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Consequences of activating the calcium-permeable ion channel TRPV1 in breast cancer cells with regulated TRPV1 expression

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Abstract

Increased expression of specific calcium channels in some cancers and the role of calcium signaling in proliferation and invasion have led to studies assessing calcium channel inhibitors as potential therapies for some cancers. The use of channel activators to promote death of cancer cells has been suggested, but the risk of activators promoting cancer cell proliferation and the importance of the degree of channel over-expression is unclear. We developed an MCF-7 breast cancer cell line with inducible TRPV1 overexpression and assessed the role of TRPV1 levels on cell death mediated by the TRPV1 activator capsaicin and the potential for submaximal activation to promote proliferation. The TRPV1 level was a determinant of cell death induced by capsaicin. A concentration response curve with varying TRPV1 expression levels identified the minimum level of TRPV1 required for capsaicin induced cell death. At no level of TRPV1 over-expression or capsaicin concentration did TRPV1 activation enhance proliferation. Cell death induced by capsaicin was necrotic and associated with up-regulation of c-Fos and RIP3. These studies suggest that activators of specific calcium channels may be an effective way to induce necrosis and that this approach may not always be associated with enhancement of cancer cell proliferation.

Keywords: Breast cancer; calcium, necrosis; TRPV1

1. Introduction

Calcium is an important regulator of a variety of cellular processes many of which are de-regulated in cancer cells, such as proliferation, migration and cell death [1, 2]. Increases in the expression of specific calcium permeable ion channels occur in a variety of cancer types [1]. These include increased levels of TRPM8 [3, 4], TRPV6 [5, 6] and TRPV2 [7] in some prostate cancers and increased TRPC3 and TRPC6 in ovarian cancer [8, 9]. In some cases, alterations in the expression of specific calcium permeable ion channels occur predominantly

in specific cancer subtypes. Examples of this are seen in breast cancers, where increases in the levels of the highly selective calcium channel TRPV6 [10] are associated with the poor prognosis basal subtype [11].

Numerous studies have now also identified that silencing and/or pharmacological inhibition of overexpressed calcium ion channels in cancer cells may attenuate key aspects of cancer progression, including proliferation and metastasis. Inhibitory RNA-mediated silencing of TRPC3 reduces the proliferation of the SKOV3 ovarian cancer cell line [8] and silencing or pharmacological inhibition of ORAI1 reduces metastasis of MDA-MB-231 breast cancer cells *in vivo* [12]. These and other studies [13-15] have provided compelling evidence that overexpression of a calcium permeable ion channel can promote key hallmarks of cancer [16] and that inhibition of calcium permeable ion channels overexpressed in specific cancers may provide a strategy for cancer therapy. An alternative approach that has been proposed (but less studied) is the activation of an overexpressed calcium permeable ion channels to induce cancer cell death [1, 17].

Calcium is a critical regulator of cell death, with sustained and high (> 1 μM) increases in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) associated with the induction of apoptotic or necrotic cell death [18]. The critical importance of elevated levels of $[Ca^{2+}]_i$ in cell death pathways has been widely studied in neurons and cardiac cells [19]. In neurons, cell death due to Ca^{2+} overload is often associated with the cleavage and in some cases inactivation of calcium efflux transporters located on the plasma membrane, [20, 21]. The consequences of this attenuation of Ca^{2+} efflux mechanisms is augmentation of elevations in $[Ca^{2+}]_i$ and promotion of cell death pathways modulated by Ca^{2+}, such as those of the mitochondria [22].

In prostate cancer cells, Zhang and Barritt [17] demonstrated that silencing TRPM8 reduces cell viability and induces apoptosis in LNCaP cells, and that activation of TRPM8 with the non-selective TRPM8 activator menthol [23] also reduces cell viability and induces apoptosis [17]. Menthol also induces cell death, but not apoptosis, in a bladder cancer cell line with elevated levels of TRPM8 [24]. Transient expression of the *drosophila* TRPL (constitutively active) in the LNCaP cells reduces cell viability and increases apoptosis [25]. Activation of overexpressed TRPV2 in a bladder cancer cell line with cannabidiol and activation of overexpressed TRPV1 with capsaicin in a lung cancer cell line both cause apoptosis [26, 27].

One potential limitation of activating an overexpressed calcium permeable ion channel as a therapeutic approach for cancer therapy is the possibility of the activator producing levels of Ca^{2+} influx that are not sufficient to induce cell death, but which activate calcium sensitive cell cycle pathways [28] and thus promote proliferation. It is also still unclear how the level of calcium channel overexpression may influence the sensitivity of cancer cells to cell death via channel activation. In these studies we explored these issues using MCF-7 breast cancer cells with inducible TRPV1 over-expression. TRPV1 is a calcium permeable ion channel expressed in sensory neurons [29], keratinocytes [30] and lung cells [26], with a role in the sensing of elevated temperatures, thermal pain [31] and regulating inflammation and neurogenesis [32]. TRPV1 is activated by the hot component of chili peppers capsaicin and is sensitive to a variety of pharmacological activators and inhibitors, which have often been developed as part of the search for novel therapies for pain [33] and overactive bladder [34]. Elevated TRPV1 expression occurs in colon [35], pancreatic [36] and prostate [37, 38] cancer cells. Studies of capsaicin have reported varying effects, which may be related to non-TRPV1-mediated responses of capsaicin or differences between the levels of TRPV1 expression amongst different cancer cell lines. In the LNCaP prostate cancer cell line

capsaicin induces cell proliferation via TRPV1 [39], whereas in PC-3 prostate cancer cells capsaicin inhibits cell proliferation via a mechanism independent of TRPV1 [40].

In these studies we investigated the hypotheses that cell death mediated by pharmacological activation of TRPV1 in MCF-7 breast cancer cells is dependent on both the level of TRPV1 overexpression and the concentration of TRPV1 activator, and that the overexpression of TRPV1 and submaximal activation of TRPV1 promotes the proliferation of MCF-7 breast cancer cells. We also sought to define the nature of cell death associated with TRPV1 activation in MCF-7 breast cancer cells.

2. Methods

2.2 Cell culture

The MCF-7 Tet-off breast cancer cell line (Clontech) and the MCF-7 parental breast cancer cell line (ATCC) were cultured in Dulbecco's Modified Eagle Medium, (high glucose) (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), 4 mM L-glutamine (Invitrogen), and penicillin G (100U/mL)/ streptomycin sulphate (100 μg/mL; Invitrogen) at 37°C with 5% CO_2. Cells were supplemented with G418 (100 μg/mL; Invitrogen), hygromycin B (110 μg/mL; Invitrogen) and doxycycline (dox; 1 μg/mL; Sigma-Aldrich) as required. Cells were passaged when the density reached approximately 80%. The MCF-7 Tet-off^{TRPV1} cell line was cultured for less than 10 passages prior to experimentation.

2.3 Molecular cloning and development of MCF-7 Tet-off^{TRPV1} cell line

TRPV1 was amplified from pcDNA3.1 D/V5-His- TOPO-TRPV1 (a generous gift from A/Prof C Reilly, University of Utah) [26] using Platinum *Pfx* DNA polymerase (Invitrogen) and the following primers; forward primer GCATATATGTCGACACCATGAAGAAATGGA-GCAGCA and reverse primer GCATATATGTCGACTCACTTCTCCCCGGAAGCGGC-AGGACTCTT. The TRPV1 PCR product was ligated into the pGEM-T Easy vector (Promega) then sub-cloned into the pBI-G Tet response plasmid (Clontech). The pGEM-TRPV1 and pBI-G-TRPV1 plasmids were verified using restriction digest and nucleotide sequencing. The pBI-G-TPRV1 plasmid expresses both TRPV1 and β-galactosidase from a bidirectional promoter (Clontech).

To generate an MCF-7 Tet-Off cell line stably expressing TRPV1, cells were co-transfected with the pBI-G-Tet-off-TRPV1 response plasmid and the pTK-Hyg selection plasmid (Clontech) using LipofectAMINE 2000 (Invitrogen). Two days after transfection hygromycin (110 μg/mL) and dox (1 μg/mL) were added to the culture medium to select for transfected cells. Hygromycin resistant colonies were expanded into 24-well plates and screened for dox regulated β-galactosidase (β-gal) activity.

2.4 β-Gal assay

The β-galactosidase Enzyme Assay System (Promega) was used to evaluate dox-regulated β-gal activity in MCF-7 Tet-Off^{TRPV1} cell lines, since β-gal is an indirect reporter for TRPV1 expression. Briefly, cells were seeded in 96-well plates (8 x 10^3 cells/well) with vehicle or dox (1 μg/mL). After 96 h cells were lysed with reporter lysis buffer for 15 min at room temperature and then incubated with assay buffer for 30 min at 37°C. Absorbance was measured at 415 nm using a Bio-Rad model 550 microplate reader.

2.5 Real-time RT-PCR

RNA was isolated from the MCF-7 Tet-Off cells using a Qiagen RNeasy plus mini kit (Qiagen). RNA was reverse transcribed using the Omniscript RT kit (Qiagen) and real-time RT-PCR was performed using TaqMan Universal PCR Master Mix or Taqman Fast Universal Master Mix and TaqMan gene expression assays; TRPV1 (Hs00218912_m1), c-Fos (Hs00170630_m1), RIP3 (Hs00819388_m1) and 18S rRNA (4319413E). The real-time PCR reactions were performed using an ABI PRISM 7500 Sequence Detector or StepOnePlus Real-Time PCR System (Applied Biosystems) with the universal cycling conditions. The mRNA levels were normalized to 18S rRNA and are presented as fold change ($2^{-\Delta\Delta Ct}$) relative to control (MCF-7 Tet-Off^{TRPV1} + dox or MCF-7 tet-off). Fold change levels were calculated using the comparative threshold cycle C*t* method [41, 42].

2.6 Measurement of intracellular free Ca^{2+} $[Ca^{2+}]_i$

MCF-7 Tet-Off^{TRPV1} cells (8 x 10^3 cells/well) were seeded in 96-well black-walled CellBIND surface plates (Corning Inc.) and treated with vehicle or various concentrations of dox. Measurement of intracellular Ca^{2+} in response to vehicle (ethanol) or various concentrations of capsaicin was performed at 96 h. Cells were loaded with Fluo-4 AM (4 µM; Invitrogen) for 30 min at 37 °C and then incubated for 15 min at room temperature in physical salt solution [PSS; 140 mM NaCl, 11.5 mM glucose, 10 mM HEPES, 5.9 mM KaCl, 1.4 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 5 mM $NaHCO_3$, 1.8 mM $CaCl_2$, pH 7.3] at room temperature. Fluorescence was measured at 470 – 495 nm excitation and 515 – 575 nm emission using a Fluorescence Imaging Plate Reader (FLIPR)^{TETRA} (Molecular Devices). Response over

baseline was assessed as a relative measure of $[Ca^{2+}]_i$ and peak relative Ca^{2+} was determined by the maximum relative fluorescence (response over baseline) value achieved during the 800 s following capsaicin addition. Concentration-response curves and EC_{50} or IC_{50} values were generated using GraphPad Prism Version 5.01 software (GraphPad Software Inc). The data were fitted to a four parameter Hill equation with variable slope.

2.7 Viable cell number assay

MCF-7 Tet-Off and MCF-7 Tet-Off^{TRPV1} cells (8 x 10^3 cells/well) were seeded in 96-well plates. Cells were treated with vehicle (ethanol) or varying concentrations of capsaicin or dox and vehicle . Proliferation was assessd at 24 h, 72 h, 96 h or 120 h by adding CellTiter 96®Aqueous One Solution Reagent (20 µL) to each well containing 100 µL of culture medium and measuring the absorbance at 490 nm using a Bio-Rad model 550 microplate Reader.

2.8 Assessment of cell death

MCF-7 Tet-Off^{TRPV1} cells (8 x 10^3 cells/well) were seeded in 96-well black-walled imaging plates (Becton Dickinson Bioscience) in the presence of dox (0 – 1000 ng/mL). Capsaicin (0 – 500 µM) or staurosporine (STS, 0 – 3 µM) were added 96 h after seeding and cell death was assessed at two time points (6 h and 24 h) after capsaicin treatment. For BAPTA experiments cells were pre-treated with vehicle or BAPTA (10 mM) for 5 min prior to treatment with vehicle, BAPTA (10 mM), capsaicin (3 µM), or BAPTA (10 mM) plus capsaicin (3 µM) for 6 h. Cells were stained simultaneously with Hoechst 3342 (10 µg/mL; Invitrogen), PI (1 µg/mL; Invitrogen) and YO-PRO-1 (0.2 µM Invitrogen) for 15 min at 37

°C. Hoechst stains all cells, YO-PRO-1 can enter cells during apoptosis and PI enters cells during the late stages of apoptosis and during necrosis [43, 44]. Images were acquired and analyzed using an ImageXpress® Micro (Molecular Devices) automated epifluorescent microscope with a 10 X objective lens. Images were captured from four fields per well. Hoechst 3342 was detected using a DAPI-1160A (Semrock) filter, PI was detected using a Cy3-4040B (Semrock) filter and YO-PRO-1 was detected using a GFP-3635B (Semrock) filter. Images were analyzed using MetaXpress® (V3.1.0.83, Molecular Devices). The Multi-wavelength Cell Scoring application module was used to calculate the percentage of cells positive for each dye. Phase contrast images were captured using a Leica DM IL LED inverted microscope, 40 X objective with a Leica DFC 295 camera (Leica). Fluorescent images were captured using ImageXpress® Micro (Molecular Devices) automated epifluorescent microscope with a 10 X objective lens.

2.9 Statistical analysis

GraphPad Prism Version 5.01 software (GraphPad Sotfware Inc) was used for the generation, 95 % confidence interval values and statistical significance were calculated as described in the figure legends.

3. Results

3.1 Generation of an MCF-7 tet-off cell line over-expressing TRPV1 (MCF-7 tet-off^{TRPV1}).

A breast cancer cell line model was developed to assess the consequences of overexpression of a calcium permeable ion channel in breast cancer cells and the optimal approach and conditions to therapeutically target such channels. The MCF-7 cell line was selected because

MCF-7 cells have low levels of TRPV1, a calcium permeable ion channel with well characterized pharmacological modulators (e.g. capsaicin). The TRPV1 over expressing MCF-7 cell line was generated using a plasmid with a bi-directional promoter that expressed both TRPV1 and β-gal in the absence of dox. More than 50 stables lines were generated; three showed clear dox-sensitive β-gal expression with TRPV1 mRNA level increases and TRPV1 calcium responses to the TRPV1 activator capsaicin as a measure of functional TRPV1 protein expression, as described for the selected cell line below and in supplementary data. To validate the stable cell lines β-gal activity was assessed as a marker of TRPV1 expression. Removal of dox increased β-gal activity in the cells indicating TRPV1 expression (Fig. 1A and Fig. S2A). Real-time RT-PCR confirmed that TRPV1 mRNA was elevated in the absence of dox (Fig. 1B and Fig. S1 and S2B), and finally to assess the activity of TRPV1, stably expressing cells were stimulated with the TRPV1 agonist capsaicin and increases in $[Ca^{2+}]_i$ measured. Induction of TRPV1 expression (absence of dox) was associated with a sustained increase in $[Ca^{2+}]_i$ with the addition of capsaicin (Fig. 1C and Fig. S2C and D). One of the stable cell lines was selected for further detailed assessment and is referred to as MCF-7 tet-off^{TRPV1}. These initial studies show that MCF-7 tet-off^{TRPV1} represents a breast cancer cell line with inducible expression of functional TRPV1.

3.2 Assessment of TRPV1-mediated increases in $[Ca^{2+}]_i$ in MCF-7 tet-off^{TRPV1} cells.

MCF-7 tet-off^{TRPV1} cells were treated with increasing concentrations of dox, to concentration-dependently decrease the levels of TRPV1 and the consequences of capsaicin (3 μM) – mediated increases in $[Ca^{2+}]_i$ were assessed. As expected, capsaicin-mediated increases in $[Ca^{2+}]_i$ decreased with decreasing TRPV1 expression levels, with a dox IC_{50} of 0.117 ng/mL (Fig. 2A). The maximal capsaicin-mediated increase in $[Ca^{2+}]_i$ was reduced at dox

concentrations greater than 0.03 ng/mL, sub-maximal increases in $[Ca^{2+}]_i$ were observed with 0.1 and 0.3 ng/mL dox. Concentrations of dox greater than 1 ng/mL were sufficient to almost abolish capsaicin-mediated increases in $[Ca^{2+}]_i$ (Fig. 2A). Similar results were seen with the other two stable clones (Fig. S3A and B).

MCF-7 tet-off^{TRPV1} cells were then treated with increasing concentrations of capsaicin in the presence or absence of dox. When TRPV1 over-expression was not induced (+ dox 1 μg/mL) a slight increase in $[Ca^{2+}]_i$ was observed at high concentrations of capsaicin with an EC_{50} of 705 nM (Fig. 2B) and similar values observed for the other two clones (Fig. S3C and D). The calcium response in the presence of dox may be caused by endogenous TRPV1 in the MCF-7 cells and/or modest "leaky" expression of TRPV1 from the pBI-G-TRPV1 plasmid (Fig. S1) and/or non-specific effects of capsaicin. However, when TRPV1 was over-expressed (- dox) a significant concentration-dependent increase in $[Ca^{2+}]_i$ was observed with increasing concentrations of capsaicin, with an EC_{50} of 175 nM (Fig. 2B). Similar values were observed for the other two clones (Fig S3C and D).

3.3 Assessment of the effects of TRPV1 activation on cell viability and dependence on TRPV1 expression levels.

The effect of capsaicin on viable cell number was assessed using an MTS assay in the MCF-7 tet-off^{TRPV1} (Fig. 3A and B) and MCF-7 tet-off parental cell lines (Fig. 3C and D) cultured with varying concentrations of capsaicin. Similar studies were conducted in the two other clonal cell lines with the results shown in supplementary figures (Fig. S4A, B, C and D). In the absence of TRPV1 overexpression, capsaicin had no effect on viable cell numbers of MCF-7 tet-off^{TRPV1} (+ dox) or parental MCF-7 tet-off cells after 24 h (Fig. 3A and C). After

96 h only the highest concentration of capsaicin (0.3 mM) affected MCF-7 cells without TRPV1 overexpression (Fig. 3B and D). In the presence of TRPV1 over-expression (- dox), increasing concentrations of capsaicin reduced viable cell numbers in a concentration-dependent manner at both 24 h (Fig. 3A) and 96 h (Fig. 3B) with EC_{50} values of 713 nM and 898 nM, respectively. Vercelli showed contrasting findings, inhibition of MCF-7 cell viability with capsaicin treatment [45], however 0.1 nM and 100 μM capsaicin had the same ability to inhibit cell viability, which is not consistent with the results of other studies [26]. Furthermore, the role of TRPV1 in the effect of capsaicin on MCF-7 cells was not conclusively demonstrated [45].

The level of TRPV1 over-expression required to affect MCF-7 viable cell numbers in the presence of capsaicin was then tested. The concentration response curve showed that TRPV1 over-expression clearly sensitized MCF-7 cells to the effects of 3 μM capsaicin (EC_{50} = 0.119 ng/mL dox) (Fig. 4, and Fig. S5A and B).

3.4 Overexpression or activation of TRPV1 does not promote proliferation of MCF-7 cells.

MCF-7 cell proliferation was assessed with increasing levels of TRPV1 over-expression without the addition of capsaicin. The growth curves show that the over-expression of TRPV1 had no effect on MCF-7 proliferation (Fig. 5A).

Activation of TRPV1 could promote proliferation if the nature of the $[Ca^{2+}]_i$ signal was appropriate for proliferative signaling. Such activation of proliferation could limit the use of ion channel activation in cancer therapy. However, at no levels of TRPV1 expression did capsaicin produce an increase in cell numbers at 48 h (Fig. 5B) or 96 h (Fig. 5C). Instead,

consistent with the results presented above, high levels of TRPV1 over-expression (e.g. 0 ng/mL and 0.1 ng/mL dox) with sub-maximal capsaicin (3 μM) reduced the number of viable MCF-7 tet-off^{TRPV1} cells (Fig. 5B and C).

3.5 Activation of TRPV1 induces cell death in MCF-7 cells that over-express TRPV1

To further assess the capsaicin-mediated reduction in cell number in TRPV1 overexpressing MCF-7 tet-off^{TRPV1} cells, a cell death assay was performed using three fluorescent dyes, which distinguish between live (Hoechst 3342 only positive), apoptotic (YO-PRO-1 and Hoechst 3342 positive, and PI negative) and necrotic (YO-PRO-1, PI and Hoechst 3342 positive) cell populations. MCF-7 tet-off^{TRPV1} cells were treated with varying concentrations of staurosporine (STS) or capsaicin in the absence of dox (TRPV1 over-expressed). staurosporin induces apoptosis in various cell types including MCF-7 cells [46, 47]. Phase contrast images show characteristic necrotic morphology for the capsaicin treated cells, compared to the apoptotic morphology observed in the staurosporin treated cells (Fig. 6A) [48]. The fluorescent images show that the capsaicin treated cells are mostly positive for YO-PRO-1 and PI, and only a few cells were positive for YO-PRO-1 but not PI, indicating necrosis (Fig 6A), whereas many of the staurosporin treated cells were positive for YO-PRO-1 but not PI, indicating early apoptosis (Fig 6A). Quantification of the staining showed that capsaicin (0.3 – 3.0 μM) and staurosporin (0.1 - 3.0 μM) both induced cell death in MCF-7 tet-off^{TRPV1} cells overexpressing TRPV1 (Fig. 6B). However, staurosporin (3 μM) was associated with 8% of cells being YO-PRO-1 positive and PI negative populations indicating apoptosis, but only 1% of cells were in this population with capsaicin (3 μM) (Fig. 6C) at 6 h. Hence, capsaicin-induced cell death observed in cells over-expressing TRPV1 occurs via a mechanism primarily mediated by a necrotic pathway.

Since capsaicin activated TRPV1 is also permeable to Na^+ and Mg^{2+} [49], the dependence of capsaicin-mediated cell death on Ca^{2+} influx was assessed. The addition of the Ca^{2+} chelator BAPTA protected MCF-7 cells against capsaicin-induced cell death, demonstrating that the cell death effect of capsaicin is indeed calcium-dependent (Fig. S6).

3.6 Capsaicin induced cell death in MCF-7 cells is concentration dependent and contingent upon the level of TRPV1.

The effect of TRPV1 expression levels and capsaicin concentration on cell death was assessed in MCF-7 tet-off^{TRPV1} cells. Figures 7A & B show 3-dimensional graphs of how capsaicin and dox concentrations affected the percentage of dead cells (YO-PRO and PI staining). Consistent with a rapid necrotic cell death mechanism, similar results were observed at both 6 h (Fig. 7A) and 24 h (Fig. 7B) of capsaicin exposure. The highest percentage of cell death (~80%) was observed with the highest TRPV1 expression level (0 ng/mL dox) and the highest concentrations of capsaicin (3 μM and 500 μM) (Fig. 7). Expression levels of TRPV1 that induced sub-maximal $[Ca^{2+}]_i$ increases (0.1 and 0.3 ng/mL dox) also resulted in a sub-maximal percentage of cell death (15-56%) when treated with capsaicin at 3 μM or 500 μM. As the expression level of TRPV1 increased, the concentration of capsaicin required to induce cell death decreased; for example with the highest level of TRPV1 expression (0 ng/mL dox) 0.3 μM capsaicin induced cell death, while at a lower level of TRPV1 (0.3 ng/mL dox) 3 μM capsaicin was required to induce cell death (Fig. 7). This suggests that the level of TRPV1 expression is a critical determinant of the capsaicin concentration required to induce cell death in breast cancer cells.

3.7 Activation of TRPV1 induced c-Fos and RIP3 expression

To further define the consequences of TRPV1 activation in MCF-7 breast cancer cells, the effect of activating overexpressed TRPV1 with capsaicin on c-Fos, a component of the early response transcription factor, AP-1 [50], was assessed in MCF-7 tet-off^{TRPV1} cells. In the absence of TRPV1 overexpression (+ dox) capsaicin had no effect on c-Fos mRNA levels (Fig. 8A). However, when TRPV1 expression was induced, c-Fos expression was elevated by capsaicin as early as 0.5 h, with highest levels observed at 3 h (Fig. 8A). To further verify the induction of necrosis by capsaicin treatment in TRPV1 overexpressing MCF-7 cells the expression of a necrotic marker, RIP3 [51, 52] was assessed. At 12 h after capsaicin treatment the expression of RIP3 was elevated almost 2-fold in the presence compared to the absence of TRPV1 over-expression (Fig 8B).

4. Discussion

The identification of ion channels with increased expression in some cancer cell lines and clinical samples, has led to the hypothesis that pharmacological modulators of these ion channels may represent potential therapies for specific cancers. Inhibition of ion channels has been the first focus for potential therapies with studies looking at the effectiveness of silencing specific ion channels or using pharmacological inhibitors to attenuate processes important in cancer progression such as proliferation, migration and invasion and to examine the importance of the calcium signal in cell cycle progression and motility. However, *activation* of ion channels to produce sustained high levels of $[Ca^{2+}]_i$ and subsequent cell death has also been proposed as a strategy and is supported by the constitutively active TRPL

[25] and the non-selective TRPM8 activator menthol [53] producing cell death in LNCaP prostate cancer cells that overexpress the Ca^{2+} permeable ion channels TRPL [25] or TRPM8 [17], respectively.

Targeting overexpressed ion channels through pharmacological activation could potentially potentiate cellular proliferation at low concentrations in cancer cells that do not overexpress the calcium channel sufficiently such that activation produces cell death [28, 39]. Our studies using tet-regulated TRPV1 expression in MCF-7 breast cancer cells, indicated that both capsaicin-mediated $[Ca^{2+}]_i$ responses and cell death are dependent on the concentration of capsaicin and the level of TRPV1. However, at no level of TRPV1 expression or capsaicin concentration was a promotion of cellular proliferation observed. Capsaicin concentrations that did not produce cell death but were associated with increases in $[Ca^{2+}]_i$ did not increase cellular proliferation. The lack of an increase in MCF-7 cell number produced by any concentration of capsaicin at any level of TRPV1 expression including those associated with increases in $[Ca^{2+}]_i$ may seem surprising given the important role of Ca^{2+} in cell cycle progression. However the nature of the change in $[Ca^{2+}]_i$ (e.g. spatial location and temporal changes) is a critical regulator of many Ca^{2+}-dependent processes including gene transcription and cell cycle progression [54-56]. Thus pharmacological activators against overexpressed Ca^{2+} permeable ion channels may not suffer from the clinical disadvantage of promotion of cellular proliferation in tumors with lower levels of ion channel overexpression.

Although TRPV1 dependent, the EC_{50} for cell death for capsaicin was higher than for peak $[Ca^{2+}]_i$. This indicates that the level of ion channel overexpression is likely to be a critical determinant of the therapeutic effectiveness of a calcium permeable ion channel activator in cancer therapy. The assessment of the degree of ion channel overexpression will therefore be

important in identifying patient groups for such therapies. Although capsaicin mediated cell death is reported in some cancer cell lines, in many cases the concentrations used were well above those used in this study and could be due to the non TRPV1-mechanisms that have been reported at high concentrations of capsaicin [26, 40].

The identification of other calcium permeable ion channels with pronounced overexpression in clinical breast cancers and cell lines and the assessment of the consequences of their activation *in vitro* and *in vivo* are now required. The study of such ion channels in breast cancer cells should also assess the consequences of gene silencing and pharmacological inhibition. Given the reported elevated levels of TRPV6 in basal-like breast cancers [10] and the recent identification of an inhibitor of TRPV6 [57], it will be particularly interesting to determine if activation of TRPV6 could promote cell death in basal-in like breast cancers *in vitro* and *in vivo*.

High content analysis of PI and YO-PRO-1 permeability and differences with staurosporin effects and the rapid nature of cell death (6 h) is consistent with a rapid loss of the maintenance of critical ion gradients, the loss of plasma membrane integrity and induction of necrosis. Furthermore, the morphology of the capsaicin treated cells compared to the staurosporin treated cells and the elevated RIP3 expression in capsaicin treated cells with TRPV1 over-expression, indicate induction of necrotic cell death [48, 51]. Necrotic cell death can be induced by increased intracellular calcium [59], consistent with our findings. Necrosis is traditionally considered uncontrolled cell death, however, programmed necrosis has been suggested [60-62]. Necrosis can be induced in cancer cells by DNA alkylating agents, irradiation and by photodynamic treatment [63-65]. Indeed, in breast cancer patients anthracyclin predominately induces necrotic cell death [66]. Photodynamic treatment induces

cell death in an apoptotic-resistant breast cancer cell line [64], suggesting that treatments that induce necrotic cell death may be effective for tumors with defective apoptotic pathways [62, 67].

The early response gene, c-Fos was initially considered as a promoter of proliferation and a proto-oncogene, however, more recent studies show that c-Fos also has a role in cell death [68, 69]. The c-Fos protein forms part of the AP-1 transcription factor and the effect of c-Fos on cell proliferation or cell death depends on various factors, including the subunits that comprise AP-1 and the type of stimuli [68, 69]. Regulation of c-Fos by MEKs and ERKs and the downstream targets of AP-1 are either be pro-proliferative (cyclin D1 and CDK) or pro-apoptotic (p52 and p21). Since rapid increases in $[Ca^{2+}]_i$ are a well-established inducer of c-Fos expression [70, 71], c-Fos expression was assessed in MCF-7 tet-off^{TRPV1} cells. The highest c-Fos expression was observed at 3 h after TRPV1 activation with capsaicin and this expression decreased at 6 h, when cell death was observed, indicating c-Fos expression is a precursor to cell death. A rapid induction of c-Fos expression has previously been observed in preneoplastic cell lines and lymphoid cells after growth factor deprivation-mediated cell death [72, 73]. Elevated c-Fos expression is a feature of the early stages of mammary gland involution [74] and tamoxifen induced cell death in SK-BR-3 breast cancer cells [75]. This suggests that the elevations in c-Fos that were observed 3 h after capsaicin treatment, may be an early indicator of cell death mediated by capsaicin in breast cancer cells that overexpress TRPV1.

This model of calcium permeable ion channel overexpression in breast cancer cells provides evidence that the targeting of calcium permeable ion channels with activators may represent an opportunity to induce death of breast cancer cells without promotion of cellular

proliferation. Further studies are required to determine whether such activation may also promote sensitivity to cell death inducers, such as seen for silencing of specific calcium efflux pump isoforms [22]. These studies also suggest that activating calcium permeable ion channels may be an effective tool to mediate breast cancer cell death, in a way that may bypass some resistance mechanisms. However, as yet no ion channel with sufficiently high endogenous levels has yet been characterised in a breast cancer cell line and the consequences of sustained ion channel activation warrants further study. The expansion of available pharmacological activators of ion channels and the identification of other Ca^{2+} channel overexpressed in specific cancers may allow the eventual development of oncology agents that act via this mechanism.

Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Characterization of MCF-7 Tet-off cells stably expressing TRPV1. Cells were treated with vehicle or dox (1 μg/mL) for 96 h. (A) β-Gal activity in MCF-7 Tet-off^{TRPV1} cells. The data are mean ± SD (n = 3). (B) Relative TRPV1 mRNA levels in MCF-7 Tet-off^{TRPV1} cells. The data are normalized to 18S RNA and expressed as mean ±SD (n = 3) relative to + dox. (C) Relative $[Ca^{2+}]_i$ response to capsaicin (10 μM) in MCF-7 Tet-off^{TRPV1} cells in the presence or absence of dox. Relative $[Ca^{2+}]_i$ (black lines) ± SD (grey lines) (n = 3).

Fig. 2. Effect of TRPV1 expression and activity levels on capsaicin-mediated increases in $[Ca^{2+}]_i$. (A) Assessment of peak relative $[Ca^{2+}]_i$ in MCF-7 Tet-off^{TRPV1} cells treated with various concentrations of dox (96 h) prior to stimulation with capsaicin (3 μM). (B) Assessment of peak relative $[Ca^{2+}]_i$ in MCF-7 Tet-off^{TRPV1} cells treated with dox (1 μg/mL; 96 h) prior to stimulation with various concentrations of capsaicin. Graphs represent the mean concentration-response curves of peak relative $[Ca^{2+}]_i$ ± SD (n = 9) from three independent assays; * denotes $p < 0.05$ using two-way ANOVA with Bonferroni post-hoc test.

Fig. 3. The TRPV1 activator capsaicin reduces the number of viable MCF-7 cells over-expressing TRPV1. (A-D) MCF-7 Tet-off^{TRPV1} cells (A and B) or MCF-7 tet-off cells (C and D) were treated with vehicle or dox (1 μg/mL) for 96 h prior to treatment with various concentrations of capsaicin for 24 h (A and C) or 96 h (B and D). Viable cell numbers were assessed using an MTS assay. Graphs represent the mean concentration-response curves of absorbance ± SD (n = 9) from three independent assays; * denotes $p < 0.05$ using two-way ANOVA with Bonferroni post-hoc test.

Fig. 4. TRPV1 expression levels effect capsaicin-meditated effects on MCF-7 cell viability. MCF-7 Tet-off^{TRPV1} cells were treated with vehicle or dox (1 μg/mL) for 96 h prior to treatment with capsaicin (3 μM; 24 h). Viable cell numbers were assessed using an MTS assay. Graph represents the mean concentration-response curves of absorbance ± SD (n = 9) from three independent assays; * denotes $p < 0.05$ using one-way ANOVA with Dunnett post-hoc test.

Fig. 5. TRPV1 over-expression or activation does not promote MCF-7 cell proliferation. (A) MTS assay of MCF-7 tet-off^{TRPV1} cells treated with vehicle or various concentrations of dox for 24 h, 72 h or 120 h. The data are mean ± SD (n = 15) from five independent assays. (B and C) MTS assay of MCF-7 tet-off^{TRPV1} cells treated with vehicle or various concentrations of dox for 24 h prior to treatment with capsaicin (3 μM) for 48 h (B) or 96 h (C). The data are mean ± SD (n = 15) from five independent assays; * denotes $p < 0.05$ compared to vehicle at each dox concentration using two-way ANOVA with Bonferroni post-hoc test.

Fig. 6. TRPV1 activation induces cell death in MCF-7 cells over-expressing TRPV1. (A,B and C) MCF-7 Tet-off^{TRPV1} cells were cultured in the absence of dox for 96 h prior to treatment with various concentrations of staurosporine (STS) or capsaicin for 6 h. Phase contrast images (A, top panel). Cells were stained with Hoechst 3342 (blue), Yo-Pro-1 (green) and PI (red) (A, bottom panel). Cells stained with both YO-PRO-1 and PI (purple) indicate a necrotic population (A and B) and cells stained for YO-PRO-1 only indicate an apoptotic population (A and C). Images are representative of 3 independent experiments and the data are mean ± SD (n = 3).

Fig. 7. Cell death is dependent on the level of TRPV1 expression and activation. (A and B) MCF-7 Tet-off^{TRPV1} cells were treated with various concentrations of dox for 96 h prior to treatment with various concentrations of capsaicin for 6 h (A) or 24 h (B). Data are presented as percentage YO-PRO-1 and PI positivity and are representative of 3 independent experiments.

Fig. 8. Effect of TRPV1 activation and over-expression on c-Fos and RIP3 expression in MCF-7 cells. MCF-7 Tet-off^{TRPV1} cells were cultured in the presence or absence of dox (1 μg/mL) for 96 h prior to treatment with capsaicin (3 μM) for various time points. Relative c-Fos (A) or RIP3 (B) mRNA expression. Data are presented as fold change and are representative of three independent assays performed in duplicate. The data are mean ± SD (n = 3) * denotes $p < 0.05$ compared using two-way ANOVA with Bonferroni post-hoc test (A) or t-test (B).

Supplementary Figure Legends

Fig. S1. Characterization of TRPV1 expression in MCF-7 tet-off cells. Cells were treated with vehicle or dox (1 μg/mL) for 96 h. Relative TRPV1 mRNA in 1) MCF-7 tet-off, 2) MCF-7 tet-off PPARalpha, 3) MCF-7 tet-off^{TRPV1} clone 30 +Dox and 4) MCF-7 tet-off^{TRPV1} clone 30 –Dox. The data are normalized to 18S RNA and expressed as mean ±SD (n = 3 technical replicates) relative to MCF-7 cells.

Fig. S2. Characterization of MCF-7 Tet-off clones 4 and 35 stably expressing TRPV1. Cells were treated with vehicle or dox (1 μg/mL) for 96 h. (A) β-Gal activity in MCF-7 Tet-off^{TRPV1} cells. The data are mean ± SD (n = 3). (B) Relative TRPV1 mRNA levels in MCF-7 Tet-off^{TRPV1} cells. The data are normalized to 18S RNA and expressed as mean ±SD (n = 3)

relative to + dox. (C) Relative $[Ca^{2+}]_i$ response to capsaicin (10 μM) in MCF-7 Tet-off^{TRPV1} cells in the presence or absence of dox. Relative $[Ca^{2+}]_i$ (black lines) ± SD (grey lines) (n = 3).

Fig. S3. Effect of TRPV1 expression and activity levels on capsaicin-mediated increases in $[Ca^{2+}]_I$ in MCF-7 Tet-off^{TRPV1} clones 4 and 35. (A) Assessment of peak relative $[Ca^{2+}]_i$ in MCF-7 Tet-off^{TRPV1} cells treated with various concentrations of dox (96 h) prior to stimulation with capsaicin (3 μM). (B) Assessment of peak relative $[Ca^{2+}]_i$ in MCF-7 Tet-off^{TRPV1} cells treated with dox (1 μg/mL; 96 h) prior to stimulation with various concentrations of capsaicin. Graphs represent the mean concentration-response curves of peak relative $[Ca^{2+}]_i$ ± SD (n = 9) from three independent assays; * denotes $p < 0.05$ using two-way ANOVA with Bonferroni post-hoc test.

Fig. S4. Effect of TRPV1 activator capsaicin on the number of viable MCF-7 Tet-off^{TRPV1} clones 4 and 35. (A-D) MCF-7 Tet-off^{TRPV1} clone 4 cells (A and B) or MCF-7 Tet-off ^{TRPV1} clone 35 cells (C and D) were treated with vehicle or dox (1 μg/mL) for 96 h prior to treatment with various concentrations of capsaicin for 24 h (A and C) or 96 h (B and D). Viable cell numbers were assessed using an MTS assay. Graphs represent the mean concentration-response curves of absorbance ± SD (n = 9) from three independent assays; * denotes $p < 0.05$ using two-way ANOVA with Bonferroni post-hoc test.

Fig. S5. TRPV1 expression levels effect capsaicin-meditated effects on viability of MCF-7 Tet-off^{TRPV1} clones 4 and 35. MCF-7 Tet-off^{TRPV1} cells were treated with vehicle or dox (1 μg/mL) for 96 h prior to treatment with capsaicin (3 μM; 24 h). Viable cell numbers were assessed using an MTS assay. Graph represents the mean concentration-response curves of

absorbance ± SD (n = 9) from three independent assays; * denotes $p < 0.05$ using one-way ANOVA with Dunnett post-hoc test.

Fig. S6. The cell death induced in MCF-7 cells with TRPV1 over-expression when TRPV1 is activated involves Ca^{2+}. MCF-7 Tet-off^{TRPV1} cells were cultured in the absence of dox for 96 h prior to the addition of capsaicin (or vehicle) ± BAPTA for 6 h. Cells stained with both YO-PRO-1 and PI indicate a necrotic population. Data are mean ± SD (n = 3) from 3 independent assays.

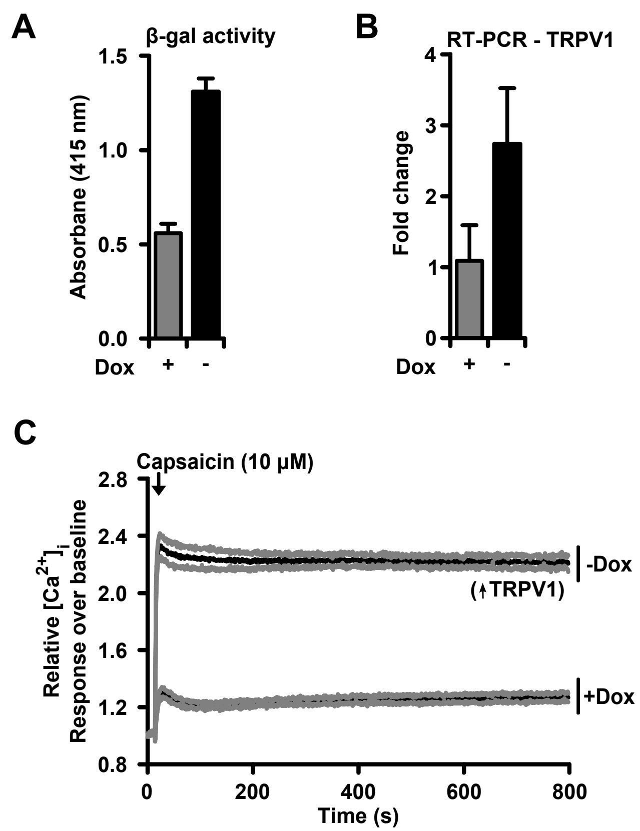


Figure 1

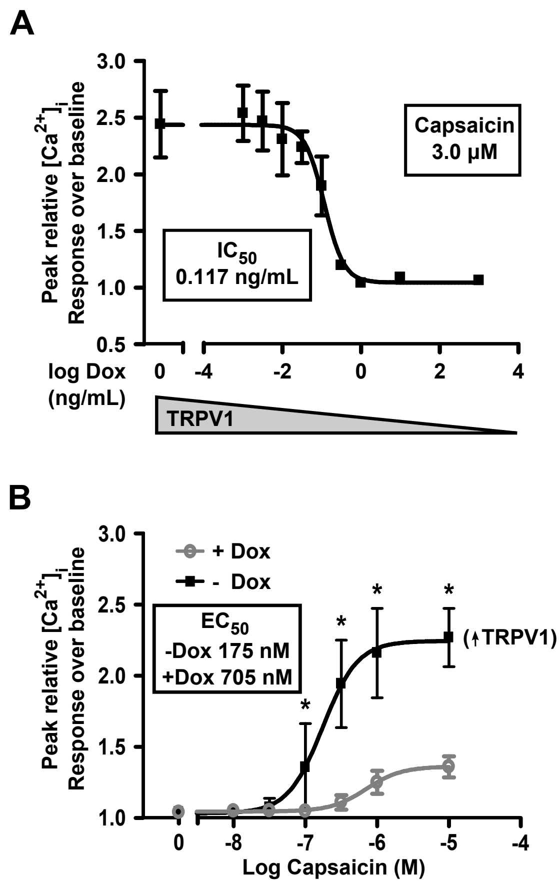


Figure 2

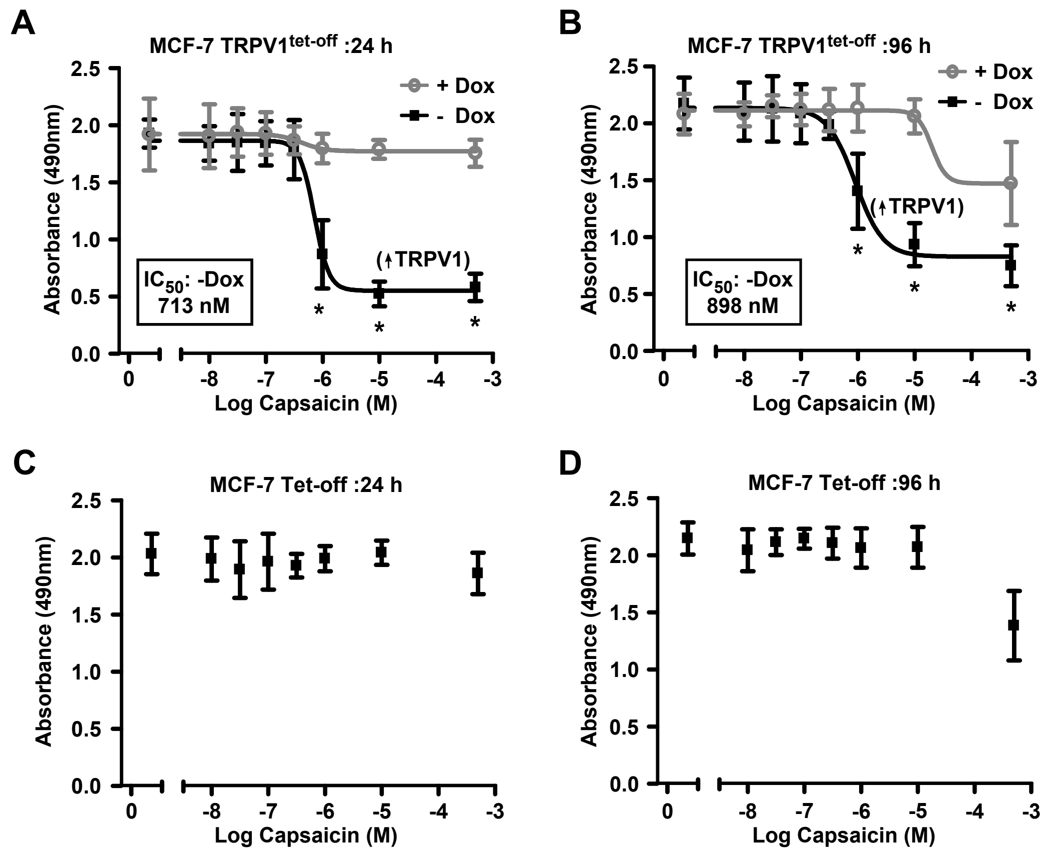


Figure 3

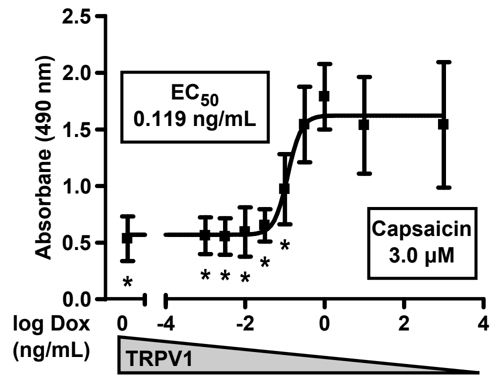
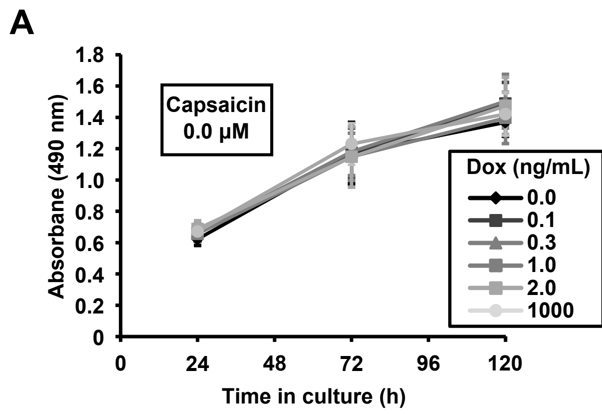
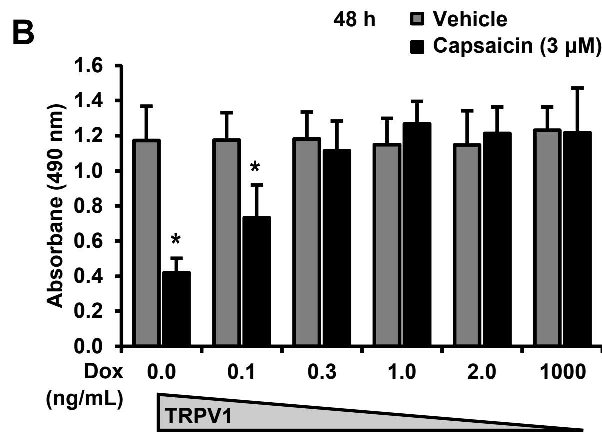


Figure 4





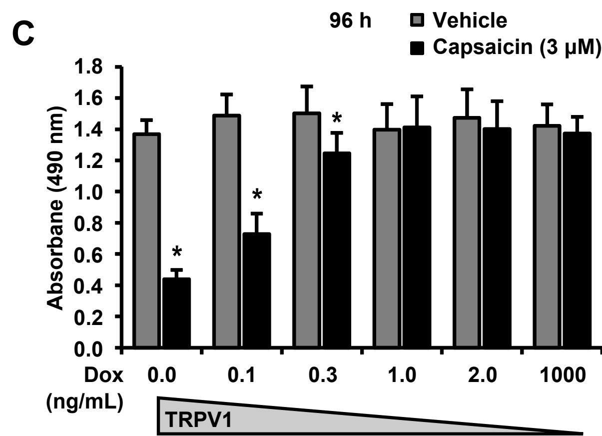
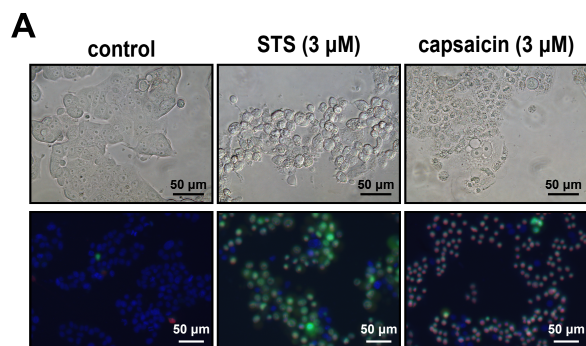


Figure 5

A



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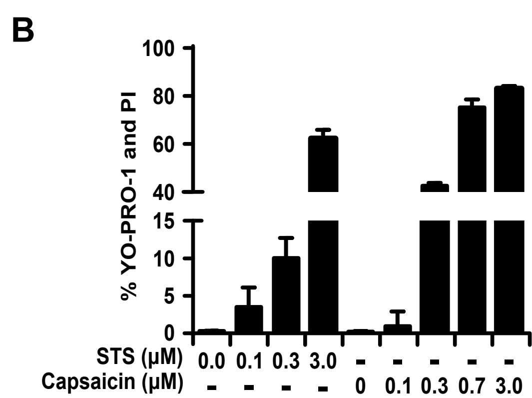

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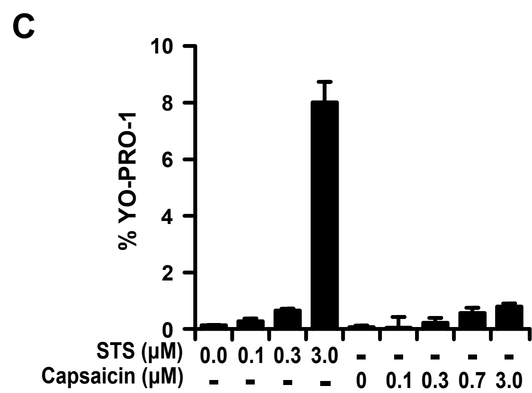
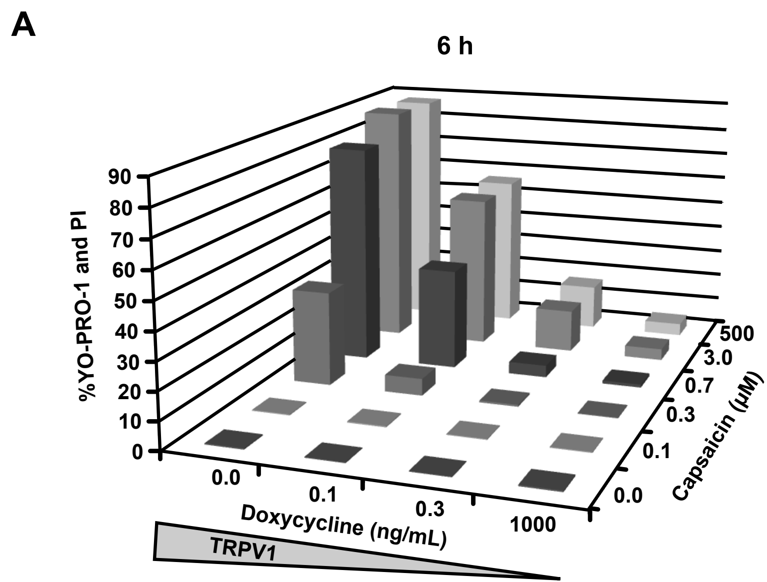


Figure 6

A



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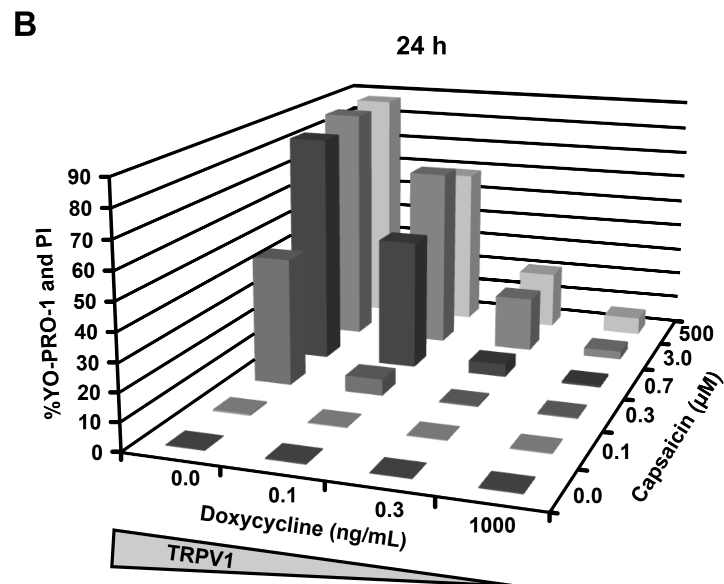


Figure 7

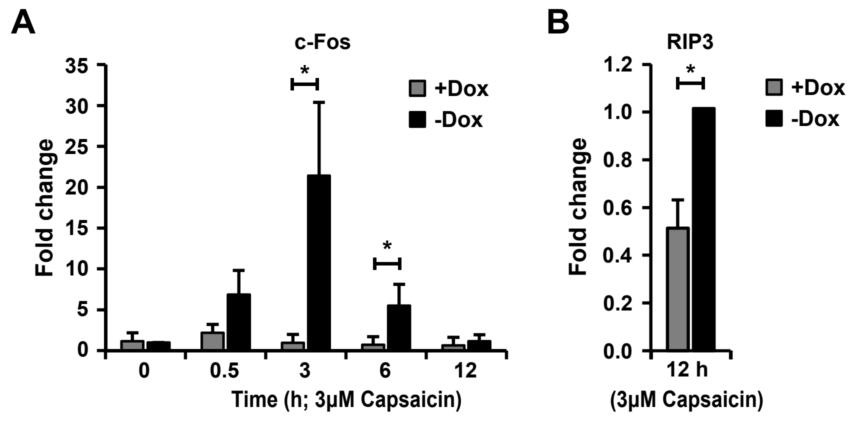


Figure 8

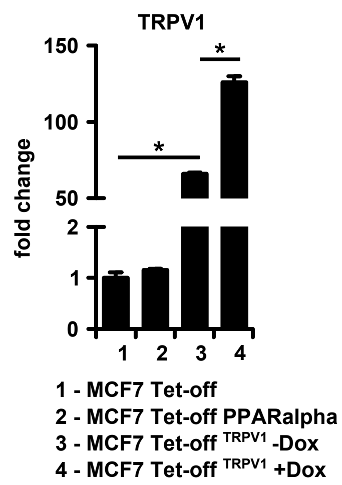
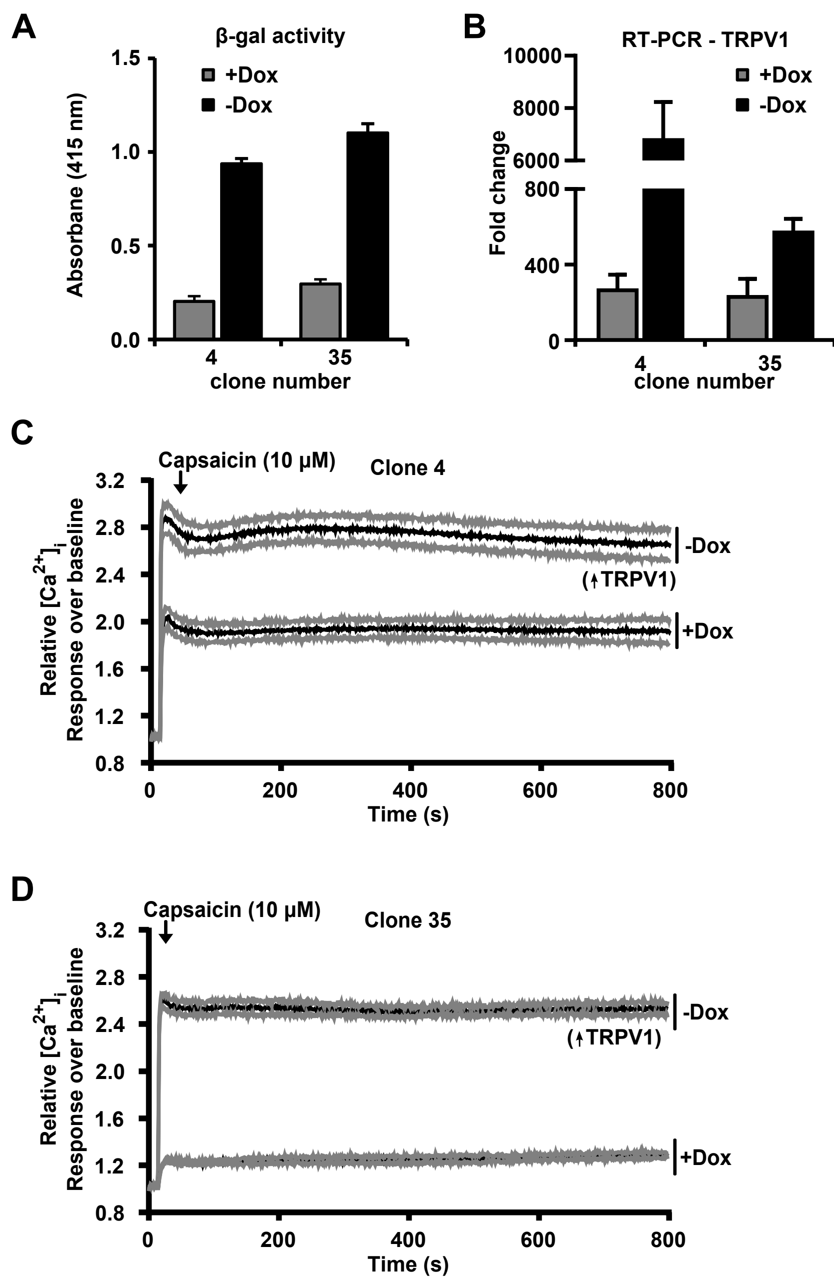


Figure s1



Supp Figure s2

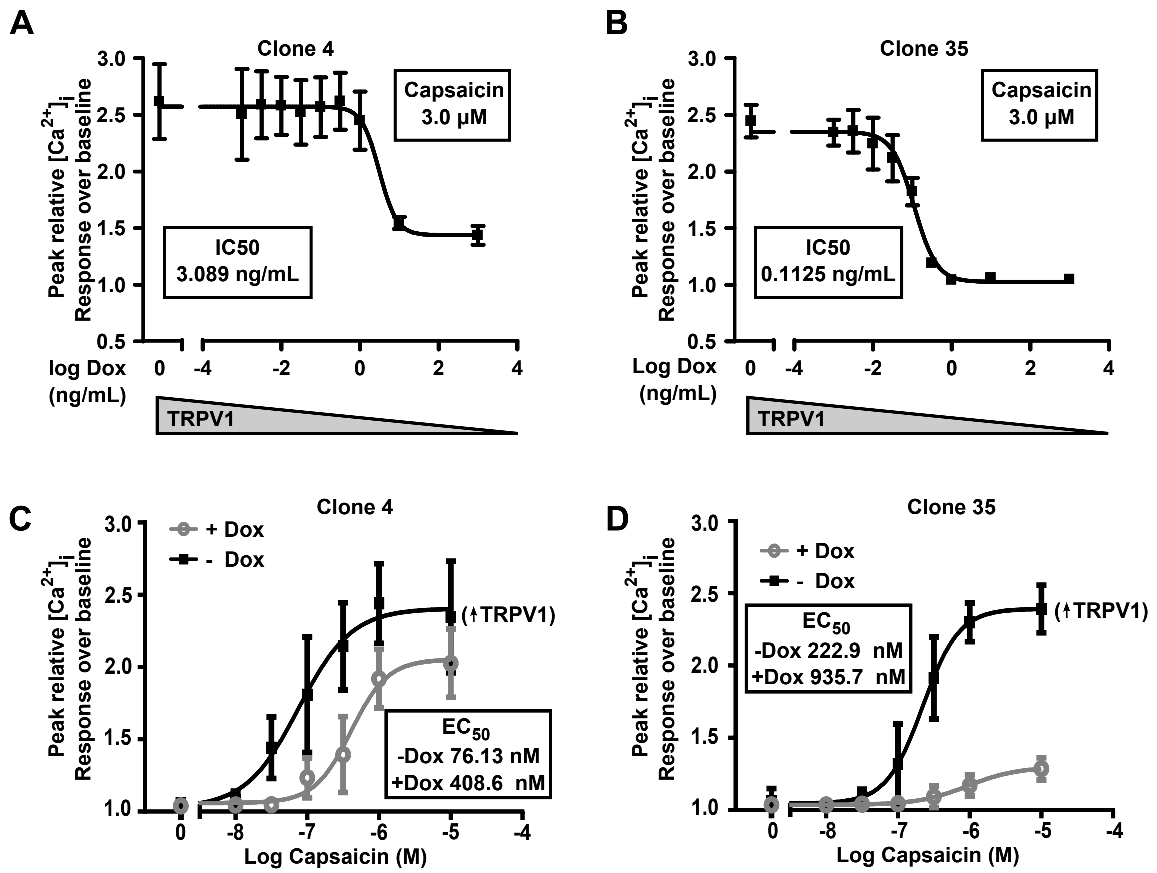


Figure s3

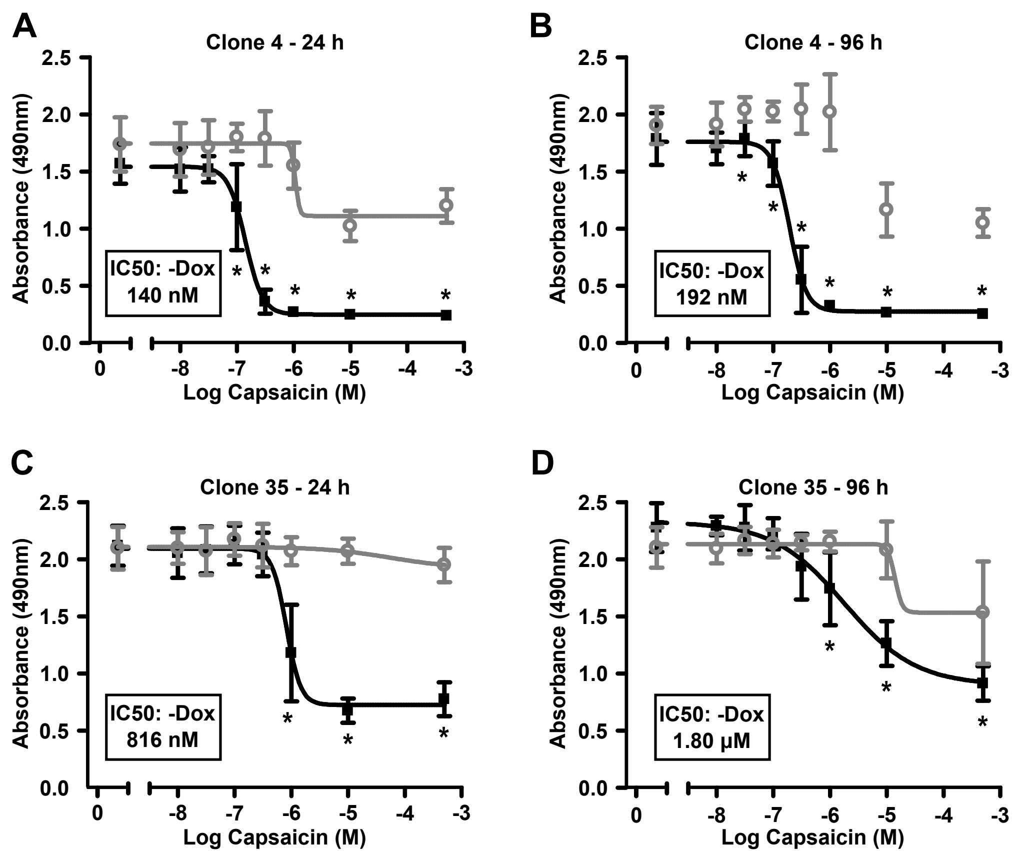
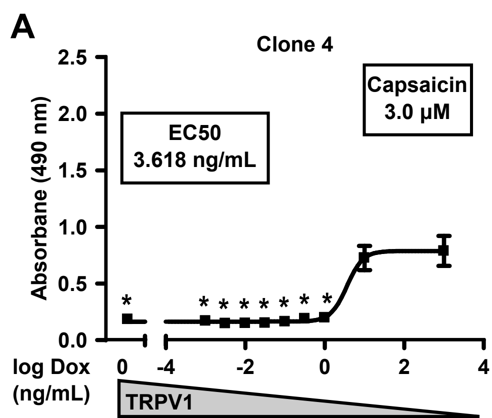


Figure s4



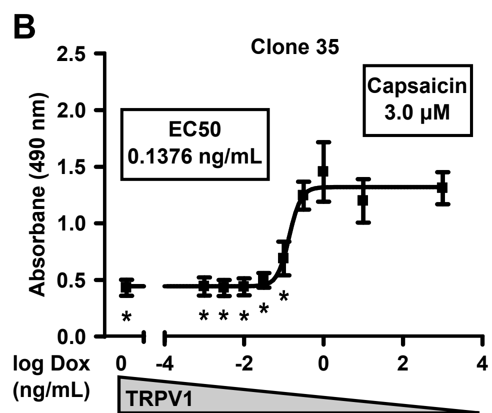


Figure s5

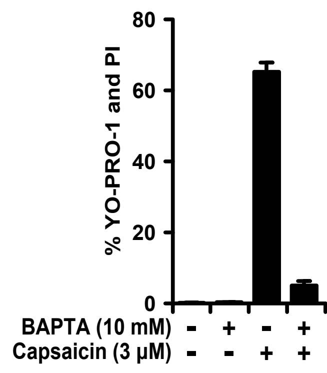


Figure S6