Accepted Manuscript

Assessment of the TRPM8 inhibitor AMTB in breast cancer cells and its identification as an inhibitor of voltage gated sodium channels

Kunsala T.D.S. Yapa, Jennifer Deuis, Amelia A. Peters, Paraic A. Kenny, Sarah J. Roberts-Thomson, Irina Vetter, Gregory R. Monteith

Please cite this article as: Kunsala T.D.S. Yapa, Jennifer Deuis, Amelia A. Peters, Paraic A. Kenny, Sarah J. Roberts-Thomson, Irina Vetter, Gregory R. Monteith , Assessment of the TRPM8 inhibitor AMTB in breast cancer cells and its identification as an inhibitor of voltage gated sodium channels. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Lfs(2017), doi:10.1016/j.lfs.2018.02.030

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Assessment of the TRPM8 inhibitor AMTB in breast cancer cells and its identification as an inhibitor of voltage gated sodium channels.

Authors: Kunsala T.D.S Yapa[a], Jennifer Deuis[b], Amelia A. Peters[a,c], Paraic A. Kenny[d], Sarah J. Roberts-Thomson[a], Irina Vetter[b], Gregory R. Monteith[a,c].

[a] The School of Pharmacy, The University of Queensland, Brisbane, QLD, Australia

[b] Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD, Australia

[c] Mater Research, The University of Queensland, Translational Research Institute, Brisbane, QLD, Australia

[d] Kabara Cancer Research Institute, Gundersen Medical Foundation, La Crosse, WI, USA

Corresponding author: Gregory R Monteith, The School of Pharmacy and Mater Research, The University of Queensland, Brisbane, QLD, Australia, telephone: +61 7 334 61855, email: gregm@uq.edu.au

1

Abstract

Aims: To assess levels of the calcium permeable transient receptor potential cation channel, subfamily melastatin, member 8 (TRPM8) in breast cancer molecular subtypes and to assess the consequences of TRPM8 pharmacological inhibition with AMTB (an inhibitor of TRPM8) on breast cancer cell lines.

Materials and Methods: Cell viability and migration of breast cancer cells was determined using MTS assays and wound healing assays, respectively. RNA-Seq analysis of breast tumours and qPCR in breast cancer cell lines were used to assess mRNA levels of ion channels. Membrane potential assays were employed to assess the effects of AMTB against specific voltage gated sodium channels (Na_V).

Key findings: TRPM8 levels were significantly higher in breast cancers of the basal molecular subtype. AMTB decreased viable cell number in MDA-MB-231 and SK-BR-3 breast cancer cell lines (30 and 100 μM), and also reduced the migration of MDA-MB-231 cells (30 μM). However, these effects were independent of TRPM8, as no TRPM8 mRNA was detected in MDA-MB-231 cells. AMTB was identified as an inhibitor of Na_V isoforms. Na_V1.1-1.9 were expressed in a number of breast cancer cell lines, with Na_V1.5 mRNA highest in MDA-MB-231 cells compared to the other breast cancer cell lines assessed.

Significance: TRPM8 levels may be elevated in basal breast cancers, however, TRPM8 expression appears to be lost in many breast cancer cell lines. Some of the effects of AMTB attributed to TRPM8 may be due to effects on Na_V channels.

Key words: AMTB, breast cancer, TRPM8, sodium channels

Introduction

Changes in cytosolic free calcium ($[Ca^{2+}]_{CYT}$) are often critical for events important in cancer progression (1, 2). Altered expression of specific calcium permeable ion channels has been widely reported in cancers and/or cancer cell lines originating from the lung, prostate and breast (3-7). In some cases, such changes are even specific to the cancer clinical subtype, such as the overexpression of the transient receptor potential cation channel subfamily V member 6 (TRPV6) channel in estrogen receptor negative breast cancers and those of the basal molecular subtype (8). The basal molecular subtype of breast cancer is of particular interest due to its overlap with breast cancers that lack the expression of receptors representing current targets for targeted therapies, such as the estrogen and human epidermal growth factor 2 (HER2) receptors (9, 10).

TRPM8 is a member of the TRP ion channel family and is expressed in primary sensory neurons where it plays an important role in cold sensation (11). The properties of TRPM8 in specific neuronal sensory pathways have seen its identification as a target for a diverse range of conditions including pain, migraine and cough (12-14). However, despite the importance of TRPM8 as a sensor in neurons, it was actually first identified through a study assessing the overexpression of proteins in the prostate, which also demonstrated elevated levels in cancers of the prostate, lung, colon and breast (15). Subsequent to this report, a variety of studies have explored in great depth the contribution of TRPM8 to prostate cancer cell growth and invasion and the potential role of TRPM8 modulators to control prostate cancer disease progression and/or TRPM8 as a biomarker (16-20).

Despite the first report of TRPM8 overexpression in breast cancer occurring in 2001 (15), there have been limited studies of TRPM8 in the context of possible associations with breast cancer subtypes, the role of TRPM8 in breast cancer cells and the suitability of this ion channel as a target for breast cancer therapy (21, 22). An agent which may be useful in defining the possible role of TRPM8 in breast cancer and as a proof of concept tool for targeting TRPM8 in breast cancer therapy is the TRPM8 inhibitor, *N*-(3-aminopropyl)-2-{[(3-methylphenyl) methyl]oxy}(20)-*N*-(2-thienylmethyl)benzamide (AMTB) (23). AMTB, a 2-benzyloxy-benzoic acid amide derivative (18), has been used as a TRPM8 pharmacological inhibitor in a variety of studies (23-25) and has been reported to inhibit the migration of LNCaP, PC3 and DU145 prostate cancer cell lines (25). TRPM8 has also been reported to regulate migration, proliferation and also angiogenesis processes (20, 26-32). However, AMTB has not been assessed in breast cancer cell lines.

3

Hence, in this study, we sought to define the expression of TRPM8 in breast cancer molecular subtypes and assess the consequences of TRPM8 pharmacological inhibition in breast cancer cell lines. These studies yielded the unexpected result that some of the reported effects of AMTB in a variety of disease models may be independent of TRPM8 and could rather be a consequence of the ability of AMTB to inhibit voltage gated sodium channels (Na_V) of the Na_V1 class.

Materials and Methods:

Analysis of TRPM8 expression in breast tumours

RNA-Seq gene expression profiles in human breast tumours (n = 506) from The Cancer Genome Atlas (TCGA) study (33) were assessed as previously described (34). Gene expression of TRPM8 in 84 basal-like, 58 HER2 enriched, 233 Luminal A, and 131 Luminal B tumours were evaluated. Statistical significance was measured using Kruskal-Wallis test with Dunn's post-test.

Cell Culture

The human breast cancer cell lines used this study: MDA-MB-231, MCF-7 and HCC1569 were purchased from American Type Culture Collection; SK-BR-3, T-47D and ZR-75-1 cell lines were a kind donation from the late Professor Rob Sutherland (Garvan Institute, Sydney) and MDA-MB-468 cells were a generous gift from Dr Chanel Smart (The University of Queensland Centre for Clinical Research). MDA-MB-231 cells and SK-BR-3 cells were cultured as previously described (35, 36) in antibiotic-free media and routine mycoplasma contamination testing was carried out using the MycoAlert Mycoplasma Detection Kit (LT07-218, Lonza Inc).

Pharmacological agents

AMTB was purchased from Sigma-Aldrich (SML0103). Stock solutions were prepared by dissolving compounds in dimethyl sulfoxide (DMSO) (D8418, Sigma-Aldrich) or sterile distilled water and aliquots were stored at 4°C or -20°C. Lidocaine (N-(2,6-dimethylphenyl)acetamide) stock solution was purchased from Sigma-Aldrich (L7757).

Cell viability assay

4

CellTiter 96® AQueous One Solution Cell Proliferation Assay (G3580, Promega) was used to determine cell viability. Cells were cultured in antibiotic-free media and plated in 96-well plates at a density of 5,000 cells per well for MDA-MB-231 and 6,000 cells per well for SK-BR-3 cells. Pharmacological compounds/toxins were diluted to the appropriate concentrations in cell culture media and added to the cells at 24 h and 72 h post plating. Control wells were treated with 0.1% vehicle (DMSO or absolute ethanol) or antibiotic-free media. MTS assays were performed 96 h post plating using CellTiter 96® AQueous One Solution Reagent (Promega). Absorbance at 490 nm was measured using a micro-plate reader ((iMark, BioRAD).

In vitro scratch assay

MDA-MB-231 cells (17 000 cells per well) were seeded in 96-well plates and incubated at 37°C, 5% CO_2 for 48 h. The WoundMaker™ instrument (Essen Biosciences) was used to produce an equal sized scratch in the cell monolayer of all wells of the plate. Cells were washed twice with fresh media and immediately treated with various concentrations of AMTB for 24 h. Images of the centre of each well were acquired with a JuLI™ Stage live cell imaging system (NanoEntek) (10X objective), 24 h post-treatment. Analysis of wound closure (confluence) was performed using JuLI™ Stage automated cell imaging system software (V0.0, NanoEntek). Confluency results were normalised to a control well (media treatment) representing the highest confluence at 24 h.

RNA isolation and real-time RT-PCR

Total RNA was isolated and purified using a RNeasy Plus Mini Kit (74136, Qiagen). Purified RNA was reverse transcribed using Omniscript RT Kit (Qiagen); random primers (C1181, Promega) and RNAase inhibitor (N2111, Promega) were purchased separately. Real-time PCR was performed using TaqMan® Universal PCR Master Mix (2X) and TaqMan® gene expression assays purchased from Life Technologies. Gene expression assays: SCN1A/Na$_V$1.1 (Hs00374696_m1), SCN2A/Na$_V$1.2 (Hs01109877_m1), SCN3A/Na$_V$1.3 (Hs00366902_m1), SCN4A/Na$_V$1.4 (Hs01109480_m1), SCN5A/Na$_V$1.5 (Hs00165693_m1), SCN8A/Na$_V$1.6 (Hs00274075_m1), SCN9A/Na$_V$1.7 (Hs00161567_m1), SCN10A/Na$_V$1.8 (Hs01045137_m1), SCN11A/Na$_V$1.9 (Hs00204222_m1), TRPM8 (Hs01066597_m1) and endogenous control for 18S rRNA (4319413E) were used in this study. Assays were performed using a StepOne Plus instrument (Applied Biosystems) under universal cycling conditions and data were analysed using StepOne Plus Software, version 2.2.2 (Applied Biosystems). The relative quantification or comparative C_T (cycle

threshold) method (37) was used to determine relative mRNA levels with data normalised to 18S rRNA.

Human TRPM8 cDNA clone (pCMV6-XL5-hTRPM8, un-tagged, homo-sapiens, clone MGC:2849, complete cds, NCBI RefSeq: BC001135.1, 10 μg, Cat#: SC13793, Origene Technologies Ltd) was used as a positive control for TRPM8 real-time RT-PCR assays.

FLIPR membrane potential assay

HEK293 cells heterologously expressing human $Na_V1.1–1.8$ (SB Drug Discovery, Glasgow, UK) were cultured in MEM containing 10% v/v FBS and selection antibiotics as recommended by the manufacturer. Cells were grown in a humidified 5% CO_2 incubator at 37°C, grown to 70–80% confluence, and passaged every 3-4 days using TrypLE Express (Invitrogen). Cells were seeded in 384-well plates (Corning) at a density of 10,000-15,000 cells per well and cultured for 48 h. Membrane potential dye (Molecular Devices) was diluted in physiological salt solution (PSS; 140 mmol/L NaCl, 11.5 mmol/L glucose, 10 mmol/L HEPES, 5.9 mmol/L KaCl, 1.4 mmol/L $MgCl_2$, 1.2 mmol/L NaH_2PO_4, 5 mmol/L $NaHCO_3$, 1.8 mmol/L $CaCl_2$, pH 7.4). Growth media was removed from the cells, 20 µL dye/well added and cells incubated in the dark at 37°C for 30 min. A two-addition FLIPR protocol consisting of addition of AMTB, incubation for 5–10 min and addition of veratridine (20-60 μM) to activate $Na_V1.1$-1.7 or deltamethrin (150 μM) was used to activate $Na_V1.8$. The change in fluorescence was measured (excitation 515–545 nm, emission 565–625 nm) every 1-2 s for 5-30 min after addition of agonists using the fluorescence imaging plate reader FLIPR TETRA (Molecular Devices). To quantitate the effect of test compounds on Na_V responses, the area under the curve (AUC) corresponding to 5 min after the addition of veratridine or maximum response corresponding to 30 min after the addition of deltamethrin was computed using ScreenWorks (Molecular Devices, Version 3.2.0.14).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.04 for Windows, unless otherwise specified.

Results:

TRPM8 levels are elevated in basal-like breast cancers

TRPM8 levels were significantly higher in basal-like breast tumours, compared to the luminal A, B and HER2-enriched subtypes (Figure 1). Considerable variation in TRPM8 levels was also observed within the basal-like subtype, with the sample showing the highest TRPM8 expression level having 476 times more TRPM8 transcript than the median level among the basal-like samples. Although the most striking elevation was detected in the basal-like breast tumour samples, the HER2-amplified group also had higher TRPM8 levels than both of the luminal subtypes.
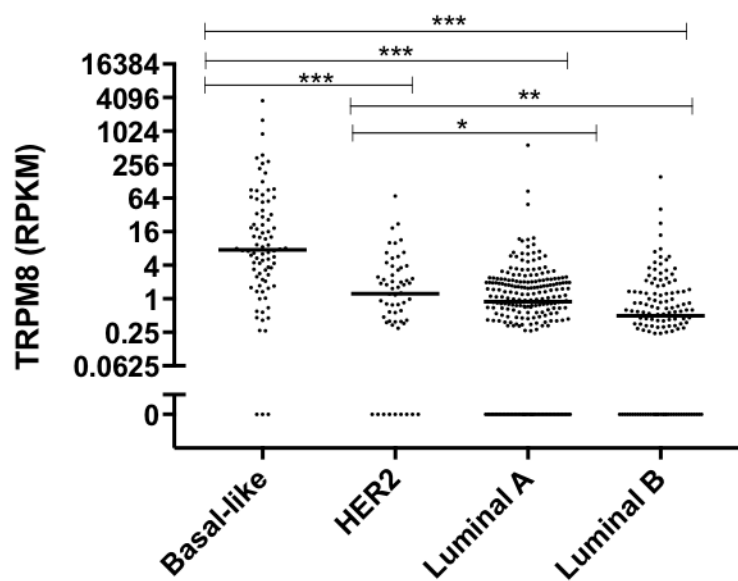

Figure 1: TRPM8 expression in breast cancer clinical samples. Gene expression (Reads Per Kilobase of transcript per Million (RPKM)) of TRPM8 in 506 breast tumours from TCGA breast cancer RNA-Seq data, classified according to breast tumour subtypes. TRPM8 is over-expressed the basal-like subtype compared to HER2 enriched and luminal A/B subtypes. Horizontal lines represent median levels for each subtype, statistical significance was determined using Kruskal-Wallis test with Dunn's post-test; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

AMTB decreases the proliferation and migration of breast cancer cells via a TRPM8 independent mechanism

Treatment of MDA-MB-231 and SK-BR-3 breast cancer cells with the TRPM8 inhibitor AMTB reduced cell viability by approximately 70% at 30 and 100 µM, with IC_{50} values of 23.7 µM and 17.3 µM in MDA-MB-231 and SK-BR-3 cells, respectively (Figure 2A and B). *In vitro* scratch assays showed that AMTB (30 µM) also significantly decreased the migration of MDA-MB-231 breast cancer cells (Fig 2C). However, this effect was not due to TRPM8, since assessment of TRPM8 mRNA levels in a variety of breast cancer cell lines representing distinct molecular subtypes (38, 39), revealed little or no TRPM8 mRNA (Table 1), including in SK-BR-3 and MDA-MB-231 cells where effects by AMTB were observed. Therefore, the effects of AMTB, a reported selective TRPM8 inhibitor (23), at a concentration (30 µM) used in other model systems (18), occurred independently of TRPM8 inhibition in these breast cancer cell lines. The possibility of off-target effects of AMTB was therefore investigated.
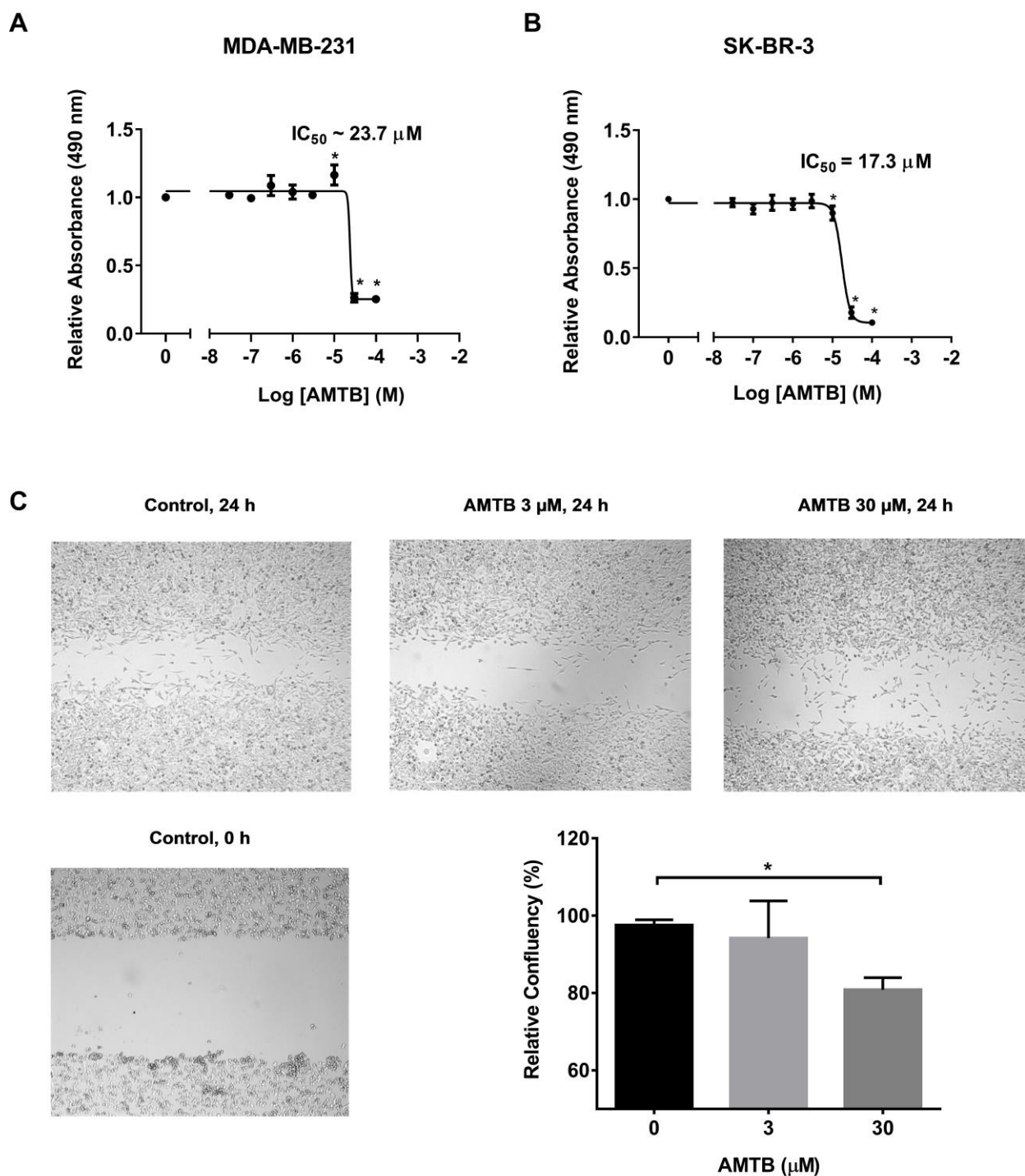
A

MDA-MB-231

B

SK-BR-3

C

Figure 2: AMTB reduces cell viability and migration of breast cancer cells. Concentration-response curves for AMTB in MDA-MB-231 (**A**) and SK-BR-3 (**B**) breast cancer cells. Cells were incubated with AMTB (0 – 100 μM) for 72 h and an MTS assay was used to approximate viable cell number. Absorbance values (490 nm) were normalised to control treated cells and plotted using non-linear regression curve fit: log (inhibitor) vs response – variable slope (four parameters). Data show mean ± SD, n = 3. Statistical analysis was performed using a one-way ANOVA with

9

Dunnett's multiple comparisons test; * $P < 0.05$ compared to control. **C**: In vitro scratch assays were performed on MDA-MB-231 cells in the absence (control, 0) and presence of AMTB (3 μM, 30 μM). AMTB (30 μM) decreased the migration of MDA-MB-231 cells by ~20%. Representative images from one experiment after initial scratch (0 h) and 24 h post-treatment. Bar graph represents the relative confluency of each treatment group normalised to control well (24 h with media only); mean ± SD, n=3, * $P < 0.05$.

Table 1: TRPM8 mRNA are very low or undetectable in breast cancer cell lines. Molecular subtypes, receptor status (estrogen receptor, ER; progesterone receptor, PR (38, 39)) and average C_T values for TRPM8 and 18S rRNA (housekeeping control) in different breast cancer cell lines, $C_T > 35$ were assigned not detected (ND); mean ± SD, n=3.

Cell Line	Molecular Subtype and Receptor Status	C_T TRPM8	C_T 18S
HCC1569	Basal, ER-, PR-, HER2+	ND	15.52 ± 0.62
MCF-7	Luminal, ER+, PR+	ND	15.92 ± 0.42
MDA-MB-231	Basal, ER-, PR-	ND	14.93 ± 0.30
MDA-MB-468	Basal, ER-, PR-	34.04 ± 0.52	15.53 ± 0.39
SK-BR-3	Luminal, ER-, PR-, HER2+	33.34 ± 0.57	14.94 ± 0.11
T-47D	Luminal, ER+, PR+	ND	15.83 ± 0.16
ZR-75-1	Luminal, ER+, PR-	34.81 ± 0.62	17.13 ± 0.18
Positive control: TRPM8 cDNA	-	12.55 ± 0.05	-

10

AMTB inhibits veratridine-induced effects on membrane potential

Strikingly, AMTB contains several structural features required for inhibition of voltage-gated sodium channels (Na_V) by local anesthetics, including a substituted benzene ring, an intermediate chain with amide linker, as well as a hydrophilic terminal (Figure 3A and B). In addition to structural similarities of AMTB to such compounds, we also observed profound analgesia of AMTB after intraplantar administration in animal models of chemotherapy-induced allodynia where local anaesthetic provided anti-allodynic effects, but TRPM8 knockout animals lacked a phenotype (data not shown) (40). We thus assessed the effect of AMTB on the function of $Na_V1.1$-1.8 using a high-throughput fluorescent imaging plate reader (FLIPR) membrane potential assay (Figure 3C). AMTB blocked veratridine-induced membrane potential changes at each Na_V1 isoform more potently than the prototypical Na_V blocker lidocaine (Figure 3C and D). The IC_{50} (~2 - 15 μM) for each Na_V placed the actions of AMTB on Na_Vs in a similar concentration range as that reported for inhibition of TRPM8 by AMTB (23). Given veratridine has no effect on membrane potential in untransfected HEK293 cells (Suppl. Fig 1), and that AMTB did not affect membrane potential in HEK cells expressing $Na_V1.1$-1.8 (data not shown and Suppl. Fig 2), these results are consistent with inhibition of $Na_V1.1$-1.8 by AMTB. These results, therefore, suggested that one of the mechanisms by which AMTB could exert effects on MDA-MB-231 cells is via effects on Na_Vs. Therefore, the presence of Na_Vs in a panel of breast cancer cell lines representing different molecular subtypes was evaluated.
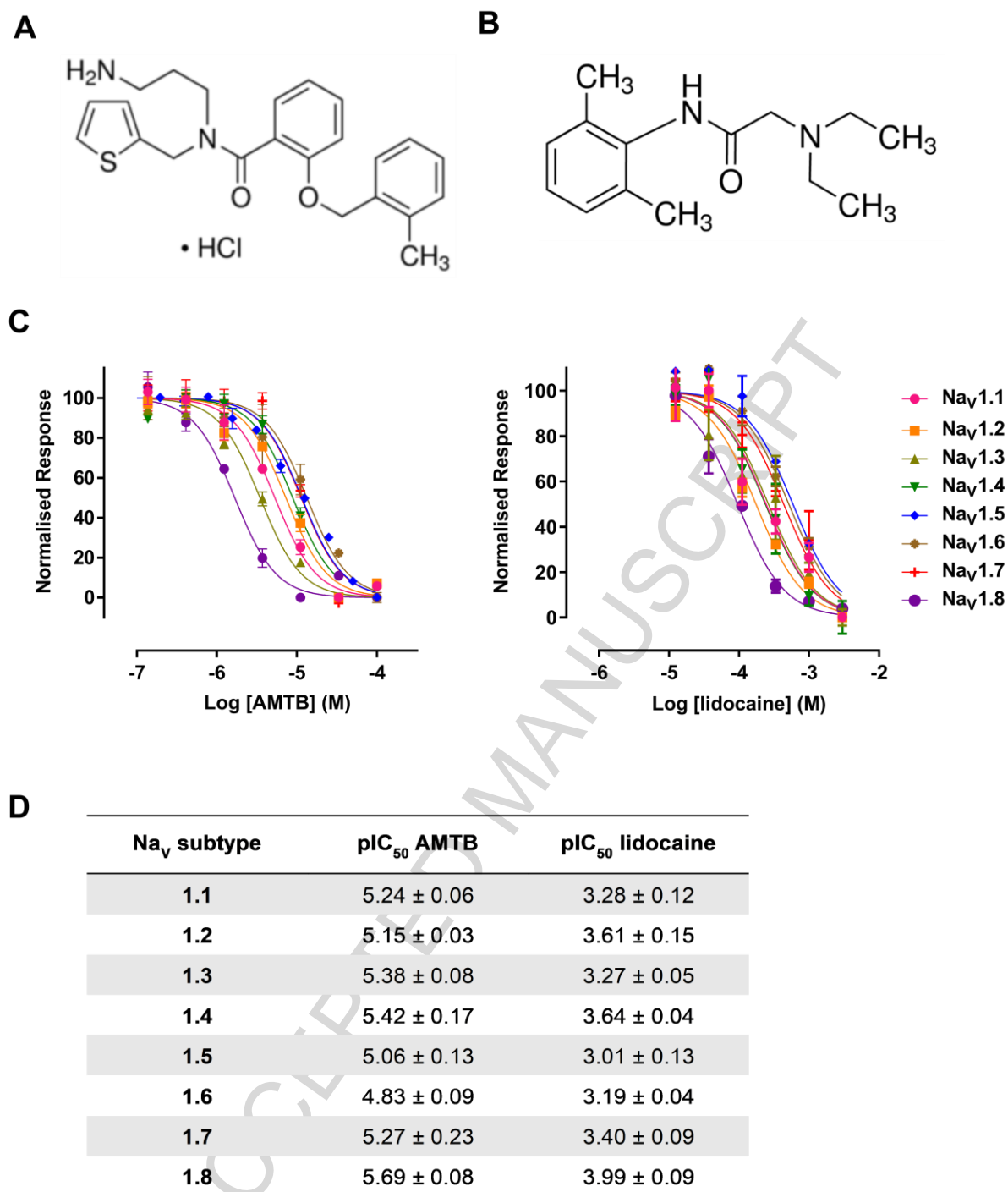
A

B

C

Na$_V$ subtype	pIC$_{50}$ AMTB	pIC$_{50}$ lidocaine
1.1	5.24 ± 0.06	3.28 ± 0.12
1.2	5.15 ± 0.03	3.61 ± 0.15
1.3	5.38 ± 0.08	3.27 ± 0.05
1.4	5.42 ± 0.17	3.64 ± 0.04
1.5	5.06 ± 0.13	3.01 ± 0.13
1.6	4.83 ± 0.09	3.19 ± 0.04
1.7	5.27 ± 0.23	3.40 ± 0.09
1.8	5.69 ± 0.08	3.99 ± 0.09

Figure 3: **AMTB is a non-selective inhibitor of voltage-gated sodium channels (Na$_V$).** (A) Chemical structure of AMTB (N-(3-aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)-benzamide). (B) Chemical structure of lidocaine (N-(2,6-dimethylphenyl)acetamide). (C) Representative concentration-response curves for AMTB and lidocaine in HEK293 cells stably expressing hNa$_V$1.1-1.8. Data are presented as mean ± SEM from 3-6 wells. (D) pIC$_{50}$ values for AMTB and lidocaine at hNa$_V$1.1-1.8 obtained from the membrane potential assays. Data are presented as mean ± SEM from 3 independent experiments.

12

Differential expression of Na$_V$s in human breast cancer cell lines

There have been reports of Na$_V$s in human breast cancer cell lines, including the consequences of silencing and/or pharmacological inhibition of specific Na$_V$1 isoforms (41-43). However, there has been no comprehensive study of Na$_V$1 isoforms across human breast cancer cell lines of different molecular subtypes. Our assessment of Na$_V$1.1-1.8 mRNA in luminal, HER2 enriched and basal-like breast cancer cell lines identified that in contrast to TRPM8, all assessed breast cancer cell lines had detectable levels of mRNA for at least one Na$_V$1 isoform.

There were significant differences in the mRNA levels of specific mRNA isoforms between breast cancer cells lines. For example, the HER2 enriched basal-like molecular subtype cell line HCC1569 (38, 39) had the highest levels of Na$_V$1.1 and Na$_V$1.6 mRNA, whereas the basal-like MDA-MB-468 cell line had the highest levels of Na$_V$1.2. The basal, triple negative MDA-MB-231 cell line (38, 39) had the highest levels of Na$_V$1.3, Na$_V$1.5 and Na$_V$1 .7. Na$_V$1.8 was undetected or at the limit detection for all breast cancer cell lines examined. Elevated levels of specific Na$_V$1 isoforms is not a characteristic specific to basal-like cell lines since the luminal and estrogen receptor positive breast cancer cell lines MCF-7 and T47D (38, 39) had the highest levels of Na$_V$1.9 and Na$_V$1.4, respectively. Indeed, there was no clear relationship between the expression of a specific Na$_V$ isoforms and the molecular subtype of the breast cancer cell line or the expression of any specific biomarker (e.g. HER2 enriched or the estrogen receptor).
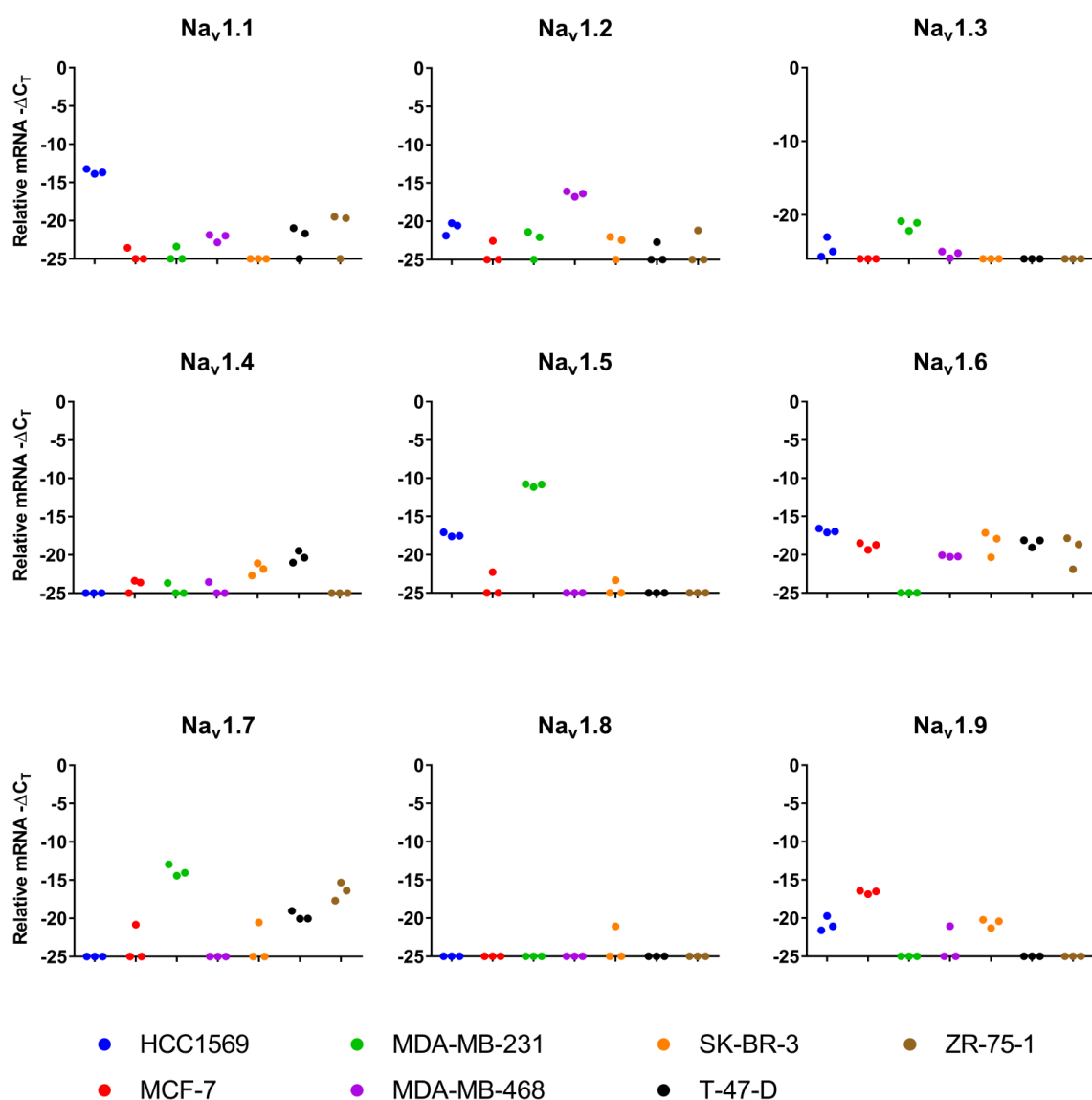
Figure 4: Levels of Na$_V$1.1-1.9 mRNA in seven breast cancer cell lines. - ΔC_T values (-C_T target-C_T 18S) from qPCR analysis of Na$_V$ isoforms in a panel of breast cancer cell lines. A more positive value is reflective of a greater level of target mRNA. Dots on the x-axis signify the target was beyond the designated limit of detection ($C_T > 35$) and was assigned a -ΔC_T value of -25. Each dot represents an independent biological replicate, (n = 3). See Table 1 for molecular subtypes and receptor status of the cell lines used.

Discussion

These studies suggest that basal-like breast tumours express significantly higher levels of TRPM8 compared to luminal A, B and HER2 enriched subtypes suggesting a possible role for TRPM8 as a therapeutic target or biomarker in breast cancer. However, TRPM8 seems to be undetectable in many breast cancer cell lines. Our studies using breast cancer cell lines that do not express TRPM8 suggest that the widely used TRPM8 inhibitor AMTB may exert effects via non-TRPM8 mechanisms in some models, specifically through inhibition Na_Vs. Hence, AMTB like the non-selective TRPM8 activator menthol should be used with caution in studies of TRPM8 because of non-TRPM8 mediated effects (44, 45).

An association between TRPM8 and breast cancer was first evident in the initial reports of TRPM8 (previously known as Trp-p8), where TRPM8 levels were reported to be elevated in breast tumours compared to corresponding normal tissues (15). TRPM8 overexpression has also been reported in human breast ductal adenocarcinoma compared to adjacent non-tumoural tissue using RT-PCR and immunohistochemistry (46). Despite these reports and the importance of breast cancer subtypes (47, 48), there has been no published assessment of TRPM8 in the context of breast cancer molecular subtypes. Our studies suggest that elevated TRPM8 mRNA is a feature of basal breast cancers, which represent a subtype with significant overlap with triple negative breast cancer cancers (49), which have been identified as a subtype requiring new therapeutic approaches (9, 10). However, future assessment of TRPM8 in basal breast cancer may be challenging since we found that TRPM8 is undetectable in the basal-like MDA-MB-231 breast cancer cell line and was also undetectable or at the limit of detection in all the other breast cancers (luminal and basal-like) that we examined. This result is in contrast to other studies of TRPM8 in human breast cancer cell lines (21, 22). Clearly, our quantitative RT-PCR assay is distinct from both the endpoint PCR assay and western blots used in the studies of Chodon et al (21). Indeed, it is notable that the short 16 and 17 bp PCR primers used in Chodon et al's study to assess TRPM8 expression lack specificity to this gene as both primers bind to the mRNAs of several genes. Liu et al (22), reported a relatively consistently high level of TRPM8 protein by western blot in a panel of 6 breast cancer cell lines, although the antibody used was not specified. We have tried to resolve this disparity by examining RNA-Seq data from an MDA-MB-231 line cultured in a different laboratory. Analysis of sequencing runs from two independent samples with 13,925,285 and 40,037,205 aligned reads found totals of 0 and 3 sequencing reads, respectively mapping to TRPM8 (data not shown), suggesting that this gene is silent in MDA-MB-231 cells and raising questions about the specificity of the antibody used which

was interpreted as showing very robust expression of TRPM8 protein in this particular cell line (22).

Variations in the origin and batch of foetal bovine serum used in cell culture could also alter TRPM8 gene expression as has been reported for other genes (50). The relatively few studies of TRPM8 in breast cancer cell lines may be reflective of issues related to TRPM8 expression. The loss of TRPM8 in cultured breast cancer cell lines is not totally unexpected. Genes that have roles in proliferation, angiogenesis, migration and invasion can display very different expression profiles in 2D *in vitro* cell cultures compared to *in vivo* conditions (51-53), leading to differences in mRNA and protein levels between clinical samples and cell lines (54). In prostate cancer (where TRPM8 is highly expressed in androgen-dependent tumours (15, 28, 30, 55), TRPM8 mRNA levels in primary cell cultures of prostate tumours are reduced when cells are cultured for extended periods of time (> 20 days) (56). In any case, the absence of detectable levels of TRPM8 mRNA in breast cancer cells indicated that AMTB was not exerting its effects on the proliferation and migration of breast cancer cells via the TRPM8 ion channel in our studies. These findings highlight the need for careful validation of results from experiments using pharmacological modulators due to unknown off-target effects. Examples of off-target effects with other TRP channel inhibitors include the selective TRPV1 channel activator capsaicin, which can modulate Na_V and potassium channel function when used at high concentrations (micro – millimolar) (57, 58).

AMTB possesses several structural features which are recognised as important for the activity of local anesthetics at Na_V channels, such as a substituted benzene ring, an intermediate chain with amide linker, as well as a hydrophilic terminal. In addition, we have previously observed *in vivo* analgesic effects of AMTB that appeared to be mediated via inhibition of Na_V channels rather than TRPM8 (unpublished data). Accordingly, findings from our study indicate that AMTB (pIC_{50} ~ 4.83 – 5.69 at Na_Vs) is a more potent inhibitor of Na_Vs (Na_V1.1-1.8) than lidocaine (IC_{50} = 210 μM / pIC50 ~ 3.67) (59). Further studies would be required to confirm inhibition of Na_V isoforms using electrophysiological approaches, and to assess whether AMTB also binds to the local anesthetic site on Na_V channels.

There was no obvious relationship between the expression profile of Na_V isoforms and common breast cancer subtype markers (e.g. estrogen receptor and HER2 overexpression). However, the basal breast cancer cell lines HCC1569 and MDA-MB-231 showed high mRNA levels for Na_V1.5

compared to the luminal breast cancer cell lines assessed. Studies assessing protein levels of Na$_V$ channels in breast cancer cell lines would enhance our understanding of potential differences between breast cancer cell lines. Na$_V$s are normally found in excitable tissues (60) but have also been reported in cancer cells (61). Altered expression of Na$_V$s have been described in prostate (62), breast (43), lung (63), cervical (64), colon (65) and ovarian cancers (66). In many of these cancers, altered mRNA and/or protein expression correlates with metastatic /invasive potential (67). For example, Na$_V$1.8 is highly expressed in several prostate cancer cell lines (DU-145, PC-3 and PC-3M) and its expression correlates with more advanced pathologic stages of the disease (62) In breast cancer, the upregulation of Na$_V$1.5 and the neonatal splice variant of Na$_V$1.5 (nNa$_V$1.5) is linked to metastatic potential (43, 68). Indeed, the highly metastatic MDA-MB-231 breast cancer cell line (38, 39) has been reported to have greater levels of Na$_V$1.5 (~1,800 times higher) compared to the weakly metastatic MCF-7 cell line, which was consistent with our data (41, 69, 70). Moreover, Na$_V$1.5 levels appear lower in non-tumourigenic cells (41, 69, 70) and increased expression of Na$_V$1.5 mRNA is a feature of some breast tumours (71).

Conclusion

These studies provide evidence that some of the effects of AMTB are not mediated by TRPM8 but instead occur via inhibition of Na$_V$s. Despite a possible association between TRPM8 levels in basal breast cancers, the absence of TRPM8 in commonly used breast cancer cell lines may represent challenges in defining the applicability of TRPM8 as a therapeutic target in this breast cancer subtype.

Acknowledgments

Conflicts of Interest

The authors declare that there are no conflicts of interest.

References

1. Monteith GR, Prevarskaya N, Roberts-Thomson SJ. The calcium-cancer signalling nexus. Nat Rev Cancer. 2017;17(6):367-80.

2. Prevarskaya N, Ouadid-Ahidouch H, Skryma R, Shuba Y. Remodelling of Ca2+ transport in cancer: how it contributes to cancer hallmarks? Philos Trans R Soc Lond B Biol Sci. 2014;369(1638):20130097.

3. Jiang HN, Zeng B, Zhang Y, Daskoulidou N, Fan H, Qu JM, et al. Involvement of TRPC channels in lung cancer cell differentiation and the correlation analysis in human non-small cell lung cancer. PLoS ONE. 2013;8(6):e67637.

4. Prevarskaya N, Skryma R, Bidaux G, Flourakis M, Shuba Y. Ion channels in death and differentiation of prostate cancer cells. Cell Death Differ. 2007;14(7):1295-304.

5. Deliot N, Constantin B. Plasma membrane calcium channels in cancer: Alterations and consequences for cell proliferation and migration. Biochim Biophys Acta. 2015;1848(10 Pt B):2512-22.

6. Aydar E, Yeo S, Djamgoz M, Palmer C. Abnormal expression, localization and interaction of canonical transient receptor potential ion channels in human breast cancer cell lines and tissues: a potential target for breast cancer diagnosis and therapy. Cancer Cell Int. 2009;9:23.

7. Azimi I, Roberts-Thomson SJ, Monteith GR. Calcium influx pathways in breast cancer: opportunities for pharmacological intervention. Br J Pharmacol. 2014;171(4):945-60.

8. Peters AA, Simpson PT, Bassett JJ, Lee JM, Da Silva L, Reid LE, et al. Calcium channel TRPV6 as a potential therapeutic target in estrogen receptor-negative breast cancer. Mol Cancer Ther. 2012;11(10):2158-68.

9. Bertucci F, Finetti P, Birnbaum D. Basal breast cancer: a complex and deadly molecular subtype. Curr Mol Med. 2012;12(1):96-110.

10. Perou CM. Molecular stratification of triple-negative breast cancers. Oncologist. 2010;15 Suppl 5(suppl 5):39-48.

11. Madrid R, Pertusa M. Intimacies and physiological role of the polymodal cold-sensitive ion channel TRPM8. Curr Top Membr. 2014;74:293-324.

12. Millqvist E. TRPV1 and TRPM8 in Treatment of Chronic Cough. Pharmaceuticals (Basel). 2016;9(3).

13. Dussor G, Cao YQ. TRPM8 and Migraine. Headache. 2016;56(9):1406-17.

14. Vetter I, Kym PR, Szallasi A. Feeling hot, feeling cold: TRP channels-a great story unfolds. Temperature (Austin). 2015;2(2):150-1.

15.   Tsavaler L, Shapero MH, Morkowski S, Laus R. Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. Cancer Res. 2001;61(9):3760-9.

16.   Bidaux G, Borowiec AS, Dubois C, Delcourt P, Schulz C, Vanden Abeele F, et al. Targeting of short TRPM8 isoforms induces 4TM-TRPM8-dependent apoptosis in prostate cancer cells. Oncotarget. 2016;7(20):29063-80.

17.   Zhang L, Barritt GJ. Evidence that TRPM8 is an androgen-dependent Ca2+ channel required for the survival of prostate cancer cells. Cancer Res. 2004;64(22):8365-73.

18.   Perez de Vega MJ, Gomez-Monterrey I, Ferrer-Montiel A, Gonzalez-Muniz R. Transient Receptor Potential Melastatin 8 Channel (TRPM8) Modulation: Cool Entryway for Treating Pain and Cancer. J Med Chem. 2016;59(22):10006-29.

19.   Prevarskaya N, Zhang L, Barritt G. TRP channels in cancer. Biochim Biophys Acta. 2007;1772(8):937-46.

20.   Peng M, Wang Z, Yang Z, Tao L, Liu Q, Yi LU, et al. Overexpression of short TRPM8 variant alpha promotes cell migration and invasion, and decreases starvation-induced apoptosis in prostate cancer LNCaP cells. Oncol Lett. 2015;10(3):1378-84.

21.   Chodon D, Guilbert A, Dhennin-Duthille I, Gautier M, Telliez MS, Sevestre H, et al. Estrogen regulation of TRPM8 expression in breast cancer cells. BMC Cancer. 2010;10(1):212.

22.   Liu J, Chen Y, Shuai S, Ding D, Li R, Luo R. TRPM8 promotes aggressiveness of breast cancer cells by regulating EMT via activating AKT/GSK-3beta pathway. Tumour Biol. 2014;35(9):8969-77.

23.   Lashinger ES, Steiginga MS, Hieble JP, Leon LA, Gardner SD, Nagilla R, et al. AMTB, a TRPM8 channel blocker: evidence in rats for activity in overactive bladder and painful bladder syndrome. Am J Physiol Renal Physiol. 2008;295(3):F803-10.

24.   Hosoya T, Matsumoto K, Tashima K, Nakamura H, Fujino H, Murayama T, et al. TRPM8 has a key role in experimental colitis-induced visceral hyperalgesia in mice. Neurogastroenterol Motil. 2014;26(8):1112-21.

25.   Valero ML, Mello de Queiroz F, Stuhmer W, Viana F, Pardo LA. TRPM8 ion channels differentially modulate proliferation and cell cycle distribution of normal and cancer prostate cells. PLoS ONE. 2012;7(12):e51825.

26.   Klumpp D, Frank SC, Klumpp L, Sezgin EC, Eckert M, Edalat L, et al. TRPM8 is required for survival and radioresistance of glioblastoma cells. Oncotarget. 2017;8(56):95896-913.

27.   Gkika D, Lemonnier L, Shapovalov G, Gordienko D, Poux C, Bernardini M, et al. TRP channel-associated factors are a novel protein family that regulates TRPM8 trafficking and activity. J Cell Biol. 2015;208(1):89-107.

28.   Wang Y, Wang X, Yang Z, Zhu G, Chen D, Meng Z. Menthol inhibits the proliferation and motility of prostate cancer DU145 cells. Pathol Oncol Res. 2012;18(4):903-10.

29.   Gkika D, Flourakis M, Lemonnier L, Prevarskaya N. PSA reduces prostate cancer cell motility by stimulating TRPM8 activity and plasma membrane expression. Oncogene. 2010;29(32):4611-6.

30.   Yang ZH, Wang XH, Wang HP, Hu LQ. Effects of TRPM8 on the proliferation and motility of prostate cancer PC-3 cells. Asian J Androl. 2009;11(2):157-65.

31.   Genova T, Grolez GP, Camillo C, Bernardini M, Bokhobza A, Richard E, et al. TRPM8 inhibits endothelial cell migration via a non-channel function by trapping the small GTPase Rap1. J Cell Biol. 2017;216(7):2107-30.

32.   Zhu G, Wang X, Yang Z, Cao H, Meng Z, Wang Y, et al. Effects of TRPM8 on the proliferation and angiogenesis of prostate cancer PC-3 cells in vivo. Oncol Lett. 2011;2(6):1213-7.

33.   Cancer Genome Atlas N. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490(7418):61-70.

34.   Azimi I, Beilby H, Davis FM, Marcial DL, Kenny PA, Thompson EW, et al. Altered purinergic receptor-Ca2+ signaling associated with hypoxia-induced epithelial-mesenchymal transition in breast cancer cells. Mol Oncol. 2016;10(1):166-78.

35.   Pera E, Kaemmerer E, Milevskiy MJ, Yapa KT, O'Donnell JS, Brown MA, et al. The voltage gated Ca2+-channel Cav3.2 and therapeutic responses in breast cancer. Cancer Cell Int. 2016;16:24.

36.   Peters AA, Jamaludin SYN, Yapa K, Chalmers S, Wiegmans AP, Lim HF, et al. Oncosis and apoptosis induction by activation of an overexpressed ion channel in breast cancer cells. Oncogene. 2017.

37.   Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.

38.   Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell. 2006;10(6):515-27.

39.   ATCC. American Type Cell Culture Product Information [Internet]. ATCC; 2012 [Accesed on 4th August 2013]. Available from: https://www.atcc.org/.

40.   Deuis JR, Zimmermann K, Romanovsky AA, Possani LD, Cabot PJ, Lewis RJ, et al. An animal model of oxaliplatin-induced cold allodynia reveals a crucial role for Nav1.6 in peripheral pain pathways. Pain. 2013;154(9):1749-57.

41.   Brackenbury WJ, Chioni AM, Diss JK, Djamgoz MB. The neonatal splice variant of Nav1.5 potentiates in vitro invasive behaviour of MDA-MB-231 human breast cancer cells. Breast Cancer Res Treat. 2007;101(2):149-60.

42.   Driffort V, Gillet L, Bon E, Marionneau-Lambot S, Oullier T, Joulin V, et al. Ranolazine inhibits Nav1.5-mediated breast cancer cell invasiveness and lung colonization. Mol Cancer. 2014;13:264.

43.   Fraser SP, Diss JK, Chioni AM, Mycielska ME, Pan H, Yamaci RF, et al. Voltage-gated sodium channel expression and potentiation of human breast cancer metastasis. Clin Cancer Res. 2005;11(15):5381-9.

44.   Naziroglu M, Blum W, Josvay K, Cig B, Henzi T, Olah Z, et al. Menthol evokes Ca(2+) signals and induces oxidative stress independently of the presence of TRPM8 (menthol) receptor in cancer cells. Redox Biol. 2018;14:439-49.

45.   Mahieu F, Owsianik G, Verbert L, Janssens A, De Smedt H, Nilius B, et al. TRPM8-independent menthol-induced Ca2+ release from endoplasmic reticulum and Golgi. J Biol Chem. 2007;282(5):3325-36.

46.   Dhennin-Duthille I, Gautier M, Faouzi M, Guilbert A, Brevet M, Vaudry D, et al. High expression of transient receptor potential channels in human breast cancer epithelial cells and tissues: correlation with pathological parameters. Cell Physiol Biochem. 2011;28(5):813-22.

47.   Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406(6797):747-52.

48.   Sørlie T. Molecular portraits of breast cancer: tumour subtypes as distinct disease entities. Eur J Cancer. 2004;40(18):2667-75.

49.   Prat A, Adamo B, Cheang MC, Anders CK, Carey LA, Perou CM. Molecular characterization of basal-like and non-basal-like triple-negative breast cancer. Oncologist. 2013;18(2):123-33.

50.   Shahdadfar A, Fronsdal K, Haug T, Reinholt FP, Brinchmann JE. In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. Stem Cells. 2005;23(9):1357-66.

51.   Luca AC, Mersch S, Deenen R, Schmidt S, Messner I, Schafer KL, et al. Impact of the 3D microenvironment on phenotype, gene expression, and EGFR inhibition of colorectal cancer cell lines. PLoS ONE. 2013;8(3):e59689.

52.   Price KJ, Tsykin A, Giles KM, Sladic RT, Epis MR, Ganss R, et al. Matrigel basement membrane matrix influences expression of microRNAs in cancer cell lines. Biochem Biophys Res Commun. 2012;427(2):343-8.

53.   Kiss DL, Windus LC, Avery VM. Chemokine receptor expression on integrin-mediated stellate projections of prostate cancer cells in 3D culture. Cytokine. 2013;64(1):122-30.

54.   Edmondson R, Broglie JJ, Adcock AF, Yang L. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. Assay Drug Dev Technol. 2014;12(4):207-18.

55.   Bidaux G, Flourakis M, Thebault S, Zholos A, Beck B, Gkika D, et al. Prostate cell differentiation status determines transient receptor potential melastatin member 8 channel subcellular localization and function. J Clin Invest. 2007;117(6):1647-57.

56.   Bidaux G, Roudbaraki M, Merle C, Crepin A, Delcourt P, Slomianny C, et al. Evidence for specific TRPM8 expression in human prostate secretory epithelial cells: functional androgen receptor requirement. Endocr Relat Cancer. 2005;12(2):367-82.

57.   Lundbaek JA, Birn P, Tape SE, Toombes GE, Sogaard R, Koeppe RE, 2nd, et al. Capsaicin regulates voltage-dependent sodium channels by altering lipid bilayer elasticity. Mol Pharmacol. 2005;68(3):680-9.

58.   Yang R, Xiong Z, Liu C, Liu L. Inhibitory effects of capsaicin on voltage-gated potassium channels by TRPV1-independent pathway. Cell Mol Neurobiol. 2014;34(4):565-76.

59.   Scholz A, Kuboyama N, Hempelmann G, Vogel W. Complex blockade of TTX-resistant Na+ currents by lidocaine and bupivacaine reduce firing frequency in DRG neurons. J Neurophysiol. 1998;79(4):1746-54.

60.   Voltage-gated sodium channels, introduction. IUPHAR/BPS Guide to PHARMACOLOGY. [Internet]. [cited 24/12/2015]. Available from: http://www.guidetopharmacology.org/GRAC/FamilyIntroductionForward?familyId=82.

61.   Roger S, Potier M, Vandier C, Besson P, Le Guennec JY. Voltage-gated sodium channels: new targets in cancer therapy? Curr Pharm Des. 2006;12(28):3681-95.

62.   Suy S, Hansen TP, Auto HD, Kallakury BV, Dailey V, Danner M, et al. Expression of Voltage-Gated Sodium Channel Nav1.8 in Human Prostate Cancer is Associated with High Histological Grade. J Clin Exp Oncol. 2012;1(2).

63.   Roger S, Rollin J, Barascu A, Besson P, Raynal PI, Iochmann S, et al. Voltage-gated sodium channels potentiate the invasive capacities of human non-small-cell lung cancer cell lines. Int J Biochem Cell Biol. 2007;39(4):774-86.

64.   Diaz D, Delgadillo DM, Hernandez-Gallegos E, Ramirez-Dominguez ME, Hinojosa LM, Ortiz CS, et al. Functional expression of voltage-gated sodium channels in primary cultures of human cervical cancer. J Cell Physiol. 2007;210(2):469-78.

65.	House CD, Vaske CJ, Schwartz AM, Obias V, Frank B, Luu T, et al. Voltage-gated Na+ channel SCN5A is a key regulator of a gene transcriptional network that controls colon cancer invasion. Cancer Res. 2010;70(17):6957-67.

66.	Gao R, Shen Y, Cai J, Lei M, Wang Z. Expression of voltage-gated sodium channel alpha subunit in human ovarian cancer. Oncol Rep. 2010;23(5):1293-9.

67.	Brackenbury WJ. Voltage-gated sodium channels and metastatic disease. Channels (Austin). 2012;6(5):352-61.

68.	Roger S, Besson P, Le Guennec JY. Involvement of a novel fast inward sodium current in the invasion capacity of a breast cancer cell line. Biochim Biophys Acta. 2003;1616(2):107-11.

69.	Gao R, Wang J, Shen Y, Lei M, Wang Z. Functional expression of voltage-gated sodium channels Nav1. 5 in human breast caner cell line MDA-MB-231. Journal of Huazhong University of Science and Technology [Medical Sciences]. 2009;29:64-7.

70.	Brisson L, Driffort V, Benoist L, Poet M, Counillon L, Antelmi E, et al. NaV1.5 Na(+) channels allosterically regulate the NHE-1 exchanger and promote the activity of breast cancer cell invadopodia. J Cell Sci. 2013;126(Pt 21):4835-42.

71.	Yang M, Kozminski DJ, Wold LA, Modak R, Calhoun JD, Isom LL, et al. Therapeutic potential for phenytoin: targeting Na(v)1.5 sodium channels to reduce migration and invasion in metastatic breast cancer. Breast Cancer Res Treat. 2012;134(2):603-15.