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Evaluation of known and novel inhibitors of Orai1-mediated store operated Ca^{2+} entry in MDA-MB-231 breast cancer cells using a fluorescence imaging plate reader assay

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ABSTRACT

The Orai1 Ca^{2+} permeable ion channel is an important component of store operated Ca^{2+} entry (SOCE) in cells. It's over-expression in basal molecular subtype breast cancers has been linked with poor prognosis, making it a potential target for drug development. We pharmacologically characterised a number of reported inhibitors of SOCE in MDA-MB-231 breast cancer cells using a convenient Fluorescence Imaging Plate Reader (FLIPR) assay, and show that the rank order of their potencies in this assay is the same as those reported in a wide range of published assays. The assay was also used in a screening project seeking novel inhibitors. Following a broad literature survey of classes of calcium channel inhibitors we used simplified ligand structures to query the ZINC on-line database, and following two iterations of refinement selected a novel Orai1-selective dichlorophenyltriazole hit compound. Analogues of this were synthesized and evaluated in the FLIPR assay to develop structure-activity relationships (SAR) for the three domains of the hit; triazole (head), dichlorophenyl (body) and substituted phenyl (tail). For this series, the results suggested the need for a lipophilic tail domain and an out-of-plane twist between the body and tail domains.

Keywords: Calcium signalling, store-operated calcium entry (SOCE), pharmacological inhibitors, pharmacophore modelling, Orai1, breast cancer

Abbreviations: AFU, arbitrary fluorescence units; CPA, cyclopiazonic acid; $[Ca^{2+}]_{CYT}$, cytosolic free Ca^{2+}; ER, endoplasmic reticulum; FLIPR, Fluorescence Imaging Plate Reader; IP3, inositol trisphophate; PSS, physiological salt solution; SAR, structure-activity relationship; SERCA, sarco/endoplasmic reticulum ATPase; SOCE, store operated Ca^{2+} entry; TRP, transient receptor potential

1. Introduction

The regulation of cytosolic free Ca^{2+} ($[Ca^{2+}]_{CYT}$) is a vital component of a variety of cellular signalling cascades that are responsible for processes as diverse as muscle contraction to hormone secretion.[1] The opening of Ca^{2+} permeable ion channels on the plasma membrane is one of the key mechanisms for increasing $[Ca^{2+}]_{CYT}$. These Ca^{2+} permeable ion channels include voltage-gated Ca^{2+} channels such as L-type Ca^{2+} channels which are the target for dihydropyridine-based Ca^{2+} channel blockers used in the treatment of hypertension;[2] transient receptor potential (TRP) channels such as the heat sensor TRPV1 activated by the red hot chilli component capsaicin;[3] and the more recently identified component of store operated Ca^{2+} entry (SOCE) - Orai1.

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SOCE describes the activation of Ca^{2+} influx through the sensing of depletion of intracellular calcium stores of the endoplasmic reticulum.[4] Such depletion occurs through the release of Ca^{2+} by activation of phospholipase C coupled G-protein coupled receptors and the associated production of inositol triphosphate (IP_3) and activation of IP_3-activated Ca^{2+} channels of the endoplasmic reticulum. SOCE can also be induced by pharmacological inhibition of the sarco/endoplasmic reticulum ATPase (SERCA), a calcium pump responsible for the active transport of Ca^{2+} into the endoplasmic reticulum.

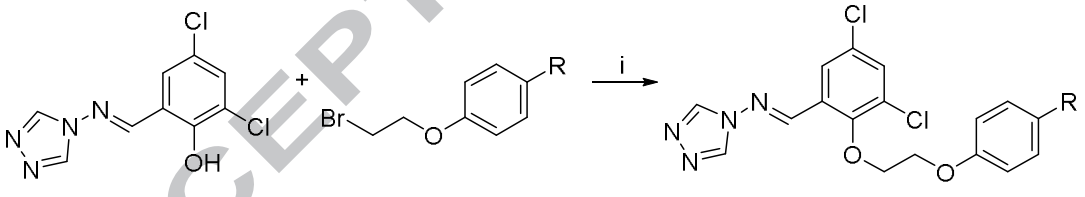
In 2006, Orai1 was identified as a plasma membrane protein responsible for SOCE.[5-7] Orai1 is implicated in a variety of physiological functions including processes important in immunity,[5] lactation[8] and the cardiovascular and respiratory systems.[9] A number of studies have also linked Orai1 with cancer.[10, 11] For example, basal molecular subtype breast cancers associated with poor prognosis express elevated levels of Orai1 and alterations in the relative levels of the Orai1 activators STIM1 and STIM2.[12] Silencing of Orai1 reduces proliferation and metastasis of breast cancer cells such as MDA-MB-231 basal-like breast cancer cells[12, 13] and pharmacological inhibition of SOCE has been shown to reduce the proliferation and metastasis of MDA-MB-231 breast cancer cells.[13, 14]

A variety of agents have been reported[15, 16] to inhibit SOCE and this pathway has been highlighted as an opportunity for therapies for the treatment of a variety of diseases.[17] In this study we assessed the effects of a number of reported inhibitors of SOCE in MDA-MB-231 breast cancer cells using a Fluorescence Imaging Plate Reader (FLIPR), and also report on a novel class of triazole-based SOCE inhibitors.

2. Results and Discussion

2.1. Chemistry

Compounds **14**-**16** of Table 2 were prepared by K_2CO_3-induced condensation of iminotriazole **25** and the appropriate 2-bromoethoxybenzenes **26**-**28** (Scheme 1). Aldehyde **20** was similarly prepared from bromide **26** and aldehyde **29**. Aldehyde **29** was also bromoethylated to give bromide **30**, which gave the pyridyl aldehyde **31** and iminotriazole **17** via (Scheme 2). Finally, the monochloro derivatives **18** and **19** were similarly prepared by condensation of bromide 26 with the known iminotriazoles **32** and **33** respectively (Scheme 3). Compound structures and purity were monitored by ^{1}H NMR spectroscopy, HPLC and combustion analysis.



25

26: R=*sec*-butyl
27: R=Me
28: R=*tert*-butyl

14: R=*sec*-butyl
15: R=Me
16: R=*tert*-butyl

Scheme 1. Reagents and conditions: (i) K_2CO_3, dry DMF, 48 h, 20 ^oC.

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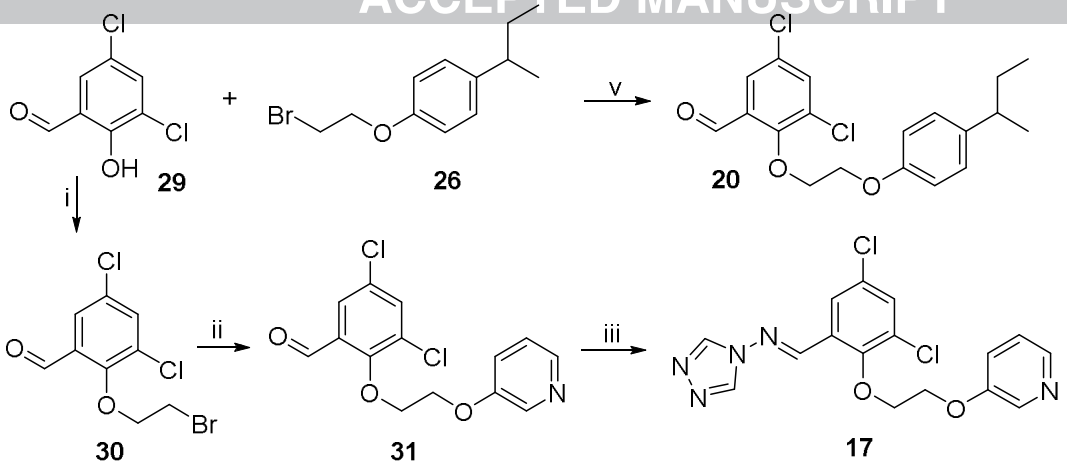
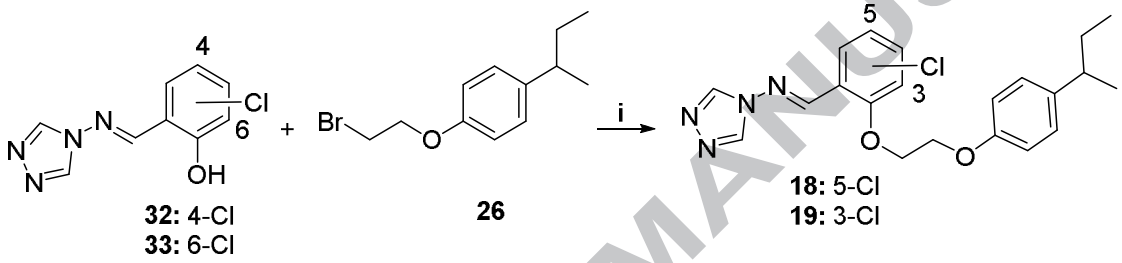


Scheme 2. Reagents and conditions: (i) dibromoethane, K_2CO_3 , dry DMF, 80 °C, 24 h; (ii) pyridin-3-ol, K_2CO_3, dry DMF, 12h, 40 °C; (iii) 4*H*-1,2,4-triazol-4-amine, MeOH/THF, 5h, reflux; (iv) K_2CO_3 , dry DMF, 48 h, 20 °C.



Scheme 3. Reagents and conditions: (i) K_2CO_3, dry DMF, 24 h, 20 °C.

2.2. Biology

Measurement of free intracellular calcium was carried out using a Fluorescence Imaging Plate Reader (FLIPR) Ca^{2+} assay in MDA-MB-231 breast cancer cells (Tables 1 and 2). This assay has been demonstrated to be sensitive to changes in Orai1 activity in MDA-MB-231[12] and is described in detail in the methods section. As shown in Figure 1, addition of the SERCA inhibitor cyclopiazonic acid (CPA, 10 μM) produced a gradual release of Ca^{2+} from internal stores indicated by peak 1. Due to the chelation of extracellular Ca^{2+} ions with the calcium-specific chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid) (BAPTA) this resulted in store depletion. The addition of extracellular Ca^{2+} produced the SOCE (peak 2). This SOCE peak was reduced by silencing of Orai1 but not the related isoforms Orai2 and Orai3 (Figure 1A) and the reported SOCE inhibitor YM-58483 (**1**) (Figure 1B). The assessment of peak 1 (Ca^{2+} release) and peak 2 (SOCE) allowed effective and rapid identification of agents which were not selective and may have affected SOCE due to a consequence of effects on Ca^{2+} store levels (equipotent against peak 1 and peak 2 (e.g., SKF-96365 (**7**) and mibefradil (**8**)) and those able to inhibit SOCE in the absence of effects on Ca^{2+} store release (no effect on peak 1, inhibition of peak 2, e.g. YM-58483 (**1**) and Synta66 (**12**)). The consistent reporting of peak 1 concentration response effects described in this FLIPR assay, represents a powerful tool for the high throughput identification of agents that may alter SOCE through mechanisms independent of Orai or STIM proteins.

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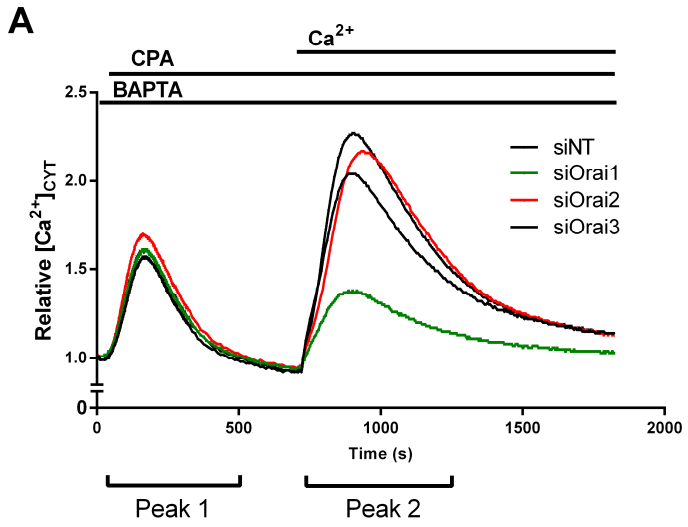


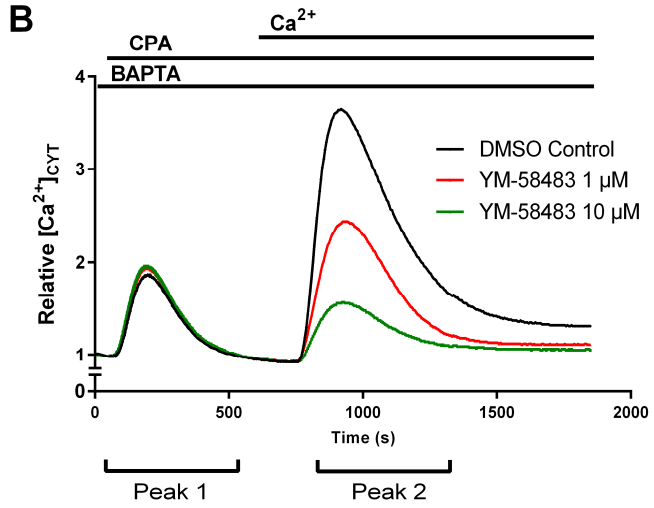
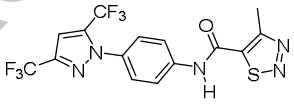
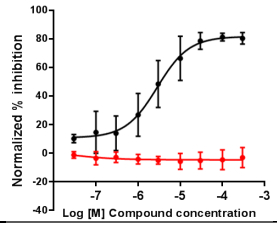
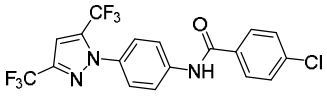
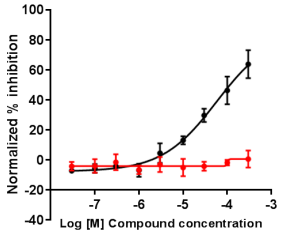


Table 1. Evaluation of known calcium channel inhibitors for Orai1 activity

No	Structure	IC$_{50}$[a]		Concentration Response Curve For SOCE
		ER release (Peak 1)	SOCE (Peak2)	Peak 1 / Peak 2
1		not active[b]	2.8±0.95	
2		not active[b]	57±39	

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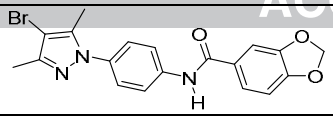
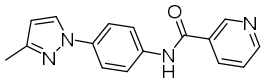
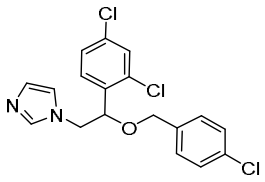
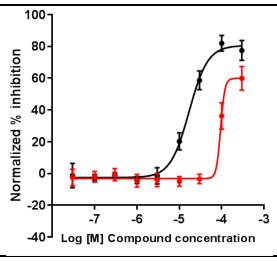
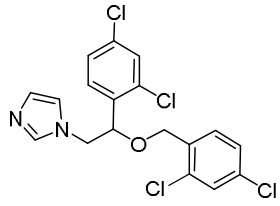
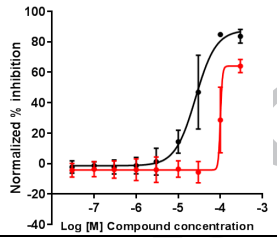
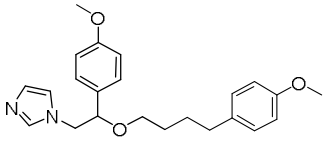
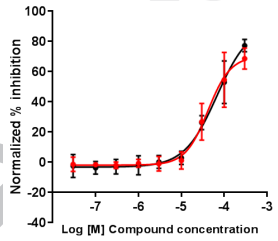
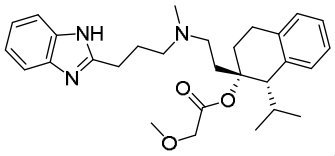
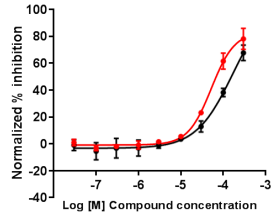
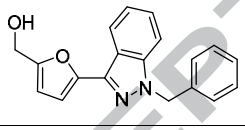
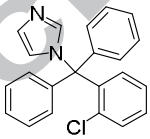
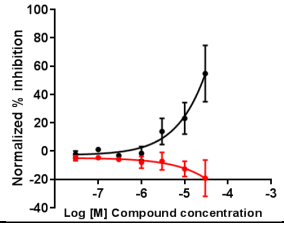
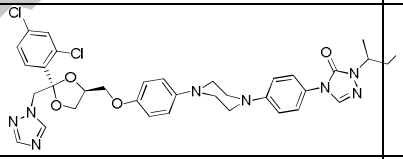
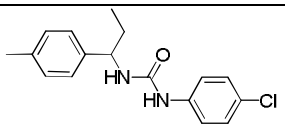
3		not active[b]	not active[b]	
4		not active[b]	not active[b]	
5		94 ±7.2	17±1.2	
6		~100	27±8.4	
7		42±0.4	67± 11.8	
8		52.1±2.5	>100	
9		not active[b]	not active[b]	
10[c]		not active[b]	55% at 30 µM (est.)[c,d]	
11[c]		not active[b]	not active[b]	
12		not active[b]	not active[b]	

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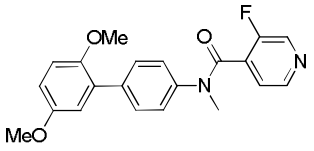
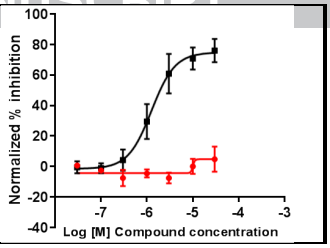
13[c]		not active[b]	1.3±1.8 [d]	

Footnotes for Table 1

[a]IC_{50} is drug concentration for a half-maximal response; [b]Less than 10% inhibition at 100 µM. [c]Estimate of activity where 100% inhibition not reached. [d]Due to low solubility, these compounds were tested only up to 30 µM.

Pyrazole analogues such as YM-58483 (**1**)[18] are known to be potent inhibitors of thapsigargin-induced SOCE Ca^{2+} influx in cells, being 30-fold more selective for SOCE over voltage-gated Ca^{2+} channels, and many analogues are known. Members of the class inhibit hypersensitivity reactions in mice, attributed to their inhibition of T-cell activation,[19] but also show activity against other Ca^{2+} channels such as TRPM4.[20] In our assay, **1** was the second most potent inhibitor of SOCE mediated by Orai1, with an IC_{50} of 2.8 µM, and highly selective for Orai1 versus the other channels studied here. The closely-related [21] **2** was also active against Orai1 but the structurally related compounds **3** and **4**, with changes in the pyrazole ring, were not active. None of the compounds were active against TRPV1, TRPM8 or $Ca_V2.2$ at concentrations up to 100 µM (data not shown).

The well-known anti-mycotic imidazole econazole (**5**) has also been shown to block SOCE by acting extracellularly[16, 22] and showed measurable Orai1 activity in our assay. The structurally related analogue miconazole (**6**)[23] was also active and showed some selectivity for Orai1. SKF-96365 (**7**), one of the first reported SOCE channel inhibitors,[24] is known to also prevent tumour metastasis[13] but is reported to be relatively non-selective, blocking many other ion channels with similar potency.[25, 26] Mibefradil (**8**) is an inhibitor of T-type Ca^{2+} channels,[27] and was briefly marketed for migraine, but was withdrawn because of drug-drug interactions due to its potent inhibition of CYP3A4.[28] In an earlier study[29] it was shown to have no effect on signal- and extracellular Ca^{2+}-dependent increases in [Ca^{2+}] stimulated by oxytocin. In our assay both **7** and **8** were active (albeit not very potent; IC_{50}s 67 and above 100 µM respectively) against Orai1-mediated Ca^{2+} influx (Table 1) but were not selective with respect to inhibition of the $Ca_V2.2$ channel (data not shown).

YC-1 (**9**) is a nitric oxide-independent activator of soluble guanylyl cyclase via allosteric binding,[30] and is reported to be an activator of large-conductance Ca^{2+}-activated potassium channels,[31] but was not active in our assay. The antifungal drug clotrimazole (**10**) is structurally similar to the Ca^{2+}-dependent potassium Gardos channel inhibitor senicapoc,[32] but was similarly not active in our assay. The antifungal drug itraconazole (**11**) is a triazole-based calcium channel inhibitor that has been shown to have anti-angiogenic activity, and is in clinical trial for non-small-cell lung cancer,[33, 34] but was not active in our assay.

The urea analogue **12** was the most potent of a number of analogues reported[35] as specific inhibitors of Orai1, but was not active in our assay. Finally, the extensively-studied biphenyl analogue Synta66 (**13**)[36-38] has been demonstrated to be a potent (IC_{50} 26 nM by patch-clamp assay) and selective Orai1 inhibitor. This was confirmed in the FLIPR assay, where it was the most potent (IC_{50} 1.3 µM) literature compound evaluated. These studies with a variety of literature compounds demonstrated the utility of the FLIPR assay to rapidly assess the properties of compounds for their effects on Orai1-controlled Ca^{2+} influx into cells.

Overall, while there is too much diversity in the assay methods to attempt a quantitative comparison between our FLIPR assay results and literature values for these known compounds, the rank order of their potencies is certainly the same. The differences in effectiveness of various agents in this model may be related to the cell line used, for example Orai1 channels are known to form heteromers with other ion channel components and this could lead to differences in sensitivity to various bioactive molecules and pharmacological modulators, this is exemplified by the remodeling of Orai1/Orai3 channels in some prostate cancer cells which are activated by arachidonic acid rather than Ca2+ store depletion (new ref 39).

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The assay was also used in a screening project seeking novel inhibitors. Following a literature survey to identify different classes of calcium channel inhibitors of all types, we used either the ligand structures or simplified versions as queries for the ZINC on-line database (SI Table 1). The results of the ZINC similarity search were combined and reduced to a smaller set of 20 compounds (based on similarity, availability and cost) that were subsequently purchased and screened in the FLIPR assay for Orai1-selective effects. From this set we discovered two compounds active in our assay; T6454376 and 6035967 (Figure S1) (IC_{50} values estimated at 48 and 75 μM from a single determination). These compounds were used, along with other literature compounds that inhibited Orai1-controlled Ca^{2+} influx in our hands (these included compounds **1**, **2**, **5**, **7**, **8** and **9**) to search for related molecules from Chembridge. The results of the search were then restricted to either the molecular weight or clogP range of the query compounds, then by availability and cost. A final collection of 2140 compounds was tested. A triazole (**14**) from the final screening library was selected as a hit for further elaboration since, although containing both a triazole unit and the dichlorobenzene unit of miconazole, it was overall novel and showed encouraging potency and selectivity for Orai1 in the FLIPR assay. The ability of FLIPR to simultaneously assess Ca2+ levels in 384 wells over the entire experimental period was critical in defining the properties of peak 1 and peak 2 for each tested compound, and would not be possible using approaches not capable to complete simultaneous assessment of all wells (new ref 40).

The results of this initial structure-activity relationship (SAR) study are shown in Table 2. For SAR purposes, **14** can be divided into three separate domains, defined as the head (triazole), body (dichlorophenyl) and tail (substituted phenyl) (Figure 2). The dichloro unit making up the body of the compound suggested the existence of an out-of-plane twist in the molecule. Overall, **14** is relatively lipophilic (clogP 4.73), with much of the lipophilicity residing in the *sec*-butyl tail unit. Each of these features was investigated. Substitutions in the tail provided an opportunity to vary the overall lipophilicity and perhaps improve solubility. Both the methyl (**15**) (clogP 3.27) and *tert*-butyl (**16**) (clogP 4.60) were prepared and showed some selectivity for Orai1 but were much less active than **14**, not achieving IC_{50}s below 100 μM. In contrast the much more hydrophilic 3-pyridyl analogue **17** (clogP 1.77) was essentially inactive.

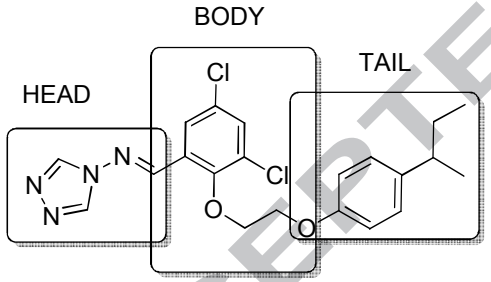

Figure 2. Defined structural domains of the triazole class of Orai1 inhibitors (Table 2)

Table 2. Evaluation of novel triazoles for Orai1 activity

No	Structure	IC_{50}[a] (μM)		Concentration Response Curve For SOCE
		ER release (Peak 1)	SOCE (Peak2)	Peak 1 Peak 2

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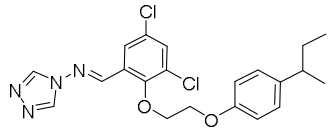
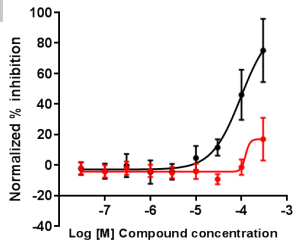
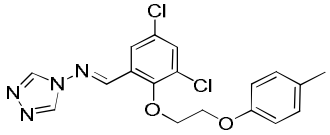
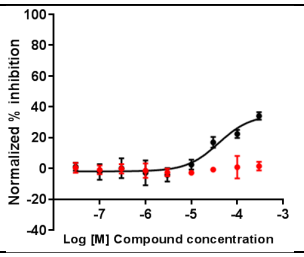
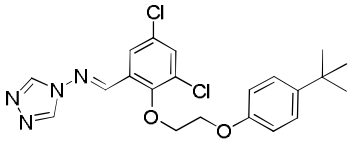
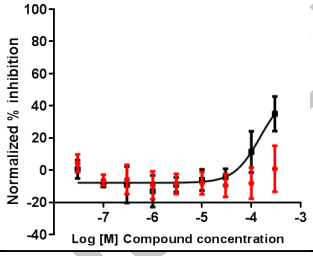
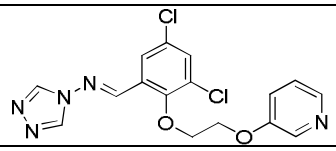
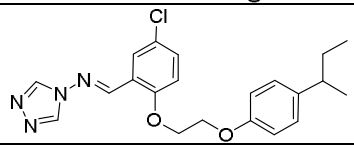
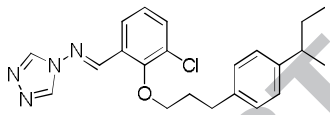
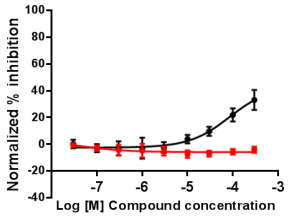
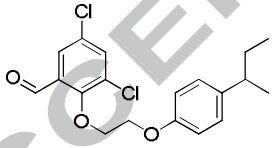
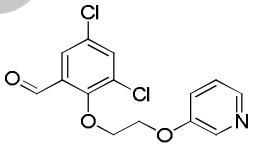
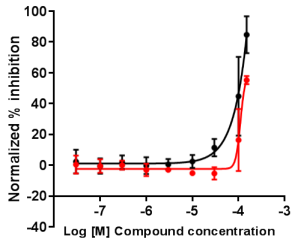
14	 35693 (lead compound from screen)	not active[b]	96.9±32	
15	 36882 (analogue of lead)	not active[b]	22% at 100 µM (est.)[c]	
16	 SN36959 (more lipophilic))	not active[b]	11% at 100 µM (est.)[c]	
17		not active[b]	not active[b]	
18		not active[b]	not active[b]	
19		not active[b]	22% at 100 µM (est.)[c]	
20		not active[b]	not active[b]	
21[c]		~100	~100	

Footnotes for Table 2

[a]IC_{50} is drug concentration for a half-maximal response; [b]Less than 10% inhibition at 100 µM. [c]Estimate of activity where 100% inhibition not reached.

To explore the influence of the chloride substituents in the central ring, both of the monochloro analogues (**18** and **19**) of the hit compound **14** were prepared and evaluated. Removal of the 3-Cl group *ortho* to the side chain in compound **18** completely abolished activity, whereas the 5-Cl analogue **19** did retain some

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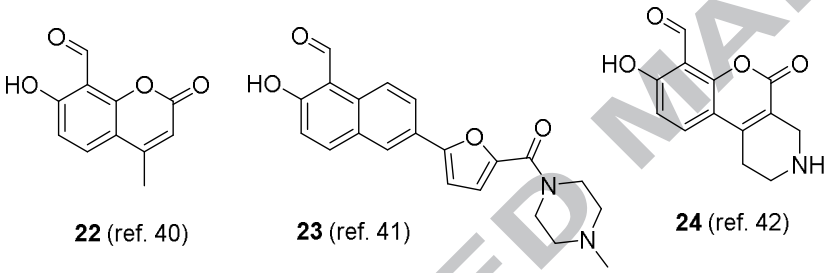
activity (albeit at >100 μM), suggesting the requirement of an out-of-plane twist between the body and tail domains.

N-benzylidene-4*H*-1,2,4-triazol-4-amines such as **14** have been reported and evaluated in various biological assays,[41] but they have potential susceptibility for acid hydrolysis to the corresponding aldehydes. The stability of **14** was therefore determined under the conditions used in the calcium influx assay, and no loss of compound or formation of the corresponding aldehyde (**20**) was seen, indicating that hydrolysis is not occurring under the FLIPR test conditions. This aldehyde itself was also inactive when tested at two concentration points, suggesting that the 4-formimidoyl triazole head group is necessary for the inhibition of Orai1 channel activity, but was extremely lipophilic (clogP 6.74). We therefore also explored the more hydrophilic aldehyde **21** (clogP 3.79), which significantly inhibited both calcium influx peaks, suggesting non-selective activity at multiple targets.

Compound **21** was explored because aromatic aldehydes have been previously evaluated as drugs (Figure 3); examples include **22**, which inhibits the endoplasmic reticulum transmembrane protein IRE1 (that mediates the unfolded protein response) by forming an unusually stable Schiff base with lysine 907.[42] Similarly, aldehyde MKC-3946 (**23**) showed growth inhibition in multiple myeloma cell lines and potentiated the cytotoxicity of the endoplasmic reticulum stress inducers bortezomib or 17-AAG,[43] and **24**, which by targeting IRE1 mimicked XBP-1 deficiency, suppressed growth of B cell chronic lymphocytic leukemia in a xenograft model.[44]

The compounds in Table 2 were also screened in the FLIPR assay for their ability to inhibit the TRPV1, TRMP8 and $Ca_V2.2$ (N-type) calcium channels, but none showed any activity up to 100 μM.



22 (ref. 40) **23** (ref. 41) **24** (ref. 42)

Figure 3. Literature examples of aromatic aldehyde drugs

2.4. Conclusions

This study explored the use of a FLIPR Ca^{2+} assay in MDA-MB-231 breast cancer cells to evaluate a number of compounds previously reported (using a variety of different assays) as inhibitors of SOCE mediated by Orai1. We also used the assay to briefly explore the SAR around a class of triazoles as potential inhibitors. Screening studies had identified the triazole **14** as a novel inhibitor of SOCE (Figure 4A) with superior ability to avoid the effects on Ca^{2+} store release compared with the SOCE inhibitor SKF-96365 (**7**) (Figure 4B) and with a similar inhibition profile (although with slightly less potency) to the SOCE inhibitor YM-58683 (**1**) (Figure 4C). Structure-activity studies suggested that a lipophilic tail unit (Figure 2) was needed (compare **14** and **17**), with a central unit that provided some non-planarity (compare monochlorides **18** and **19**). Finally, while the free aldehyde (**20**) of the parent compound was inactive, the aldehyde (**21**) of the (inactive) 3-pyridyl derivative (**17**) did show activity but was not selective for effects on SOCE (peak 2) versus release of Ca^{2+} from internal stores (peak 1).

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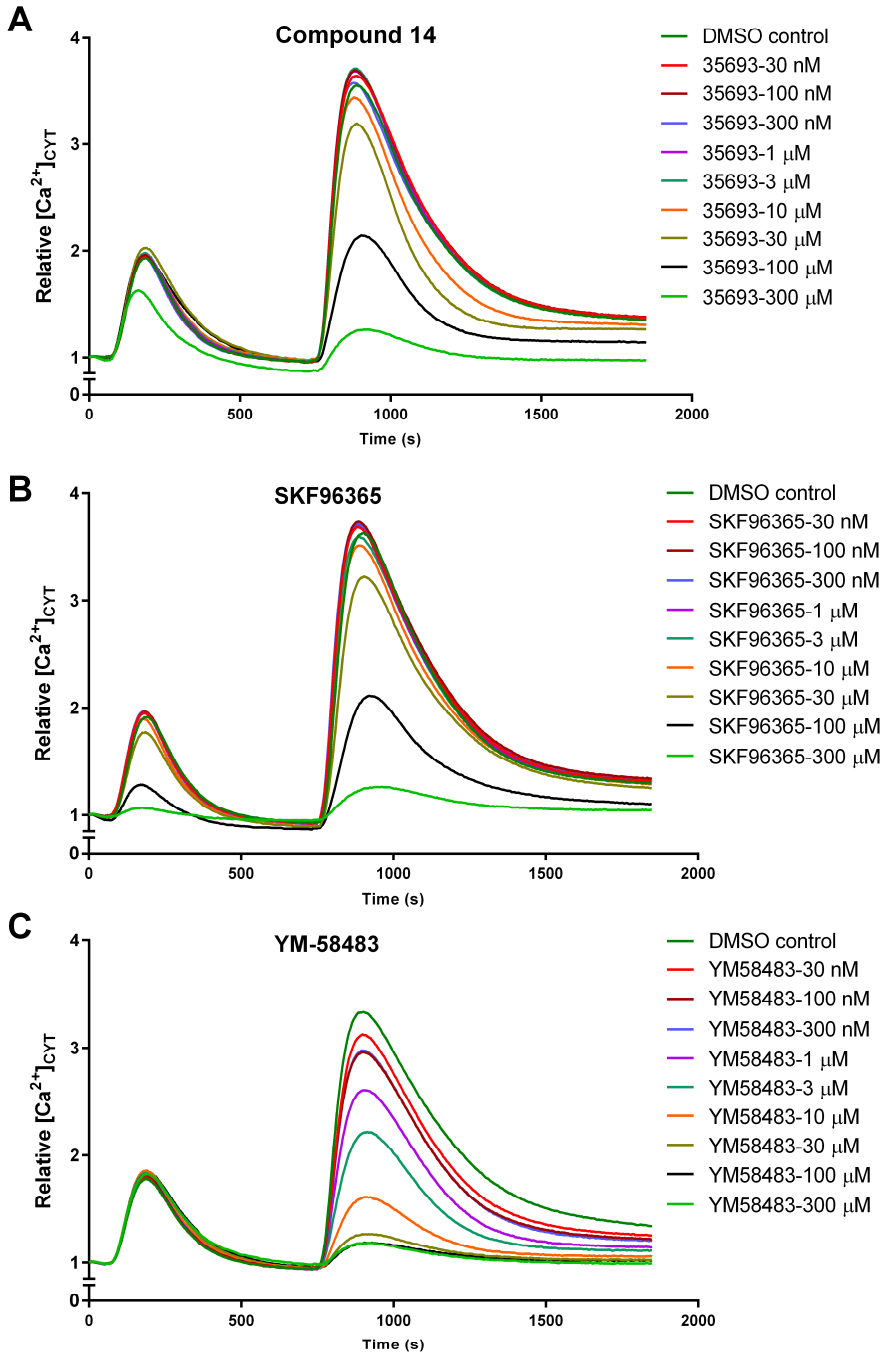

3. Experimental

3.1. Chemistry

Final products were analysed by reverse-phase HPLC (Alltima C18 5 µm column, 150 × 3.2 mm; Alltech Associated, Inc., Deerfield, IL) using an Agilent HP1100 equipped with a diode-array detector. Mobile phases were gradients of 80% CH_3CN/20% H_2O (v/v) in 45 mM NH_4HCO_2 at pH 3.5 and 0.5 mL/min. Purity was determined by monitoring at 330 ± 50 nm and was ≥95% for all final products. Final product purity and structure were also assessed by combustion analysis carried out in the Campbell Microanalytical Laboratory, University of Otago, Dunedin, New Zealand. Melting points were determined on an Electrothermal 9100 melting point apparatus. NMR spectra were obtained on a Bruker Avance 400 spectrometer at 400 MHz for ^{1}H.

3.1.1. (*E*)-1-(2-(2-(4-(*sec*-Butyl)phenoxy)ethoxy)-3,5-dichlorophenyl)-*N*-(4*H*-1,2,4-triazol-4-yl)methanimine (14) (Scheme 1). A mixture of *(E)*-2-(((4*H*-1,2,4-triazol-4-yl)imino)methyl)-4,6-

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dichlorophenol[45] (**25**) (100 mg, 0.39 mmol) and 1-(2-bromoethoxy)-4-(*sec*-butyl)benzene[44*46] (**26**) (120 mg, 0.47 mmol) and K_2CO_3 (160 mg, 1.17 mmol) were stirred in dry DMF (2 mL) for 48 h. EtOAc was added and the system was washed with water and dried (Na_2SO_4). Flash chromatography (petroleum ether/EtOAc; 1:1) followed by petroleum ether then Et_2O trituration gave **14** (32 mg, 19%) as a white solid; mp 109-110 °C; ^{1}H NMR ($CDCl_3$) δ 9.04 (s, 1H), 8.40 (s, 2H), 7.97 (d, *J* = 2.6 Hz, 1H), 7.58 (d, *J* = 2.6 Hz, 1H), 7.09 (d, *J* = 8.6 Hz, 2H), 6.77 (d, *J* = 8.7 Hz, 2H), 4.50 (dt, *J* = 4.1, 2.0 Hz, 2H), 4.28 (dt, *J* = 4.1, 2.0 Hz, 2H), 2.54 (sext, *J* = 7.0 Hz, 1H), 1.53 (quint, *J* = 7.3 Hz, 2H), 1.20 (d, *J* = 7.0 Hz, 3H), 0.80 (d, *J* = 7.4 Hz, 3H); Anal. Calcd for $C_{21}H_{22}Cl_2N_4O_2$: C, 58.21; H, 5.12; N, 12.93. Found: C, 58.48; H, 5.18; N, 12.87.

3.1.2. (*E*)-1-(3,5-Dichloro-2-(2-(*p*-tolyloxy)ethoxy)phenyl)-*N*-(4*H*-1,2,4-triazol-4-yl)methanimine (15). Similar reaction of **25** (100 mg, 0.39 mmol) and 1-(2-bromoethoxy)-4-methylbenzene[47] (**27**) (100 mg, 0.47 mmol) and K_2CO_3 (160 mg, 1.17 mmol) in dry DMF (2 mL) for 24 h, followed by workup, flash chromatography (CH_2Cl_2/MeOH; 100:0 then 197:3) and recrystallization (CH_2Cl_2/*i*-Pr_2O) gave **15** (48 mg, 32%) as a white solid; mp 144-145 °C; ^{1}H NMR ($CDCl_3$) δ 9.01 (s, 1H), 8.37 (s, 2H), 7.97 (d, J = 2.6 Hz, 1H), 7.58 (d, J = 2.6 Hz, 1H), 7.07 (d, J = 8.2 Hz, 2H), 6.73 (d, J = 8.6 Hz, 2H), 4.50 (dt, J = 4.0, 1.9 Hz, 2H), 4.27 (dt, J = 4.0, 1.9 Hz, 2H), 2.80 (s, 3H). Anal. Calcd for $C_{18}H_{16}Cl_2N_4O_2$: C, 55.26; H, 4.12; N, 14.32. Found: C, 55.16; H, 4.09; N, 14.17.

3.1.3. (*E*)-1-(2-(2-(4-(*tert*-Butyl)phenoxy)ethoxy)-3,5-dichlorophenyl)-*N*-(4*H*-1,2,4-triazol-4-yl)methanimine (16). Similar reaction of **25** (300 mg, 1.17 mmol), 1-(2-bromoethoxy)-4-(*tert*-butyl)benzene[48] (**28**) (360 mg, 1.40 mmol) and K_2CO_3 (480 mg, 3.50 mmol) were stirred in dry DMF (3 mL) for 24 h. EtOAc was added and the system was washed with water, brine and dried (Na_2SO_4). Flash chromatography (petroleum ether/EtOAc; 1:1 to 2:3) followed by recrystallization (CH_2Cl_2/*i*-Pr_2O) gave **16** (63 mg, 13%) as a white solid; mp 85-87 °C; ^{1}H NMR ($CDCl_3$) δ 9.03 (s, 1H), 8.38 (s, 2H), 7.98 (d, *J* = 2.6 Hz, 1H), 7.58 (d, *J* = 2.6 Hz, 1H), 7.29 (d, *J* = 8.9 Hz, 2H), 6.77 (d, *J* = 8.9 Hz, 2H), 4.51 (dt, *J* = 4.0, 1.9 Hz, 2H), 4.28 (dt, *J* = 4.0, 1.9 Hz, 2H), 1.29 (s, 9H). Anal. calcd. for $C_{21}H_{22}Cl_2N_4O_2$: C, 58.21; H, 5.12; N, 12.93. Found: C, 58.55; H, 5.10; N, 12.65.

3.1.4. (*E*)-1-(3,5-Dichloro-2-(2-(pyridin-3-yloxy)ethoxy)phenyl)-*N*-(4*H*-1,2,4-triazol-4-yl)methanimine (17) (Scheme 2). A mixture of 3,5-dichloro-2-hydroxybenzaldehyde (**29**) (3.0 g, 15.7 mmol) and K_2CO_3 (10.8 g, 78.5 mmol) in 1,2 dibromoethane (15 mL) and DMF (5 mL) was heated to 80 °C for 24 h. EtOAc was added and the system was washed with water and dried (Na_2SO_4). Flash chromatography (petroleum ether/EtOAc; 100:0 then 99:1) gave 2-(2-bromoethoxy)-3,5-dichlorobenzaldehyde (**30**) (3.9 g, 83%) as a white solid; mp 58-60 °C; ^{1}H NMR ($CDCl_3$) δ 10.45 (s, 1H), 7.73 (d, *J* = 2.6 Hz, 1H), 7.63 (d, *J* = 2.7 Hz, 1H), 4.45 (t, *J* = 5.7 Hz, 2H), 3.72 (t, *J* = 5.7 Hz, 2H). Anal. calcd. for $C_9H_7BrCl_2O_2$: C, 36.28; H, 2.37. Found: C, 36.34; H, 2.31.

A mixture of **30** (200 mg, 0.67 mmol), pyridin-3-ol (77 mg, 0.81 mmol) and K_2CO_3 (280 mg, 2.02 mmol) were stirred in dry DMF (10 mL) at 40 °C for 12 h. EtOAc was added and the system was washed with water, brine and dried (Na_2SO_4). Flash chromatography (petroleum ether/EtOAc; 1:1 to 2:3) gave 3,5-dichloro-2-(2-(pyridin-3-yloxy)ethoxy)benzaldehyde (**31**) (96 mg) as a white solid; ^{1}H NMR ($CDCl_3$) δ 10.42 (s, 1H), 8.33 (d, *J* = 2.5 Hz, 1H), 8.27 (dd, *J* = 4.3, 1.5 Hz, 1H), 7.74 (d, *J* = 2.6 Hz, 1H), 7.64 (d, *J* = 2.6 Hz, 1H), 7.27-7.19 (m, 2H), 4.53 (dt, *J* = 4.2, 2.4 Hz, 2H), 4.40 (dt, *J* = 4.2, 2.4 Hz, 2H). The HCl salt was prepared by stirring 30 mg of the free base in HCl/MeOH (1.25 M, 2 mL) and $CHCl_3$ (1 mL) for 2 h and filtration of the precipitate of **31**.**HCl** as a white solid; mp 182-184 °C; Anal. calcd. for $C_{14}H_{12}Cl_3NO_3$: C, 48.24; H, 3.47; N, 4.02. Found: C, 48.52; H, 3.38; N, 4.02.

A solution of aldehyde **31** (32 mg, 0.10 mmol) and 4*H*-1,2,4-triazol-4-amine (9 mg, 0.10 mmol) in MeOH (2 mL) and THF (1 drop) was heated under reflux for 5 h. After cooling, solvents were removed and residue was subjected to flash chromatography (CH_2Cl_2/MeOH; 100:0 to 93:7) to give **17** (31 mg, 80%) as a white solid; mp 149-152 °C; ^{1}H NMR ($CDCl_3$) δ 9.01 (s, 1H), 8.52 (s, 2H), 8.34 (s, 1H), 8.30 (d, *J* = 4.2 Hz, 1H), 7.98 (d, *J* = 2.5 Hz, 1H), 7.60 (d, *J* = 2.5 Hz, 1H), 7.29-7.16 (m, 2H), 4.58-4.49 (m, 2H), 4.48-4.37 (m, 2H). Anal. calcd. for $C_{16}H_{13}Cl_2N_5O_2 \cdot H_2O$: C, 48.50; H, 3.82; N, 17.68. Found: C, 48.10; H, 3.64; N, 17.66.

3.1.5. 2-(2-(4-(*sec*-Butyl)phenoxy)ethoxy)-3,5-dichlorobenzaldehyde (20) (Scheme 2). A mixture of 3,5-dichloro-2-hydroxybenzaldehyde (**29**) (200 mg, 1.05 mmol), bromide **26** (320 mg, 1.26 mmol) and K_2CO_3

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(430 mg, 3.14 mmol) were stirred in dry DMF (2 mL) for 48 h. EtOAc was added and the system was washed with water, brine and dried (Na_2SO_4). Flash chromatography (petroleum ether/EtOAc; 100:0 to 99:1) gave **20** (70 mg, 18%) as a white solid; mp 73-74 ^oC; ^1H NMR ($CDCl_3$) δ 10.45 (s, 1H), 7.74 (d, *J* = 2.6 Hz, 1H), 7.62 (d, *J* = 2.6 Hz, 1H), 7.09 (d, *J* = 8.6 Hz, 2H), 6.81 (d, *J* = 8.7 Hz, 2H), 4.51 (dt, *J* = 4.3, 2.7 Hz, 2H), 4.31 (dt, *J* = 4.3, 2.7 Hz, 2H), 2.54 (sext, *J* = 7.0 Hz, 1H), 1.55 (quint, *J* = 7.4 Hz, 2H), 1.21 (d, *J* = 7.0 Hz, 3H), 0.81 (t, *J* = 7.4 Hz, 3H). Anal. calcd. for $C_{19}H_{20}Cl_2O_3$: C, 62.14; H, 5.49. Found: C, 62.20; H, 5.60.

3.1.6. (*E*)-1-(2-(2-(4-(*sec*-Butyl)phenoxy)ethoxy)-5-chlorophenyl)-*N*-(4*H*-1,2,4-triazol-4-yl)methanimine (18) (Scheme 3). A mixture of (*E*)-2-(((4*H*-1,2,4-triazol-4-yl)imino)methyl)-4-chlorophenol[49] (**32**) (100 mg, 0.45 mmol), bromide **26** (140 mg, 0.54 mmol) and K_2CO_3 (190 mg, 1.35 mmol) were stirred in dry DMF (2 mL) for 24 h. EtOAc was added and the system was washed with water, brine and dried (Na_2SO_4). Flash chromatography (CH_2Cl_2/MeOH; 100:0 then 197:3) followed by recrystallization (EtOAc /petroleum ether) gave **18** (110 mg, 61%) as a white solid; mp 127-128 ^oC; ^1H NMR ($CDCl_3$) δ 8.91 (s, 1H), 8.52 (s, 2H), 8.02 (d, *J* = 2.7 Hz, 1H), 7.47 (dd, *J* = 8.9, 2.7 Hz, 1H), 7.12 (d, *J* = 8.6 Hz, 2H), 7.02 (d, *J* = 8.9 Hz, 1H), 6.85 (d, *J* = 8.6 Hz, 2H), 4.44 (dt, *J* = 4.8, 2.6 Hz, 2H), 4.33 (dt, *J* = 4.8, 2.6 Hz, 2H), 2.56 (sext, *J* = 7.0 Hz, 1H), 1.56 (quint, *J* = 7.3 Hz, 2H), 1.21 (d, *J* = 6.9 Hz, 3H), 0.81 (d, *J* = 7.4 Hz, 3H). Anal. calcd. for $C_{21}H_{23}ClN_4O_2$: C, 63.23; H, 5.81; N, 14.05. Found: C, 63.26; H, 5.69; N, 14.10.

3.1.7. (*E*)-1-(2-(2-(4-(*sec*-Butyl)phenoxy)ethoxy)-3-chlorophenyl)-*N*-(4*H*-1,2,4-triazol-4-yl)methanimine (19) (Scheme 3). A mixture of *E*)-2-(((4*H*-1,2,4-triazol-4-yl)imino)methyl)-2-chlorophenol[50] (**33**) (100 mg, 0.45 mmol), bromide **26** (140 mg, 0.54 mmol) and K_2CO_3 (190 mg, 1.35 mmol) were stirred in dry DMF (2 mL) for 48 h. EtOAc was added and the system was filtered, washed with water, brine and dried (Na_2SO_4). Recrystallization (CH_2Cl_2/*i*-Pr_2O) gave (70 mg, 39%) as a white solid; mp 102-103 ^oC; ^1H NMR ($CDCl_3$) δ 9.13 (s, 1H), 8.42 (s, 2H), 8.00 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.59 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.20 (t, *J* = 7.9 Hz, 1H), 7.09 (d, *J* = 8.6 Hz, 2H), 6.79 (d, *J* = 8.7 Hz, 2H), 4.52 (dt, *J* = 4.0, 2.0 Hz, 2H), 4.29 (dt, *J* = 4.0, 2.0 Hz, 2H), 2.54 (sext, *J* = 7.0 Hz, 1H), 1.54 (quint, *J* = 7.3 Hz, 2H), 1.21 (d, *J* = 7.0 Hz, 3H), 0.81 (d, *J* = 7.3 Hz, 3H). Anal. calcd. for $C_{21}H_{23}ClN_4O_2$: C, 63.23; H, 5.81; N, 14.05. Found: C, 63.30; H, 5.73; N, 14.01.

3.2 Biology

3.2.1. Compound library development

A ligand based virtual screening approach was used in two phases for the discovery of SOCE channel inhibitors. Initially, known inhibitors where extracted from the literature, including patents, and were either kept as the complete molecule or reduced to a core scaffold, and used to query the ZINC on-line compound database[51] using the similarity search facility. The ligand structures and search query are listed in Supplementary Information Table 1. A small virtual library was created by combination of the hit lists for each compound and removal of duplicates using the ligand preparation module implemented in SYBYL. DIVERSE SOLUTIONS (v6.3.2) was then used to reduce the set size from 675 compounds down to 57. This involved calculating a binary fingerprint for each compound using the 166 MACCS keys option, comparing these using the Hamming distance calculation method, and selecting the final subset using the elimination method option. This smaller set was queried against the ZINC database to find ,suppliers, and a final set of 32 compounds was selected, of these 20 were purchased and tested. In the second round, the known SOCE channel blockers **1**, **2**, **5**, **7**, **8**, and **9** along with active molecules identified from the first round (T6454376, Enamine; 6035967, Chembridge) were used to build a larger library of approximately 2140 compounds from Chembridge. This involved collecting sets of at least 1000 but up to 1316 compounds from the Hit2lead on-line vendor (www.hit2lead.com) by submitting each compound to the similarity search tool. The hit lists were combined and duplicates were removed using the ligand preparation module within SYBYL, leaving a set of 8468 compounds. Reduction of this set was performed firstly by selecting compounds that fell within either the molecular weight range (304 to 478) or the clogP range (3.3 to 6.2) of the query compounds as calculated in SYBYL. The combined, non-redundant set had 7197compounds that occupied a MW range 186.25 to 667.62 and clogP range -3.65 to 9.15, from this a set of 2140 compounds occupying a MW range 214.3 to 667.62 and clogP range -1.79 to 9.15 were selected based on cost and

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availability. The screening cascade used the primary, medium throughput (96-well plate format) (FLIPR) assay,[50] 50for measuring store operated calcium influx in the basal like breast cancer cell line MDA-MB-231.

3.2.2. Measurement of intracellular free Ca^{2+} by FLIPR assay

MDA-MB-231 triple negative breast cancer cells were plated at a density of 2 x 10^3 cells per well in 384-well black plates (Corning Costar, *Cambridge, MA, USA).* Three days post seeding, intracellular free Ca^{2+} levels were measured in a Fluorescence Imaging Plate Reader ($FLIPR^{TETRA}$, Molecular Devices, Sunnyvale, CA, USA) using the PBX Calcium Assay Kit (640175, BD Biosciences, Franklin Lakes, NJ, USA) as described previously.[52] Briefly, cells were first loaded for 1 hour at 37°C with a dye-loading solution comprising of 2 µM PBX Calcium Assay dye, 5% (v/v) PBX Signal Enhancer and 500 µM probenecid in physiological salt solution (PSS, 5.9 mM KCl, 1.4 mM $MgCl_2$, 10 mM HEPES, 1.2 mM NaH_2PO_4, 5 mM $NaHCO_3$, 140 mM NaCl, 11.5 mM glucose, pH 7.3). Cells were then treated for 15 min at room temperature with different concentrations of compound in a solution containing 5% (v/v) PBX Signal Enhancer and 500 µM probenecid in PSS. For assessment of store-operated Ca^{2+} entry (SOCE), which has been previously shown to be mediated by Orai1 proteins in MDA-MB-231 cells,[12] the following solutions in PSS were added in order inside the $FLIPR^{TETRA}$ using a robotic arm: 500 µM BAPTA (Invitrogen, Carlsbad, CA, USA) for chelation of extracellular Ca^{2+}; 10 µM cyclopiazonic acid (CPA; Sigma-Aldrich, St Louis, MO) for inhibition of sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) pump and depletion of endoplasmic reticulum (ER);[53] 0.5 mM $CaCl_2$ (700 s after the addition of CPA) for assessment of store-operated Ca^{2+} influx. Fluorescence was measured at 470-495 nm excitation and 515-575 nm emission. ScreenWorks Software (v2.0.0.27, Molecular Devices) was used for data analyses. Ca^{2+} levels were assessed through the change in relative fluorescence of the Ca^{2+} dye. The percentage inhibition of the maximum peak height for each concentration of each compound normalized to its corresponding DMSO control was calculated and plotted separately for peak 1 (a measure of the release of endoplasmic reticulum calcium store) by addition of CPA to assess potential non-specific effects on Ca^{2+} homeostasis and peak 2 (a measure of store-operated Ca^{2+} influx).[35,54] Where the % inhibition of peak 2 (SOCE) did not exceed 10% at 100 µM in initial assessments, these compounds were defined as not active.

3.2.3. Selectivity screen

TRPV1 and TRPM8 responses were assessed in HEK293 cells (American Tissue Culture Collection, Manassas, VA, USA) 48 h after transfection with plasmid DNA of rTRPV1 (D. Julius, Department of Physiology, University of California, Berkeley, CA, USA) or rTRPM8 (K. Zimmermann, Department of Anesthesiology, Friedrich-Alexander-University, Erlangen-Nuremberg, Erlangen, Germany) using Lipofectamine 2000 as previously described.[55] $Ca_V2.2$ responses were assessed in SH-SY5Y neuroblastoma cells in the presence of nifedipine (10 µM) according to established protocols.[56] HEK293 cells were routinely maintained in DMEM containing 10% foetal bovine serum, 2 mM L-glutamine, pyridoxine and 110 mg/ml sodium pyruvate. SH-SY5Y cells (European Collection of Authenticated Cell Cultures, Salisbury, UK) were cultured in RPMI 1640 antibiotic-free medium (Invitrogen) supplemented with 10% heat-inactivated FBS and 2 mM GlutaMAX™ (Invitrogen). Cells were split every 3-6 days in a ratio of 1:5 using 0.25% trypsin/EDTA. Cells were plated on 384-well black-walled imaging plates (Corning) at a density of 10,000 cells/well (HEK293) or 50,000 cells/well (SH-SY5Y) and used for Ca^{2+} experiments 24 hours after plating.

Growth media was removed and replaced with 20 µl/well Calcium 4 No-Wash dye diluted according to the manufacturer's instructions in physiological salt solution (PSS; NaCl 140 mM, glucose 11.5 mM, KCl 5.9 mM, $MgCl_2$ 1.4 mM, NaH_2PO_4 1.2 mM, $NaHCO_3$ 5 mM, $CaCl_2$ 1.8 mM, HEPES 10 mM) and incubated for 30 min at 37°C/5% CO_2. Ca^{2+} responses were measured using a $FLIPR^{TETRA}$ (Molecular Devices, Sunnyvale, CA, USA) fluorescent plate reader with excitation at 470-495 nM and emission at 515-575 nM. Camera gain and intensity were adjusted for each plate to yield a minimum of 1500-2000 arbitrary fluorescence units (AFU) baseline fluorescence. Test compounds were added 300 s prior to stimulation with capsaicin (100 nM; TRPV1), menthol (100 µM, TRPM8) and KCl (90 mM)/$CaCl_2$ (5 mM; $Ca_V2.2$). Data was analysed using Screenworks 3.2 and $FLIPR^{TETRA}$ data was plotted using GraphPad $Prism^{TM}$ software (Version 6.00).

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3.2.4. Determination of the stability of 14 under simulated conditions of the FLIPR assay

Probenecid (714 mg, 2.50 mmol) was placed in a 50 mL graduated cylinder. Portions of 1 M NaOH (50 µL) were added till all probenecid dissolved. Water (5 mL) was added and pH adjusted to 7.4 with HCl giving a total volume of 10 mL. Compound **14** (1.08 mg, 2.4 µmol) was dissolved in DMSO (25 µL). The solution was allowed to stand at room temperature for 25 min. A portion (0.9 µL) of this solution was added to phosphate-buffered saline (300 µL, pH 7.4), followed by addition of a portion (20 µL) of the probenecid solution and the mixture was allowed to stand at room temperature for 1 h. A portion of this solution was then subjected to HPLC analysis on a Zorbax Eclipse XDB C8, 5u, 4.6 x 150 mm column, using a flow rate of 1.2 mL/min and a gradient of water and MeCN as follows: 30% - 100% MeCN over 18 min, then 100% - 30% MeCN over 2 min, followed by 30%MeCN post run over 3 min. Compound **20** (0.91 mg, 2.4 µmol) was dissolved in DMSO (25 µL). A portion (0.9 µL) of this solution was added to DMSO (320 µL) to be used as a standard for the detection of any hydrolysis of **14** to **20**.

Conflict of Interests

G.R.M and W.A.D are associated with QUE Oncology Inc.

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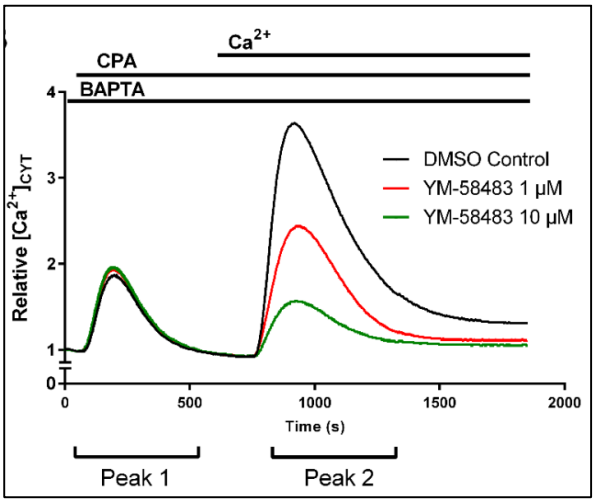

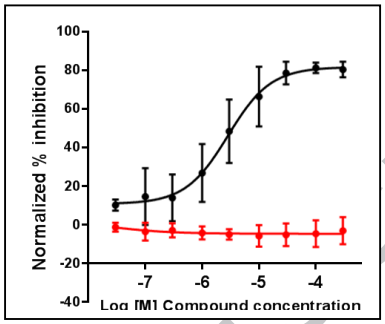

Peak 1	ER release
Peak 2	SOCE

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Highlights

- A fluorescence imaging plate reader assay for calcium channel inhibitors
- Inhibitors of the Orai1 SOCE Ca2+ channel that do not affect Ca2+ store release
- Similarity-based screening assay to identify triazole-based Orai1 inhibitors

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