated in the presence of increasing quantities of ATP that induced a time-dependent decrease of the basal BRET (Fig. 11B). Dose-response analysis revealed that ATP activated the nLuc-P2X2-mNeonG intramolecular BRET probe with EC_{50} fitting the known potency of ATP to activate native P2X2 (Fig. 11C), as measured using conventional techniques (North and Surprenant, 2000). These results further suggest the suitability of our intramolecular BRET probe to efficiently measure the conforma-
tional changes occurring in various ion channel during their gating.
Discussion

Taking advantage of our recently published intra- and intermolecular BRET probes targeting TRPV1 ion channels activation in real-time and on live cells (Ruigrok et al., 2017), we have 1) assessed whether such BRET biosensors can effectively be used for high-throughput purposes, 2) performed a comparative screen of the Prestwick chemical library with both our BRET probes and ACA, followed by the analysis of the hits using APC, and 3) exemplified the use of intramolecular BRET probe to measure other ion channel activation.

Both intra- and intermolecular BRET probes made it possible to account for the agonist or antagonist ability of different reference compounds to modulate TRPV1 activity in live HEK293T cells (Fig. 1). Although the measured potencies of each agonist obtained from each of our two BRET probes are in full agreement with the data reported in the literature and with the values measured with ACA, the pIC_{50} values measured from most of the antagonists were found to be lower by 0.5–1.5 log units (Fig. 2 and Table 1). Several experimental differences between our study and the ones in the literature may explain this discrepancy, such as the technique used to measure TRPV1 activity, the pH and temperature of the assay, the amount of CAPS needed to trigger TRPV1 activation in the presence of the various antagonists, as well as the cell model used which has been shown to directly impact both efficacy and potency of TRPV1 ligands (Bianchi et al., 2006). Species-specific differences in TRPV1 functionality may also matter (Abbas, 2020). The potencies measured for each reference compound using both intra- and intermolecular BRET probes were however very close to the one measured using ACA, which is often used as the primary screen for calcium ion channels.

Most studies using APC to study TRPV1, if not all, measure the outward current at nonphysiologic membrane potentials (between +60 and +100 mV) and might thus highlight compounds that are not relevant for a therapeutic effect. We have shown that the choice of the membrane potential to measure the chemical activation of TRPV1 not only impacts the potency of several TRPV1 agonists and antagonists, but also drastically affects the ability of many of the tested drugs to activate or inhibit TRPV1. One likely explanation for this observation is that different ternary or quaternary conformational states of TRPV1 are stabilized when the membrane potential is clamped at various values. This observation is in agreement with the allosteric model for gating of thermo-TRPs, such as TRPV1, in which voltage, temperature, and ligands are independently coupled, either positively or negatively, to channel gating (Matta and Ahern, 2007). This is of prime importance since ligand binding is expected to be intrinsically dependent on its receptor conformation state (de Boer, 2020), thereby rehabilitating nonelectrophysiological methods such as our novel BRET-based assay for ion channel HTS. Both intra- and intermolecular BRET assays achieved, moreover, excellent Z’-factors, further indicating that both BRET assays are fit enough for high-throughput screening (Fig. 3).

We then aimed at comparing the effectiveness of both intra- and intermolecular BRET assays with that of HTS conventional methods (ACA and APC) to screen the Prestwick small-sized chemical library. Primary screening using ACA, intra- and intermolecular BRET assays indicated that 59 drugs activated TRPV1 or inhibited CAPS-induced TRPV1 activation. The results were heterogeneous, since 82.6% of the hits were found by only one method. No hits were found by all three methods to behave as TRPV1 activator, and only 3 drugs were found by the three assays to behave as an inhibitor of TRPV1(CAPS). Of note, out of the 33 drugs found by ACA to modulate TRPV1 activity, 8 were found to both activate and inhibit TRPV1, indicating that almost a quarter of the hits found with ACA yielded ambiguous results (Supplementary Fig. 6 and Table 3). In sharp contrast, no hits were detected as both activator and inhibitor of TRPV1 using either intra- or intermolecular BRET probe.

The secondary screen of the 59 identified drugs with APC highlighted important facts. Since only 14 drugs were confirmed as TRPV1 activator or inhibitor by APC, our results mean either that both BRET probes and ACA are prone to yield a lot of false positive hits or that APC measurement is prone to yield a lot of false-negative hits. A careful review of the literature highlighted interesting clues pointing to a more balanced conclusion: First, as mentioned in the result section, 13 compounds over the 59 detected hits (~22% of the hits identified) are well known calmodulin antagonists, and 12 of them belongs to only three clusters (E, G, and J). Seven other compounds belonging to clusters E, G, or J, share structural similarities with known CaM antagonists (see result section for details). Over these 20 drugs, 15 were identified as TRPV1(CAPS) inhibitors by either one or both BRET probes while only 7 and 6 were respectively detected by ACA and APC (Fig. 8). Also, to the best of our knowledge, 19 other compounds known to be CaM antagonists were part of the Prestwick chemical library but were not included in the lists of 59 drugs identified during the primary screening (Supplemental Table 4). Reanalysis of the results obtained with these compounds revealed that the average inhibition efficacy of 8 of them was either very close to the drug efficacy cut-off fixed at 30%, or above but with no reproducibility between n1 and n2. These compounds, mostly detected with the intramolecular and intermolecular BRET assay, might be therefore false negatives that could have been included as positive hits using a data-driven analysis of the primary screen results instead of a subjective cut-off of the efficacy magnitude. Such workflow will be included in our further studies. Overall, these results indicate that most of the CaM antagonists included in the Prestwick chemical library were detected as inhibitor of TRPV1 activation by CAPS. Interestingly, although calmodulin is classically viewed as a negative modulator of TRPV1, regulating its desensitization (Numazaki et al., 2003; Rosenbaum et al., 2004; Lishko et al., 2007; Lau et al., 2012), CaM inhibition using various chemical compounds, including chlorpromazine (#3) and fluphenazine (#23), has already been shown to inhibit CAPS-induced TRPV1 activation with a potency in the μM range (Oläh et al., 2007). Whether CaM antagonists can inhibit TRPV1 through direct interaction with TRPV1 itself, by preventing the physical interaction between TRPV1 and CaM, or by another means, remains to be determined. In the light of our BRET-based repurposing drug screening results, and in the quest for new TRPV1 inhibitors, considering the CaM-TRPV1 physical and/or functional interaction as druggable is a tempting hypothesis that deserves further attention.
Ambrosino P, Soldovieri MV, Di Zazzo E, Paventi G, Iannotti FA, Mosca I, Miceli shown their adaptability to various TRPs and non-TRPs easy to use with a low cost of implementation and have the CaM-TRPV1 protein-protein interaction as a druggable readout of ion channel activity, and 3) brought back to light BRET probes 1) have proven to be as reliable as ACA or a thorough bibliographic analysis of our results, both pledged to a multiparametric analysis to optimize future ion--
data (Fig. 8). No single technique detected all hits in a single HTS run. Our study, therefore, highlights the need for benefitting from the output of different HTS platforms coupled to a multiparametric analysis to optimize future ion--channel drug screening processes. In conclusion, based on a thorough bibliographic analysis of our results, both BRET probes 1) have proven to be as reliable as ACA or APC in identifying potential hits, 2) provide a very specific readout of ion channel activity, and 3) brought back to light the CaM-TRPV1 protein-protein interaction as a druggable target for TRPV1 inhibition. Since BRET biosensors are easy to use with a low cost of implementation and have shown their adaptability to various TRPs and non-TRPs ion channels (Figs. 9–11), they may advantageously be included in ion channel drug screening campaigns.

Acknowledgments
The authors thank Bernard Veyret for his wise advice and proof-reading of the manuscript.

Authorship Contributions
Participated in research design: Franchet, Percheran.
Conducted experiments: Chappe, Michel, Jousshomme, Barbeau, Pierredon, Baron, Garenne, Poullier de Gannes, Hurtier.
Performed data analysis: Chappe, Michel, Barbeau, Pierredon, Garenne, Mayer, Lagrue, Quignard, Ducrot, Compan, Franchet, Percheran.
Wrote or contributed to the writing of the manuscript: Chappe, Michel, Lagrue, Quignard, Ducrot, Compan, Franchet, Percheran.

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**Supplementary Figure 1:** Capsaicin-induced activation of TRPV1 current. (A) Whole-cell patch-clamp recording showing the representative trace of 10 μM Capsaicin-activated current elicited by 650 ms voltage ramps from -60 to +60 mV in HEK293T cells transfected with empty vector, TRPV1 WT or mNeonGreen-TRPV1- nLuc. The holding potential was -60 mV. Currents were normalized to cell capacitance for each cell and are expressed as pA/pF. (B) Summary data for capsaicin-induced mean current densities at +60 mV recorded in cells transfected with the different constructions. Data are expressed as mean value ± S.D. and 3 < n < 5. Mann-Whitney test was performed to compare the TRPV1 WT and mNeonGreen-TRPV1-nLuc currents.
Supplementary Figure 2: The distance from each point to the \( y=x \) equation line was computed as shown on the inset for each method (intramolecular, intermolecular, and calcium assays) and both for agonist and antagonist conditions (complete data are shown in figure 4 G-H-I and figure 5 G-H-I). The smaller is the distance (greens line), the higher is the reproducibility between n1 and n2 assays. Statistical significance between the different conditions were analysed using a Kruskal-Wallis and Conover post-hoc tests, *: p-value < 5%, n.s.: not significant.)
**Supplementary Figure 3:** Four measures frequently used to evaluate dispersion were computed: the median absolute deviation (mad), the difference between the largest and smallest values (range), the quartile coefficient of dispersion (qcod), and the interquartile range (iqr). The higher these metrics, the larger the data dispersion. Data were pooled (n1 and n2) in the 2 different assays conditions: (A) agonist and (B) antagonist. The four metrics were computed, normalized, and plotted on radar charts.
**Supplementary Figure 4**: Number of hits detected within each data-driven group as a function of structure-driven clusters.
**Supplementary Figure 5:** Data-driven hierarchical clustering of the effect of the hit compounds performed without the BRET data (left panel) or ACA data (right panel).
Supplementary Figure 6: Venn diagram of the hits detected with the automated Calcium-flux assay in the activation and the inhibition mode.
**Supplementary Table 4:** List of known Calmodulin antagonists that are part of the Prestwick Chemical library and that were not selected during the primary screen.

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<th>Chemical name</th>
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<th>Intermolecular BRET</th>
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<th>Cluster</th>
<th>Class</th>
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<td>9.40</td>
<td>9.0</td>
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<td>Azole derivatives</td>
<td>(Hegemann et al., 1993)</td>
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<td>-8.94</td>
<td>6.53</td>
<td>17.1</td>
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<td>(Lugnier et al., 1984)</td>
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<td>-7.91</td>
<td>17.88</td>
<td>5.97</td>
<td>G</td>
<td>phenothiazine</td>
<td>(Acs et al., 1995)</td>
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<td>Amiodarone</td>
<td>-8.15</td>
<td>31.75</td>
<td>0.6</td>
<td>C</td>
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<td>(Nokin, n.d.)</td>
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<td>Fendiline</td>
<td>7.13</td>
<td>35.36</td>
<td>3.62</td>
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