



Triple-negative breast cancer cell line sensitivity to englerin A identifies a new, targetable subtype

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Received: 11 March 2019 / Accepted: 15 June 2019 / Published online: 22 June 2019
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Abstract

Purpose Triple-negative breast cancers (TNBCs) represent a heterogeneous group of tumors. The lack of targeted therapies combined with the inherently aggressive nature of TNBCs results in a higher relapse rate and poorer overall survival. We evaluated the heterogeneity of TNBC cell lines for TRPC channel expression and sensitivity to cation-disrupting drugs.

Methods The TRPC1/4/5 agonist englerin A was used to identify a group of TNBC cell lines sensitive to TRPC1/4/5 activation and intracellular cation disruption. Quantitative RT-PCR, the sulforhodamine B assay, pharmacological inhibition, and siRNA-mediated knockdown approaches were employed. Epifluorescence imaging was performed to measure intracellular Ca^{2+} and Na^{+} levels. Mitochondrial membrane potential changes were monitored by confocal imaging.

Results BT-549 and Hs578T cells express high levels of TRPC4 and TRPC1/4, respectively, and are exquisitely, 2000- and 430-fold, more sensitive to englerin A than other TNBC cell lines. While englerin A caused a slow Na^{+} and nominal Ca^{2+} accumulation in Hs578T cells, it elicited rapid increases in cytosolic Ca^{2+} levels that triggered mitochondrial depolarization in BT-549 cells. Interestingly, BT-549 and Hs578T cells were also more sensitive to digoxin as compared to other TNBC cell lines. Collectively, these data reveal TRPC1/4 channels as potential biomarkers of TNBC cell lines with dysfunctional mechanisms of cation homeostasis and therefore sensitivity to cardiac glycosides.

Conclusions The sensitivity of BT-549 and Hs578T cells to englerin A and digoxin suggests a subset of TNBCs are highly susceptible to cation disruption and encourages investigation of TRPC1 and TRPC4 as potential new biomarkers of sensitivity to cardiac glycosides.

Keywords Triple-negative breast cancer · Englerin A · TRPC1/4/5 · Digoxin · Cation disruption

Background

Triple-negative breast cancers (TNBCs) represent 15–20% of all breast cancers and are defined by the lack of estrogen receptor (ER), progesterone receptor (PR), and human

epidermal growth factor receptor (HER2) amplification. While targeted therapies have improved patient outcomes for receptor-positive breast cancers, there are no targeted therapies for TNBCs. These cancers are currently treated with cytotoxic chemotherapy and unfortunately, these therapies provide only 23–55% relapse-free 10-year survival, highlighting the need for new treatment options [1].

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10549-019-05324-7>) contains supplementary material, which is available to authorized users.

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A major challenge with TNBC is tumor heterogeneity [2, 3]. Efforts have been undertaken to identify subtypes of TNBC and targeted therapies for these subtypes. Success in this approach includes ongoing clinical trials of androgen receptor antagonists bicalutamide or enzalutamide in androgen receptor expressing TNBCs [4]. Our goal is to identify new molecular liabilities of TNBC subtypes by evaluating natural products in a panel of diverse TNBC cell lines [5]. Compounds with selective cytotoxic activity against a subgroup of these cells are investigated further to identify molecular liabilities. Multiple compounds with selective activities against TNBC cell lines have been identified and their molecular targets defined [6–8].

We investigate natural products from higher plants and fungi because nature provides a rich source of bioactive compounds. Analyses show that 55% of small molecule anticancer agents approved by the FDA from 1981–2014 were natural product-derived [9]. Englerin A (EA) is a sesquiterpene isolated from the Tanzanian plant *Phyllanthus engleri* that demonstrated potent and selective effects on renal cell carcinoma cell lines in the NCI-60 panel [10]. Studies with EA implicated multiple mechanisms of action in renal carcinoma cells, including activation of canonical transient receptor potential cation channels (TRPC1/4/5) and PKC θ activation [11, 12]. Effects of TRPC1/4/5 activation by EA appear to be dictated by the homomeric (TRPC4/4) and heteromeric (TRPC1/4) channel composition, in that TRPC4 homomer activation causes primarily Ca²⁺ influx while TRPC1/4 heteromer activation initiates a greater Na⁺ influx [13–16]. EA has also been evaluated in Ewing sarcoma cell lines, where it increased intracellular Ca²⁺ and inhibited EWS-FLI1-driven transcription [17]. The NCI-60 data showed differential antiproliferative and cytotoxic activity across 6 breast cancer cell lines [10], but EA had not been evaluated extensively in TNBC cell lines. Based on the ability of EA to activate TRPC1/4/5 channels in renal and Ewing sarcoma cancer cells, we investigated TRPC1/4/5 expression in TNBC cells and their sensitivity to EA. The results show that BT-549 cells have high TRPC4 expression and that Hs578T cells have high TRPC1 and TRPC4 expression, and both cell lines are exquisitely sensitive to EA as compared to other TNBC cells. The underlying molecular liabilities that confer sensitivity to EA were determined to identify a new molecular subgroup of TNBC that could be highly susceptible to cation-disrupting drugs.

Materials and methods

Chemicals and reagents

Englerin A was isolated from the plant *Phyllanthus engleri* [10]. Pico145 was generously provided by Dr. David Beech of the University of Leeds [18]. Digoxin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The TNBC cell lines, MDA-MB-453, Hs578T, MDA-MB-231, HCC1806, and HCC1937 were purchased from the American Type Culture Collection (Manassas, VA, USA). CAL-51 cells were purchased from Creative Bioarray (Shirley, NY, USA). BT-549 cells were obtained from the Lombardi Cancer Center of Georgetown University (Washington, DC, USA). A-498 renal cell carcinoma cells were provided by Dr. Brian Reeves (University of Texas Health Science Center at San Antonio). Cell line identity was confirmed by DNA short tandem repeat analyses by Genetica DNA Laboratories (Burlington, NC, USA). BT-549, CAL-51, HCC1937, and HCC1806 cells were grown in RPMI-1640 supplemented with 10% FBS and 50 μ g/mL gentamicin. MDA-MB-231 and MDA-MB-453 cells were grown in IMEM supplemented with 10% FBS and 25 μ g/mL gentamicin. Hs578T cells were grown in DMEM supplemented with 10% FBS and 50 μ g/mL gentamicin. A-498 cells were grown in DMEM without sodium pyruvate and supplemented with 10% heat inactivated FBS and 50 μ g/mL gentamicin. All cells were maintained at 37 °C with 5% CO₂ and used within 40 passages after revival from liquid nitrogen.

Sulforhodamine B assay

The antiproliferative and cytotoxic effects of indicated compounds were evaluated after 24 or 48 h treatments using the sulforhodamine B (SRB) assay [19, 20] as previously described [21]. The IC₅₀ is defined as the concentration that causes a 50% reduction in absorbance as compared to vehicle (DMSO) control by nonlinear regression in Prism 6.01 (GraphPad Software, La Jolla, CA, USA).

RNA isolation, cDNA preparation, and qRT-PCR

RNA was isolated and concentration and purity assessed using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). A high capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) was used to make cDNA and qRT-PCR completed with SYBR[®] Green PCR Master Mix (Applied

Biosystems). Primer pair sequences for TRPC1, TRPC4, TRPC5 [22], and GAPDH are provided (Supplementary Table S1).

Gene knockdown

Two siRNAs targeting each TRPC1 (SASI_Hs02_00335399 and SASI_Hs01_00060664) and TRPC4 (SASI_Hs01_00129366 and SASI_Hs01_00129367) (Sigma-Aldrich, St Louis, MO, USA) were transfected into BT-549 and Hs578T cells for 48 h at a final concentration of 9.4 nM TRPC1 siRNA, 18.8 nM TRPC4 siRNA and 0.025% lipofectamine RNAi-MAX (ThermoFisher Scientific). Mock transfections contained only lipofectamine RNAi-MAX. Following transfection, cells were treated with EA for 24 h, or RNA isolated to determine knock-down efficiency at the time of EA addition.

Intracellular cation imaging

BT-549, Hs578T, and HCC1806 cells were plated on glass coverslips, grown to 60–80% confluency and loaded with 2.5 μM Cal-520 AM (ATT Bioquest, Sunnyvale, CA, USA) or 5 μM CoroNA Green AM (ThermoFisher Scientific) for imaging of intracellular Ca^{2+} and Na^+ , respectively. Cell culture media was supplemented with 0.2% Pluronic F-127 (Biotium, Fremont, CA, USA) to facilitate dye loading. Cells were incubated with dye solution for 30 min at 37 °C, washed with Hank's Balanced Salt Solution (HBSS) and incubated at room temperature for 15 min in the dark. Dye-loaded cells were stored at 37 °C if imaged later than 1 h after dye loading. Unless otherwise indicated, all cells were imaged in standard HBSS with 1.8 mM Ca^{2+} within 4 h of dye loading. To investigate TRPC1/4/5 activity, cells were pretreated, after dye loading, for 5 min with 10 nM Pico145. Imaging was performed with a Nikon Eclipse TE2000-U inverted microscope with a 20 \times objective. Images were acquired with an Andor iXon camera and evaluated using Metamorph software. Fluorescent intensity was analyzed cell-by-cell using ImageJ software with personnel blinded to experimental conditions. Fluorescence intensity data are represented as corrected total cell fluorescence (CTCF) = Integrated density – (cell area \times mean background fluorescence).

Simultaneous measurements of cytosolic Ca^{2+} and mitochondrial membrane potential ($\Delta\Psi\text{m}$)

BT-549 and HCC1806 cells were grown on collagen-coated glass coverslips. Cells were loaded with Fluo-4 AM

and TMRE (tetramethylrhodamine, ethyl ester) for 20 min to measure intracellular Ca^{2+} and mitochondrial membrane potential, respectively. Time-lapse imaging was performed using a Leica SP8 DMi8 confocal S with a 63 \times objective in an environmental chamber. Cells were left untreated or treated with 200 nM EA after a 60 s baseline recording. Cytosolic Ca^{2+} and $\Delta\Psi\text{m}$ changes were recorded and analyzed using Leica Application Suite X.

Statistical analysis

qRT-PCR data are reported as fold difference compared to MDA-MB-231 cells, and statistical significance was determined using a one-way ANOVA with Dunnett's multiple comparisons test. For intracellular ion sensing in BT-549 cells with Cal-520 and CoroNa green, statistical significance was determined by two-way ANOVA with a Dunnett's or Bonferroni's multiple comparisons test. For CoroNa green fluorescence in Hs578T cells, an unpaired *t* test was sufficient to determine significance. An unpaired *t* test was used to determine the significance of Ca^{2+} and $\Delta\Psi\text{m}$ changes between non-treated and EA-treated conditions for each cell line. A one-way ANOVA with Dunnett's multiple comparisons evaluation was used to test for significant differences in sensitivity of BT-549 cells to digoxin as compared to other TNBC cell lines. All error bars represent the mean \pm SEM. All statistical analyses were performed using GraphPad Prism 6.01.

Results

mRNA Expression of TRPC1/4/5 in TNBC cell lines

Because EA has demonstrated an ability to activate TRPC1/4/5 channels in some cell types [13–15, 23], the relative expression of TRPC1, TRPC4, and TRPC5 mRNA was evaluated in 4 TNBC cell lines: MDA-MB-231, HCC1806, BT-549, and Hs578T. The results show that Hs578T cells express 4- and 110-fold higher levels of TRPC1 and TRPC4, respectively, than MDA-MB-231 cells (Fig. 1a, b). BT-549 cells express 290-times more TRPC4 than MDA-MB-231 cells, but equivalent levels of TRPC1 (Fig. 1a, b). Expression levels of TRPC4 and TRPC5 in HCC1806 cells are 81- and 20-fold higher than in MDA-MB-231 cells (Fig. 1b, c). The sensitivity of these cells to EA-mediated cytotoxicity was then evaluated and the results show that BT-549 and Hs578T cells are exquisitely sensitive to EA, with IC_{50} values of 5.4 and 16 nM, respectively. MDA-MB-231 and HCC1806 cells are resistant to EA with IC_{50} values of 11 and 8.9 μM , respectively (Fig. 1d). Evaluation of three additional TNBC cell lines shows that BT-549 and Hs578T

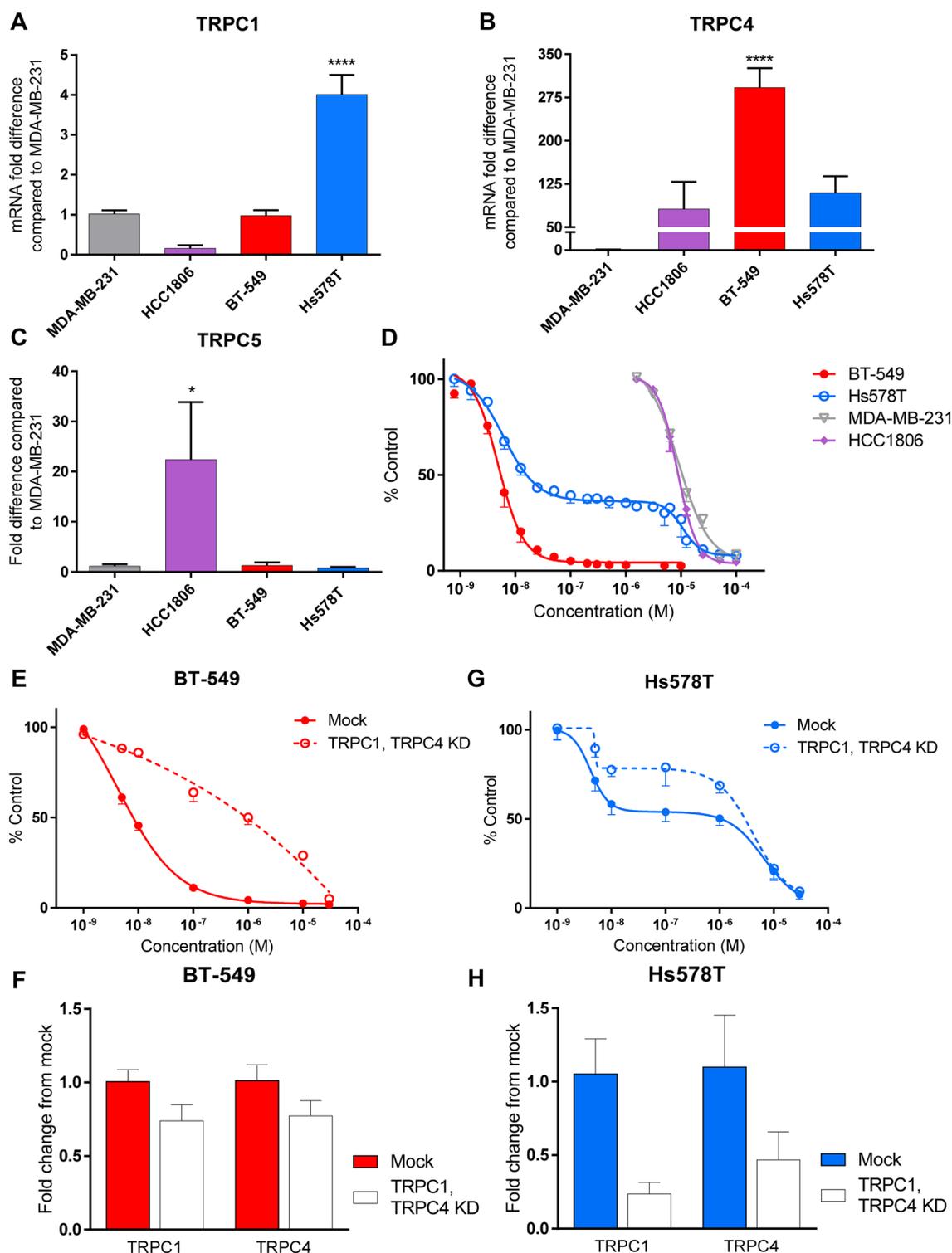


Fig. 1 TRPC1/4 expression correlates with TNBC cell line sensitivity to EA. **a** TRPC1 **b** TRPC4 and **c** TRPC5 expression was determined by qRT-PCR analysis of MDA-MB-231, HCC1806, BT-549, and Hs578T cells. Fold difference was analyzed as compared to MDA-MB-231 expression levels. Significance determined by one-way ANOVA with Dunnett's multiple comparisons test * $p=0.02$, **** $p<0.0001$ **d** The effects of EA on BT-549, Hs578T, MDA-MB-231, and HCC1806 cell survival were measured by the SRB

assay after 48 h treatment ($n=3-15$). The effects of reduced TRPC1 and TRPC4 expression on EA-mediated cytotoxicity were assessed by SRB assay following 48 h siRNA-mediated knockdown of TRPC1 and TRPC4 and 24 h EA treatment in **e** BT-549 and **g** Hs578T cells ($n=3$). The efficiency of TRPC1 and TRPC4 knockdown was determined by qRT-PCR analysis of **f** BT-549 and **h** Hs578T cells compared to the expression in mock transfected cells ($n=3$)

Table 1 IC₅₀ values of EA in TNBC cell lines

Cell line	IC ₅₀ (nM) ± SEM
BT-549	5.4 ± 1.3
Hs578T	16 ± 1.8
CAL-51	6900 ± 2000
MDA-MB-231	11,000 ± 1300
MDA-MB-453	8600 ± 1500
HCC1937	11,000 ± 1900
HCC1806	8900 ± 910

cells are 430- to 2000-fold more sensitive to EA-mediated cytotoxicity than the other TNBC cell lines (Table 1). Interestingly, the EA concentration response curve in Hs578T cells is biphasic with the first phase closely aligned with BT-549 cells, consistent with a shared mechanism of action. However, the distinct plateau and second phase of the EA concentration response curve in Hs578T cells that aligns with the resistant TNBC cell lines suggests mechanistic differences between these two sensitive cell lines.

The effects of siRNA-mediated TRPC1/4 depletion on EA sensitivity in BT-549 and Hs578T cells were evaluated. Even a small reduction in TRPC1 and TRPC4 mRNA expression by 26% and 23%, respectively, in BT-549 cells decreased the potency of EA by 115-fold compared to mock controls, demonstrating the critical nature of TRPC1/4 channels to EA sensitivity in these cells (Fig. 1e, f). In Hs578T cells, an siRNA-mediated 76% decrease in TRPC1 and 53% decrease in TRPC4 expression caused a 2.6-fold decrease in sensitivity to EA (Fig. 1g, h). Taken together, these data suggest TRPC1/4 channel expression mediates cell line sensitivity to EA, albeit with differences between cell lines.

TRPC1/4/5 inhibition decreases the potency of EA in BT-549 and Hs578T cells

Pico145, a selective inhibitor of TRPC1/4/5 channels [18], was used to determine if the cytotoxic effects of EA are due to TRPC1/4/5 activation in sensitive TNBC cell lines. Pico145 reduced the potency of EA in BT-549 and Hs578T cell lines as evidenced by a 98- and 69-fold increase in the IC₅₀, respectively (Fig. 2a, Supplementary Fig. S1a) demonstrating the importance of TRPC1/4/5 activity to the sensitivity of these cells to EA. In contrast, Pico145 did not decrease the potency of EA in MDA-MB-231 cells (Supplementary Fig. S1b).

TRPC channels are nonselective, tetrameric, cation channels composed of homomers or heteromers of 2 TRPCs [24–26]. The effects of EA on cytosolic levels of Na⁺ and Ca²⁺ were evaluated with fluorescent sensors selective for either Na⁺ or Ca²⁺. In BT-549 cells, over the course of 5 min, 100 nM EA caused a maximum increase in intracellular Na⁺

of 2.1-fold over baseline levels and did not increase further with higher EA concentrations (Supplementary Fig. S2a, b, Supplementary Table S2). A similar response was seen in Hs578T cells (Supplementary Fig. S2c, d). Pretreatment of BT-549 cells with Pico145 blocked the EA-mediated increase in intracellular Na⁺, suggesting that it is mediated by TRPC1/4/5 channel activation. In BT-549 cells, EA initiated a rapid increase in intracellular Ca²⁺ that was more robust than the increase in Na⁺. EA increased intracellular Ca²⁺ in a concentration (3–1000 nM) and time (0–120 s)-dependent manner (Fig. 2b, Supplementary Table S3). With 3 nM EA treatment, a 3.9-fold increase in intracellular Ca²⁺ compared to baseline was detected within 120 s, while 100 nM EA caused a 7.6-fold increase in Ca²⁺ within 30 s that increased to 8.7-fold by 60 s (Fig. 2c). Treatment with 1 μM EA, caused an immediate increase in intracellular Ca²⁺, 7.2 times higher than baseline (Supplementary Fig. S3a, b, Table S3). In BT-549 cells, pretreatment with Pico145 completely attenuated the EA-induced increase in intracellular Ca²⁺ (Fig. 2d–f), reinforcing that EA-induced cation influx is due to TRPC1/4/5 activation. Importantly, in Ca²⁺-free imaging solution EA caused no detectable change in intracellular Ca²⁺, demonstrating that the EA-induced cytosolic Ca²⁺ increase is primarily due to influx from the extracellular environment, and not released from intracellular stores (Fig. 2g, h). In stark contrast to BT-549 cells, EA had no effect on intracellular Ca²⁺ levels in Hs578T cells (Supplementary Fig. S4a, b). Of note, EA also had no effect on intracellular Ca²⁺ levels in EA-resistant HCC1806 cells at concentrations up to 250 μM (Supplementary Fig. S5a, b). These data suggest that EA activates TRPC1/4/5 channels and disrupts intracellular cation levels in the cells most sensitive to the cytotoxic effects of EA.

EA causes mitochondrial depolarization in BT-549 cells

The effects of the EA-mediated increase in cytosolic Ca²⁺ on mitochondria were measured in BT-549 cells because dysregulated levels of Ca²⁺ can cause a loss of mitochondrial membrane potential ($\Delta\Psi_m$) and subsequent cell death. EA caused a rapid, within 60 s, and robust 7.3-fold increase in intracellular Ca²⁺ compared to non-treated cells that was damped to a 5.7-fold increase when measured in Ca²⁺-free imaging media (Fig. 3a, b and Supplementary Figure S6a, b). The Ca²⁺ increase in BT-549 cells led to significant mitochondrial depolarization as measured by TMRE fluorescent intensity within 10 min after EA addition (Fig. 3c, d and Supplementary Figure S6c). Consistent with the aforementioned EA-mediated increases in intracellular Ca²⁺ in BT-549 cells, these data demonstrate that EA causes Ca²⁺ entry into the cytoplasm from the extracellular environment. Interestingly, BT-549 cells had baseline unsynchronized

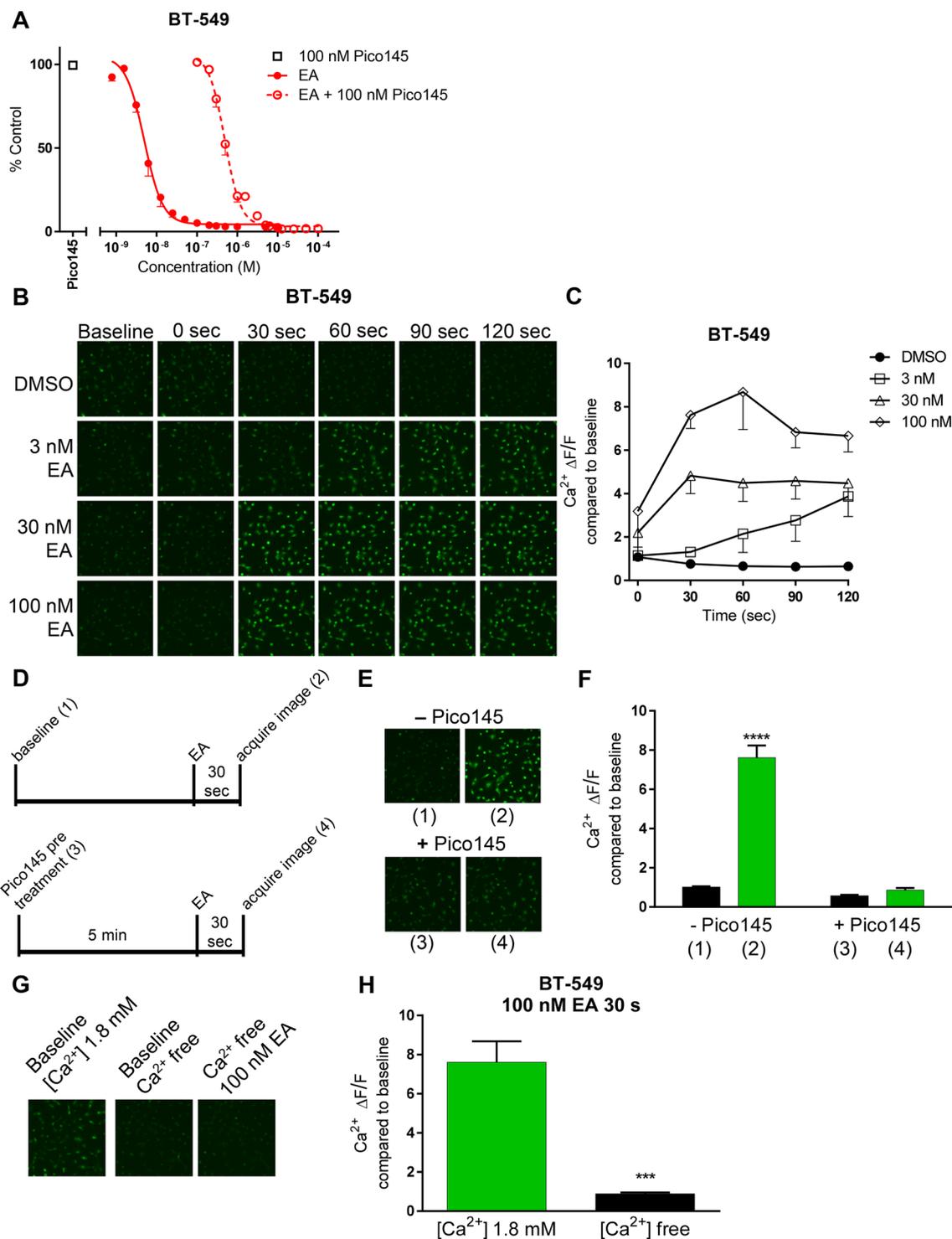


Fig. 2 Evidence that EA activity is mediated by TRPC1/4/5 channels. **a** Effects of Pico145 (100 nM) on EA concentration response in BT-549 cells were measured by SRB assay 48 h after combined treatment. EA treatment alone data are also presented in Fig. 1. 100 nM Pico145 and 100 nM Pico145 plus EA ($n=4-8$) **b** Representative images and **c** quantification of Ca²⁺ in BT-549 cells as a function of time and EA concentration measured by Cal-520 AM dye ($n=2-4$) Significance indicated in Supplementary Tables 2. $\Delta F/F = \text{CTCF}_{\text{treatment}} \div \text{CTCF}_{\text{baseline}}$ **d** Schema of evaluation of

Pico145 (10 nM) effects on EA-induced Ca²⁺ influx. Parenthetical numbers correspond to representative images in **e** and quantification in **f** of Cal-520 fluorescence ($n=3-13$). Statistical significance determined by two-way ANOVA with Bonferroni's multiple comparisons is indicated for baseline versus EA treatment \pm 10 nM Pico145, **** $p < 0.0001$ **g** Representative images and **h** quantification of 30 s 100 nM EA exposure on Ca²⁺ in BT-549 cells in normal Ca²⁺ (1.8 mM) HBSS or Ca²⁺ free HBSS ($n=3$) significance determined by unpaired *t* test, *** $p = 0.0004$

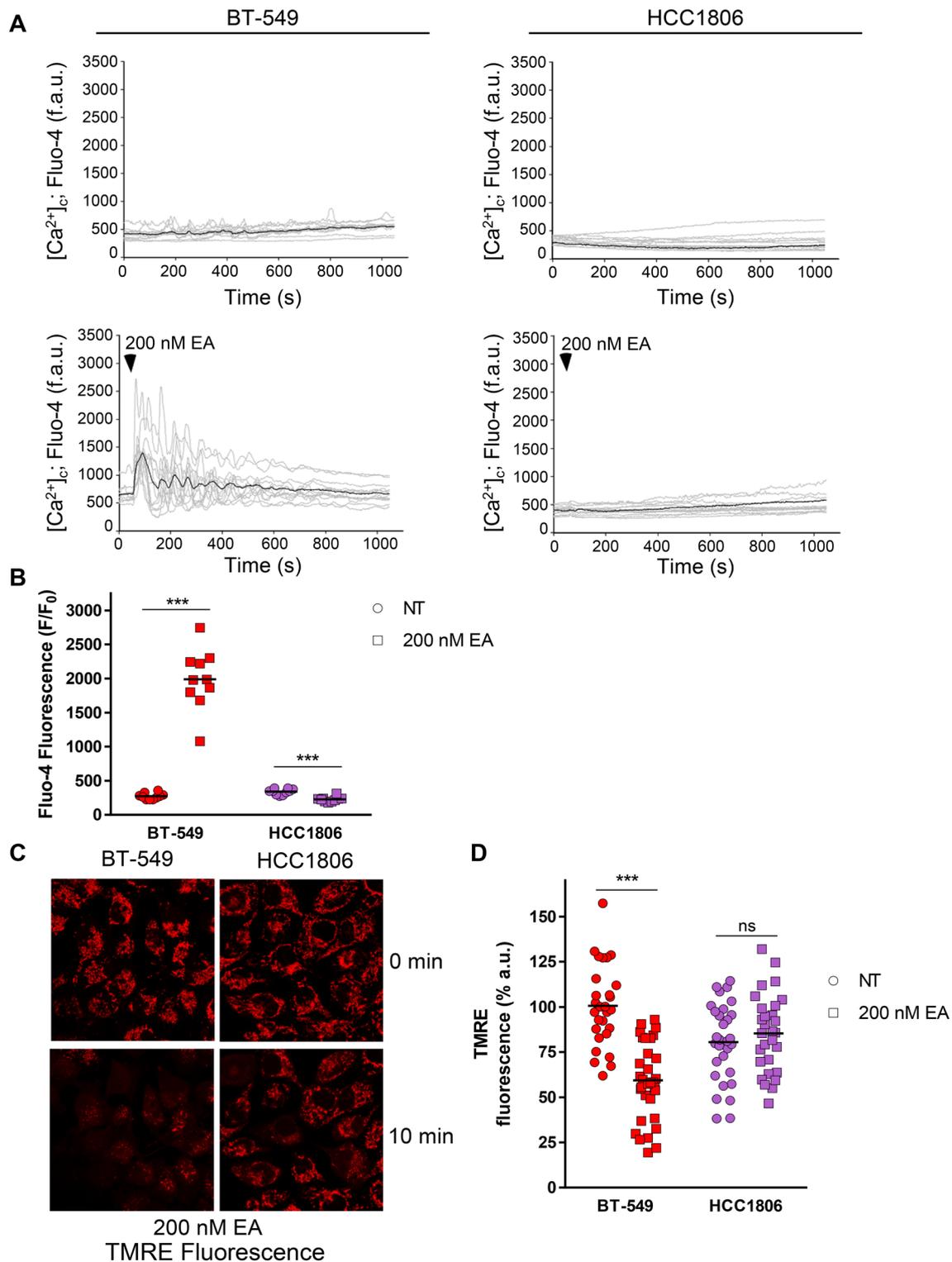


Fig. 3 EA-induced Ca^{2+} influx corresponds with mitochondrial depolarization in the presence of extracellular Ca^{2+} . **a** Ca^{2+} traces over time and **b** quantification of peak amplitude in BT-549 and HCC1806 cells in the presence of extracellular Ca^{2+} with or without 200 nM EA treatment at 60 s. Intracellular Ca^{2+} is measured by Fluo-4 AM dye ($n=4-8$) Significance determined by unpaired t test between

non-treated and EA-treated conditions in each cell line, $***p < 0.001$ **c** Representative images and **d** quantification of TMRE fluorescence at 10 min following 200 nM EA treatment of BT-549 and HCC1806 cells ($n=4-8$) Significance determined by unpaired t test between non-treated and EA-treated conditions in each cell line, $***p < 0.001$

Ca²⁺ oscillations that were maintained following EA treatment with and without extracellular Ca²⁺ (Fig. 3a and Supplementary Figure S6a). In contrast, in the resistant HCC1806 cells, EA did not increase intracellular Ca²⁺ or cause mitochondrial depolarization (Fig. 3a, b and Supplementary Figure S6a, b, c). These data show that EA-induced increases in intracellular Ca²⁺ rapidly initiate mitochondrial depolarization and this likely contributes to EA cytotoxicity in BT-549 cells.

BT-549 and Hs578T cells are also sensitive to other cation-disrupting drugs

The overall goal of our work is to identify pharmacological liabilities of subgroups of TNBCs as a mechanism to develop new treatment options for TNBC. EA is not a clinical candidate because it causes cardiotoxicity [23] and reduction of locomotor activity mediated by TRPC4/5 [27]. Based on our findings that BT-549 and Hs578T cells are highly sensitive to EA-mediated cation disruption, we considered other FDA-approved drugs that alter intracellular cation concentrations. Cardiac glycosides improve cardiac contractility by increasing intracellular Ca²⁺ through inhibition of the Na⁺/K⁺ ATPase [28]. Interestingly, Ludlow et al. [14] and Wu et al. [11] both reported that EA synergizes with cardiac glycosides in renal cancer and Ewing sarcoma cells, consistent with the compounds acting through different mechanisms. Consistent with the effects of EA, BT-549 and Hs578T cells were the most sensitive TNBC cell lines to digoxin with IC₅₀ values of 56 and 100 nM, respectively (Fig. 4a). MDA-MB-231 and HCC1937 cells were significantly less sensitive to digoxin with IC₅₀ concentrations of 300 and 520 nM, respectively (Fig. 4a). The ability of digoxin to increase intracellular Ca²⁺ was confirmed in BT-549 cells where a 4.5-fold increase in the intracellular Ca²⁺ fluorescence occurred within 6 h (Fig. 4b, c). These data suggest BT-549 and Hs578T cells represent a group of TNBCs that are particularly sensitive to multiple mechanisms of cation disruption.

Discussion

Based on previous studies, we hypothesized that some TNBC cell lines would be sensitive to EA based on TRPC1/4/5 expression. Herein, we demonstrate that cell lines with high TRPC1/4 expression are highly sensitive to EA. While BT-549 and Hs578T cells are susceptible to EA-induced cytotoxicity because of TRPC1/4 expression, they have different TRPC1/4 expression levels, EA concentration response curves, and EA-mediated cation disruptions. While regarded as nonselective, the permeability of TRPC1/4/5 channels to Ca²⁺ and Na⁺ differs depending on the tetramer

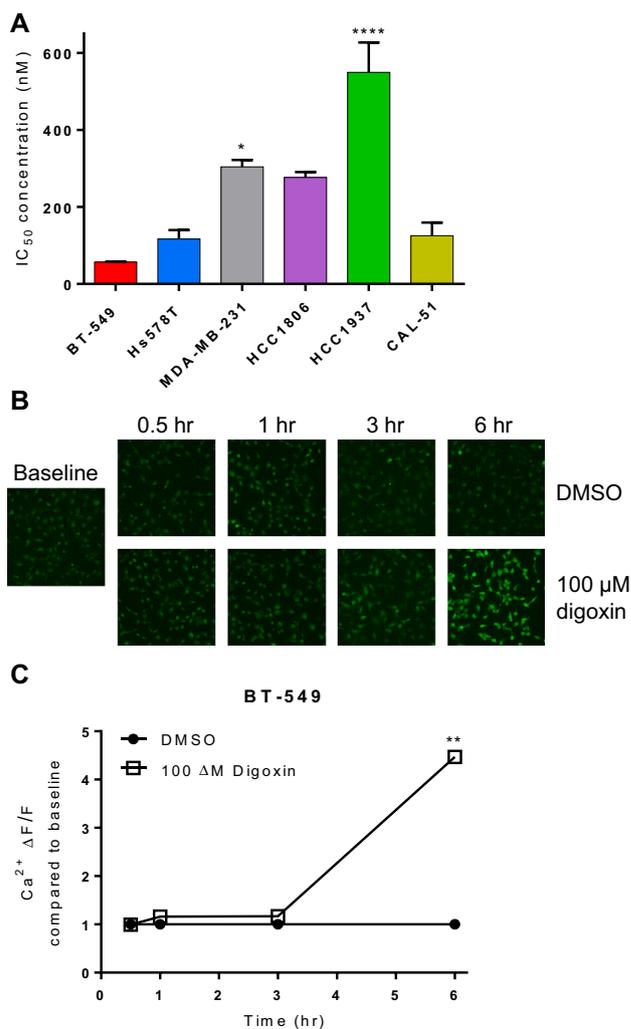


Fig. 4 Effects of digoxin on TNBC cells. **a** BT-549, Hs578T, MDA-MB-231, HCC1806, HCC1937, and CAL-51 TNBC cells were treated with digoxin for 48 h, and IC₅₀ determined by SRB assay. Significant difference of IC₅₀ in each cell line compared to BT-549 IC₅₀ determined by one-way ANOVA with Dunnett's multiple comparisons test (**p* < 0.05, ****p* < 0.001, *****p* < 0.001 (*n* = 3–5)). **b** Representative images and **c** quantification of Ca²⁺ fluorescence in BT-549 cells treated with 100 μM digoxin measured by Cal-520 AM dye. Significance between DMSO and digoxin treatment at each time point determined by two-way ANOVA with Bonferroni's multiple comparisons test (***p* < 0.0001) (*n* = 111–990 cells)

composition, for example, TRPC1/4 heteromers are less permeable to Ca²⁺ than TRPC4 homomers [16]. The differences between the effects of EA in BT-549 and Hs578T cells are likely due to the high levels of TRPC4 in BT-549 cells that form homomers that primarily conduct Ca²⁺ as compared to the high levels of TRPC1 and TRPC4 that might exist in heteromeric forms in Hs578T cells and conduct more Na⁺ [16]. There was no difference in Na⁺ influx between the 2 cell lines because each of the TRPC1/4 channel configurations are permeable to Na⁺. Interestingly, elevated levels of

TRPC4/5 co-expression in HCC1806 cells did not lead to EA sensitivity, further suggesting that specific TRPC heteromer composition influences sensitivity to EA.

Our data suggest that Hs578T cells, like the renal carcinoma A-498 cells [14] and the synovial sarcoma SW982 cells [15], are sensitive to EA-mediated Na^+ influx through the activation of TRPC1/4 heteromeric channels. When comparing the concentration response curves of EA in Hs578T and A-498 cells, both cell lines respond in a biphasic manner (Supplementary Fig. S7). Furthermore, Hs578T cells have more TRPC1 than the EA-resistant TNBC cell lines, and EA caused a marked increase in intracellular Na^+ , but not Ca^{2+} . We postulate that Na^+ influx through TRPC1/4 heteromeric channels drives the long-term accumulation of intracellular Ca^{2+} . This is analogous to the mechanism of action of cardiac glycosides which inhibit the Na^+/K^+ ATPase pump, initially causing an increase in intracellular Na^+ that is compensated for by inhibition or reversal of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, leading to a secondary increase in intracellular Ca^{2+} [29]. In contrast, our data suggest that BT-549 cells are sensitive to EA due to the acute activation of homomeric TRPC4 channels and rapid Ca^{2+} influx. Additionally, the unsynchronized, periodic oscillations in intracellular Ca^{2+} observed in BT-549, but not HCC1806 cells, suggests that BT-549 cells are continuously fine-tuning their intracellular Ca^{2+} levels. Our demonstration that even a slight decrease in TRPC1/4 expression can decrease the sensitivity to EA, further supports the delicate balance of Ca^{2+} regulation in BT-549 cells. EA-mediated rapid Ca^{2+} influx and subsequent mitochondrial depolarization demonstrates that these cells have no capacity to buffer the extensive EA-mediated Ca^{2+} influx. Additional evidence that BT-549 and Hs578T cells are different in how they modulate intracellular Ca^{2+} levels is their high expression of PMCA1 and PMCA4 Ca^{2+} pumps in comparison with other TNBC, luminal, and HER2 overexpressing breast cancer cell lines [30]. Notably, dysregulated Ca^{2+} signaling has been implicated in many of the “hallmarks of cancer”, including proliferation, migration, and metastasis [31].

This is the first study to identify a distinct group of TNBC cell lines that are exquisitely sensitive to EA and describe the mechanism underlying this sensitivity. While EA itself is not a clinical candidate, ongoing studies are aimed to identify new EA analogs with an improved therapeutic window [11]. In the meantime, we determined that EA-sensitive TNBC lines were also sensitive to the clinically approved cation-disrupting compound digoxin. For decades, it has been proposed that cardiac glycosides should be investigated for activity in breast cancer based on clinical observations. Breast cancer patients taking a cardiac glycoside for cardiac indications had more uniform and smaller breast tumors [32]. There was lower breast

cancer mortality among breast cancer patients using digitalis as compared to those that were not [33]. Preclinical studies show that cardiac glycosides can induce apoptosis [34, 35], inhibit topoisomerase II [36], initiate p21^{Cip1} expression [37], and cause immunogenic cell death [38] in cancer cells. While cardiac glycosides can also bind and activate the estrogen receptor, curbing further investigation in ER-positive breast cancer [39–41], this is not a concern in TNBC. Consequently, digoxin use was associated with a high risk of relapse in the first year after diagnosis in patients with ER-positive breast cancer, but a low risk of relapse in patients with ER-negative breast cancers [40]. Two other studies found digoxin users had a lower risk of developing ER-negative disease than ER-positive disease [42, 43]. Although there is no evidence suggesting digoxin acts through the TRPC1/4/5 channels, our results demonstrating that TNBC cell lines with high TRPC1/4 expression are particularly sensitive to cardiac glycosides suggests that there is a subgroup of TNBC with defective Ca^{2+} handling abilities that could benefit from digoxin. Mining the METABRIC breast cancer data set [44] shows that 2.2% of patients (47/2173) have amplified copy number alterations in TRPC1 or TRPC4, and half of those were ER-negative [45, 46]. Thus, a population of breast cancer patients express these potential biomarkers of response to cardiac glycosides. Furthermore, it has been shown that TRPC1 expression is high in TNBCs of the mesenchymal phenotype, which is associated with poorer patient prognosis [47]. These studies, taken together with our findings, support future investigations of TRPC1 and TRPC4 as biomarkers of response to cardiac glycosides or other cation disruptors. This would allow for the selection of a more defined group of TNBC patients for this intervention, therefore increasing the chances of therapeutic benefit.

Acknowledgements We thank Dr. Mark Shapiro (University of Texas Health Science Center at San Antonio) for providing instruments and space for intracellular ion imaging experiments. We also thank Dr. David J. Beech (University of Leeds) for providing us with Pico145.

Authors' contributions CVG, ALR, JAB, and SLM conceived and designed the experiments. CVG, CMC, MM, and KR performed the experiments. CVG analyzed the sulforhodamine B assay and qRT-PCR data. CMC and SDH analyzed the intracellular Ca^{2+} and Na^+ imaging data. MM and KR analyzed the mitochondrial imaging data. All authors contributed to data interpretation. CVG and SLM wrote the main manuscript text. All authors critically reviewed the manuscript. All authors read and approved the final manuscript.

Funding This study was funded by grants to SLM and Robert Cichewicz from the National Cancer Institute (U01CA182740), and by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research (IZIA BC1146907).

Compliance with ethical standards

Conflict of interest Corena V. Grant declares that she has no conflict of interest. Chase M. Carver declares that he has no conflict of interest. Shayne D. Hastings declares that she has no conflict of interest. Karthik Ramachandran declares that he has no conflict of interest. Madesh Muniswamy declares that he has no conflict of interest. April L. Risinger declares that she has no conflict of interest. John A. Beutler declares that he has no conflict of interest. Susan L. Mooberry has and continues to receive research funding, unrelated to this study, from Eisai, Inc.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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