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Development of CAPER peptides for the treatment of triple negative breast cancer

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ABSTRACT

Triple negative breast cancer (TNBC) is a heterogeneous disease, which lacks expression of the estrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor 2 receptor (HER2). This subtype of breast cancer has the poorest prognosis with limited therapies currently available, and hence additional options are needed. CAPER is a coactivator of the activator protein-1 (AP-1) (interacting specifically with the c-Jun component) and the ER and is known to be involved in human breast cancer pathogenesis. Recent published data have demonstrated a role for CAPER in TNBC and, as such, disrupting the function of CAPER with c-Jun could be a novel approach to treat TNBC patients. The data presented here shows the development and *in vitro* testing of CAPER-derived peptides that inhibit the coactivator activity of CAPER with c-Jun. These CAPER peptides result in a decrease in cell number and an increase in apoptosis in two TNBC cell lines, MDA-MB-231 and BT-549, while having no effect on the non-tumorigenic cell line MCF 10A. Additionally, two modes of action were demonstrated which appear to be cell line dependent: 1) a modulation of phosphorylated c-Jun leading to a decrease in Bcl-2 in MDA-MB-231 cells and a decrease in p21 in BT-549 cells and 2) a decrease in DNA repair proteins, leading to impaired DNA repair function in MDA-MB-231 cells. The data presented here supports further development of CAPER-derived peptides for the treatment of TNBC.

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Introduction

Breast cancer (BC) is one of the most prevalent cancers among women and is a major cause of death worldwide. Comprising 15–20% of all breast cancer cases, triple negative breast cancer (TNBC) does not express the estrogen receptor (ER) or progesterone receptor (PR), and there is an absence of overexpression of the human epidermal growth factor 2 receptor (HER2) [1]. This type of BC is typically more aggressive resulting in a poorer prognosis which leads to higher rates of relapse, metastases and consequently death [2]. There are currently limited targeted therapies available for TNBC, and due to the receptor status, endocrine therapies are ineffective. While significant advancements have been made in the treatment of ER+ and HER2+ breast cancers, additional treatment options are still urgently needed for TNBC patients [3,4].

CAPER (coactivator of activator protein-1 (AP-1) and ER), also known as RNA binding protein-39 (Rbm39) and hepatocellular carcinoma-1.4 (HCC1.4) is a known regulator of steroid hormone receptor-mediated transcription and alternative splicing [5]. For its coactivator activities, CAPER has been shown to interact with ERα, ERβ, PR, and AP-1, binding to the c-Jun component specifically of the AP-1 dimer. It has been demonstrated that CAPER directly interacts with ERα/β and c-Jun and stimulates their transcriptional activity *in vitro* [6]. Additionally, it has been shown that breast cancer samples have a higher level of CAPER expression when compared to normal breast tissue and that CAPER also plays a role in the progression of breast cancer [7,8]. More recently, a publication from Campbell et al. (2018) has shown a role for CAPER in TNBC, as lentiviral-mediated knockdown of CAPER

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expression resulted in reduced proliferation of the human TNBC cell lines MDA-MB-231 and BT-549 *in vitro* [7]. Not only has CAPER been implicated in breast cancer but its overexpression has also been reported in other human cancers, such as colorectal adenomas and carcinomas, non-small cell lung cancer, and acute myeloid leukemia, with the higher expression of CAPER enhancing the survival of colorectal cancer cells [9–11].

Given CAPER's role in breast cancer, the development of a novel therapeutic to inhibit its coactivator activity with the c-Jun component of AP-1 could serve as a useful targeted approach for the treatment of TNBC. Being a proto-oncogene, c-Jun is an attractive target for TNBC as it has been implicated in many aspects of cancer development, such as proliferation, invasiveness, and angiogenesis [12]. In the initial publication by Jung et al. in which CAPER's coactivator functions with AP-1 and ER were identified, the authors also pinpointed amino acid sequence 356–400 of CAPER isoform HCC1.3 as exhibiting a dominant negative phenotype with ERα transactivation [6]. Since this dominant negative phenotype was only investigated with the ER in that publication, the effect of this sequence on c-Jun has not been reported. We therefore set out to investigate if the dominant negative effect of this sequence could work as a starting point as a potential therapeutic with anti-cancer effects. To accomplish this, we developed two peptides based on amino acids 356–400 of full-length CAPER isoforms HCC1.3 and HCC1.4, which utilize cell penetrating peptide HIV-TAT for cellular entry and nuclear localization. The data presented here show that both peptides bind to c-Jun with nM affinity and competitively alter the binding of full-length CAPER to c-Jun. Additionally, we have shown that upon treatment with either peptide, both MDA-
MB-231 and BT-549 cell lines show a significant decrease in cell number and an increase in apoptotic cells with no significant change to the non-tumorigenic cell line MCF 10A. Western blotting data from TNBC cells treated with the CAPER peptides shows two potential modes of action which appear to be cell line dependent; 1) modulation of phosphorylated c-Jun leading to a decrease in pro-survival protein Bcl-2 in MDA-MB-231 cells and a decrease in p21 in BT-549 cells and 2) a decrease in DNA repair protein c-Abl and RAD51, leading to impaired DNA repair function in MDA-MB-231 cells.

Materials and methods

Materials

Cell lines MDA-MB-231 (cat# ATCC HTB-26), BT-549 (cat# ATCC HTB-122) and MCF 10A (cat# ATCC CRL-10317) were purchased from American Type Culture Collection (ATCC, Manassas, VA).

The following primary antibodies were purchased from Cell Signaling Technology (Danvers, MA): rabbit monoclonal anti-c-Jun (cat# 9165), rabbit polyclonal anti-phospho c-Jun (Ser63) II (cat# 39261), rabbit monoclonal anti-phospho c-Jun (Ser73) (D47G9) XP (cat# 3270), rabbit monoclonal anti-RAD51 (cat# 8875), rabbit monoclonal anti-p21 (cat# 2947), mouse monoclonal anti-Bcl-2 (cat# 15071), rabbit monoclonal anti-c-Abl (cat# 2862), rabbit monoclonal anti-phospho-Histone H2AX (Ser139) (cat# 9718). Mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, cat# 10R-G109a) was purchased from Fitzgerald Industries (Acton, MA). Rabbit polyclonal anti-Cyclin D1 (cat# Ab16663) and rabbit polyclonal anti-Lamin A (cat# Ab26300) were purchased from Abcam (Cambridge, MA). Rabbit polyclonal anti-histone H2AX (cat# NB100-638) was purchased from Novus Biologicals (Centennial, CO).

Secondary antibodies: IRDye® 680RD Goat anti-mouse IgG (H + L) (cat# 926–68070) and IRDye® 800CW Goat anti-rabbit IgG (H + L) (cat# 926–32211) were purchased from LI-COR Biosciences (Lincoln, NE).

Cell culture

MDA-MB-231 cells were cultured in Dulbecco minimum essential medium (DMEM) (cat# 11965, Life Technologies, Carlsbad, CA), containing 10% fetal bovine serum (FBS, cat# 16140, Life Technologies), 1% penicillin/streptomycin (cat# 15140, Life Technologies) and 1% sodium pyruvate (cat# 11360–070, Life Technologies). BT-549 cells were cultured in RPMI 1640 (cat# A10491, Life Technologies), supplemented with 10% FBS,

0.023 IU/mL insulin (cat# I0516, Sigma-Aldrich) and 1% penicillin/streptomycin. Non-tumorigenic breast epithelial cells, MCF 10A, were cultured in DMEM/F12 medium (cat# 11320–033, Life Technologies) supplemented with 5% horse serum (cat# 2020–03, Life Technologies), 20 µg/mL of EGF (cat# AF-100-12, Peprotech, Rocky Hill, NJ), 0.5 mg/mL hydrocortisone (cat# H0888, Sigma-Aldrich), 10 µg/mL insulin, 100 ng/mL cholera toxin (cat# c8052, Sigma-Aldrich) and 1% penicillin/streptomycin.

Peptides

Peptides were custom synthesized by LifeTein LLC (Somerset, NJ) as crude purity with a single biotin on the N-terminus. Peptides were stored lyophilized at −80°C until reconstitution in sterile water containing 2% DMSO (Fisher Scientific). The concentration of the reconstituted peptides was verified by absorbance using a Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA) at A280. Sequences used:

CAPER Peptide HCC1.4: YGRKKRRQRRRR LQLMARLAEGTGLQIPPAAQQALQMSGSLAF-GAVAEFSFVIDLQ

CAPER Peptide HCC1.3: YGRKKRRQRRRR LQLMARLAEGTGLQIPPAAQQALQMSGSLAF-GAVADLQTRLSQQ

CAPER Peptide Scrambled: YGRKKRRQRRR VGDALQGLRLFSTQASIGAQMEQLAAQPLRA-GQMLQLAQASPLRT

TAT Control Peptide: YGRKKRRQRRR

Binding kinetics

The binding kinetics of the peptides and full-length recombinant CAPER (R&D Systems, Minneapolis, MN) with c-Jun (Abcam, Cambridge, MA) were determined using biolayer interferometry (BLI) on the Octet HTX system (Pall ForteBio, Fremont, CA). Amine-Reactive 2nd Generation biosensors (cat# 18–5092, Pall ForteBio) were activated with 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride and N-hydroxysulfosuccinimide (EDC/NHS) (Amine Coupling Kit II, Cat# ACK-001-025, Sierra Sensors, Billerica, MA) and conjugated with an anti-6X His tag antibody (cat# ab9136, Abcam). The reaction was then quenched with 1M ethanolamine (Amine Coupling Kit II, Cat# ACK-001-025, Sierra Sensors). His-tagged recombinant c-Jun was then bound to the tips. The peptides were tested at a series of 2-fold dilutions including a blank buffer. The binding of full-length recombinant CAPER was also tested. Association and dissociation steps were performed for 900s each in 1X HBS-EP+ buffer (cat# BR100669, GE Healthcare Lifesciences, Pittsburgh, PA) supplemented with 450 mM NaCl (Sigma). The background was subtracted from each run, and kinetics data was analyzed using ForteBio's Data Analysis Software version 10.0.3.1 using a 1:1 model.

Competition assays

Competition assays were performed using BLI on the Octet HTX system (Pall ForteBio). Amine-Reactive 2nd Generation biosensors were activated and conjugated with an anti-6X His tag antibody as described above. Recombinant c-Jun containing a His-tag was then bound to the tips. The peptides were allowed to saturate the receptor at a concentration of 250 nM (approximately 10X the highest K_D). After receptor saturation, binding of full-length CAPER (250 nM) to the receptor was measured, and the signal was compared back to full-length CAPER binding to the receptor in the absence of the peptide. Binding steps were performed for 900s each in 1X HBS-EP+ buffer supplemented with 450 mM NaCl. The background was subtracted from each run, and the signal was represented as a % inhibition compared to full-length CAPER binding in the absence of the peptide.

Immunofluorescence

Coverslips were placed at the bottom of a 6-well plate, and 150,000 cells/well were added. The plates were incubated overnight at 37°C with 5% CO_2 to allow the cells to attach. The next day, cells were treated with the peptides at 10 µM for 1 hr. After the indicated time, media was removed, and the cells were rinsed three times with PBS, and fixed with ice cold 70% MeOH at −20°C for 10 min. Cells were washed 3 times with PBS and then incubated with Streptavidin, Alexa-fluor 488 conjugate (cat# S32354, Life Technologies) for 1 hr at 37°C. Cells were then mounted with Prolong Gold Antifade Mountant with DAPI (cat# P36941, Life Technologies) and imaged on an EVOS FL

Cell Imaging System (cat# AMEFC4300, Thermo Fisher Scientific, Waltham, MA) using 10X magnification.

Fractionation

MDA-MB-231, BT-549 and MCF 10A cells were added to 10 cm cell culture dishes and allowed to attach overnight in a 37°C incubator with 5% CO_2. The next day, cells were treated with the peptides at 20 μM for 1 hr. After the indicated time, media was removed, and the cells were rinsed with PBS and trypsinized using 0.05% trypsin. The cells were then washed two times with PBS, and a hypotonic lysis buffer was added. The hypotonic lysis buffer was comprised of 10 mM HEPES, 1.5 mM MgCl, 10 mM KCl, 1 mM DTT, 0.1 mM EDTA, with protease and phosphatase inhibitors. The cells were allowed to sit on ice for 10 min and then passed through a gauge needle, and allowed to sit on ice an additional 10 min. The lysate was then centrifuged for 10 min at 13,000 rpm. The supernatant was removed and added to a fresh tube (cytosolic fraction). The pellet was then rinsed twice with PBS. After the final rinse, the PBS was removed and the pellet was taken up in a hypertonic buffer containing 20 mM HEPES, 1.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 20% glycerol pH 7.9 with the addition of protease and phosphatase inhibitors. The lysate was then sonicated at 30 amp and homogenized. The lysate was then allowed to sit on ice for 30 min, with periodic vortexing. The lysate was then centrifuged for 10 min, and the supernatant was added to a fresh tube (nuclear fraction). All lysates were stored at −80°C until use.

Treatment of cell lines with CAPER peptides and cell count

Dose response curves were generated by plating MDA-MB-231 and BT-549 cells into the wells of a 24-well plate at 1,000 cells per well. The cells were allowed to adhere overnight and the next day cells were treated with CAPER peptides and TAT only control at a series of 2-fold dilutions ranging from 20 μM to 1.25 μM along with a DMSO control. Cells were dosed daily for 7 days. At the end of the 7-day dosing period, adherent cells were trypsinized and counted via a hemocytometer. Each condition was tested in one well per plate, with the entire experiment being repeated 3 times per cell line.

MDA-MB-231, BT-549 and MCF 10A cells were plated onto 10 cm plates at 50,000 cells per plate, 100,000 cells per plate and 25,000 cells per plate, respectively. The cells were allowed to adhere overnight; the next day cells were treated with 20 μM of CAPER peptides along with DMSO (vehicle) and TAT only controls. Cells were dosed daily for 7 days. At the end of the 7-day dosing period, floating cells were collected, and adherent cells were trypsinized and counted via a hemocytometer.

Apoptosis assay

Apoptosis was evaluated using the Muse Annexin V kit (cat# MCH100105, EMD Millipore). Cells were treated for 7 days as described above. At the end of the 7-day dosing period, floating cells were collected, and attached cells were washed 1X with PBS and then trypsinized with 0.05% trypsin. Cells were collected and combined with the floating cells. Cells were then counted and diluted to 20,000 cells/mL. 100 μL of cells was added to 100 μL of Annexin V reagent for 30 min at room temperature. Cells were then analyzed on the Muse Cell Analyzer (Millipore).

Cell cycle assay

The MDA-MB-231 and BT-549 cells were cell cycle synchronized using nocodazole (cat# M1404, Sigma-Aldrich) for 24 hours. The floating cells (dead) were aspirated off the plate, the plate was then rinsed with media, and the loosely attached cells were collected (G2/M fraction). The G2/M cells were then plated as described above. A fraction of the treated cells was processed for cell cycle to confirm synchronization. All cell lines were dosed for 7 days as described above. At the end of the 7-day dosing period, floating cells were collected, and attached cells were washed 1X with PBS and then trypsinized with 0.05% trypsin. Cells were collected and combined with the floating cells. Cells were counted and prepared according to the Muse Cell Cycle Kit (cat# MCH100106, EMD Millipore) and fixed in 70% ethanol at −20° C for a minimum of 3 hrs. Cells were then washed with PBS, and 200 μl of cell cycle reagent was

added for 30 min. Cells were then analyzed on the Muse Cell Analyzer (Millipore).

Western blot analysis

After the 7-day treatment period, cells were trypsinized and washed twice with cold PBS. Cells were then lysed in RIPA buffer containing protease inhibitor cocktail (cat# 11-836-153-001, Roche, Basel, Switzerland) and Halt phosphatase inhibitor single-use cocktail (cat# 78428, Fisher Scientific, Waltham, MA). The pellet was then sonicated on ice for 30s at 30 amp and centrifuged for 10 min at 10,000 X g at 4°C. After centrifugation, the supernatant was removed and stored at −80°C until use. Total protein concentration was determined via bicinchoninic acid (BCA, cat# PI23250, Fisher Scientific, Waltham, MA) assay and equal amounts of protein were added to the wells of a sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane. Membranes were blocked for 1 hr at room temperature with either 5% bovine serum albumin (BSA, cat# BP1600, Fisher Scientific) or 5% nonfat milk in 10 mM Tris, 150 mM NaCl (1X TBS) with 0.05% Tween-20 buffer. After blocking, membranes were incubated with the primary antibody overnight at 4°C. The next day, membranes were washed 3X for 10 min each with TBS with 0.1% Tween-20 (1X-TBST) and then incubated with appropriate IRDye secondary antibody (cat# 926–32211 or cat# 926–68070, LICOR Biosciences) for 1 hr. Membranes were washed 3X for 10 min each with TBST and then read using the LICOR Odyssey CLx Imager. LICOR Image Studio Version 5.2 was used to quantify the bands. The protein of interest was then normalized to the GAPDH protein loading control. Each probe was repeated for a minimum of three independent runs.

Statistical analysis

Data for cell counts, Annexin, Cell Cycle, and the competition assays were expressed as mean plus/minus S.E.M and differences between groups were evaluated and analysis was performed by one-way ANOVA with Tukey's multiple comparisons test. IC_{50} values were obtained by plotting the dose response curves in GraphPad Prism 8.0.2 using a nonlinear regression. For Western blotting, a two-sided one-sample t test or two-sided Wilcoxon Signed-Rank test was used where appropriate. Statistical significance is marked as ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.005$, *$p < 0.01$, # $p < 0.05$, not significant (n. s.) $p > 0.05$.

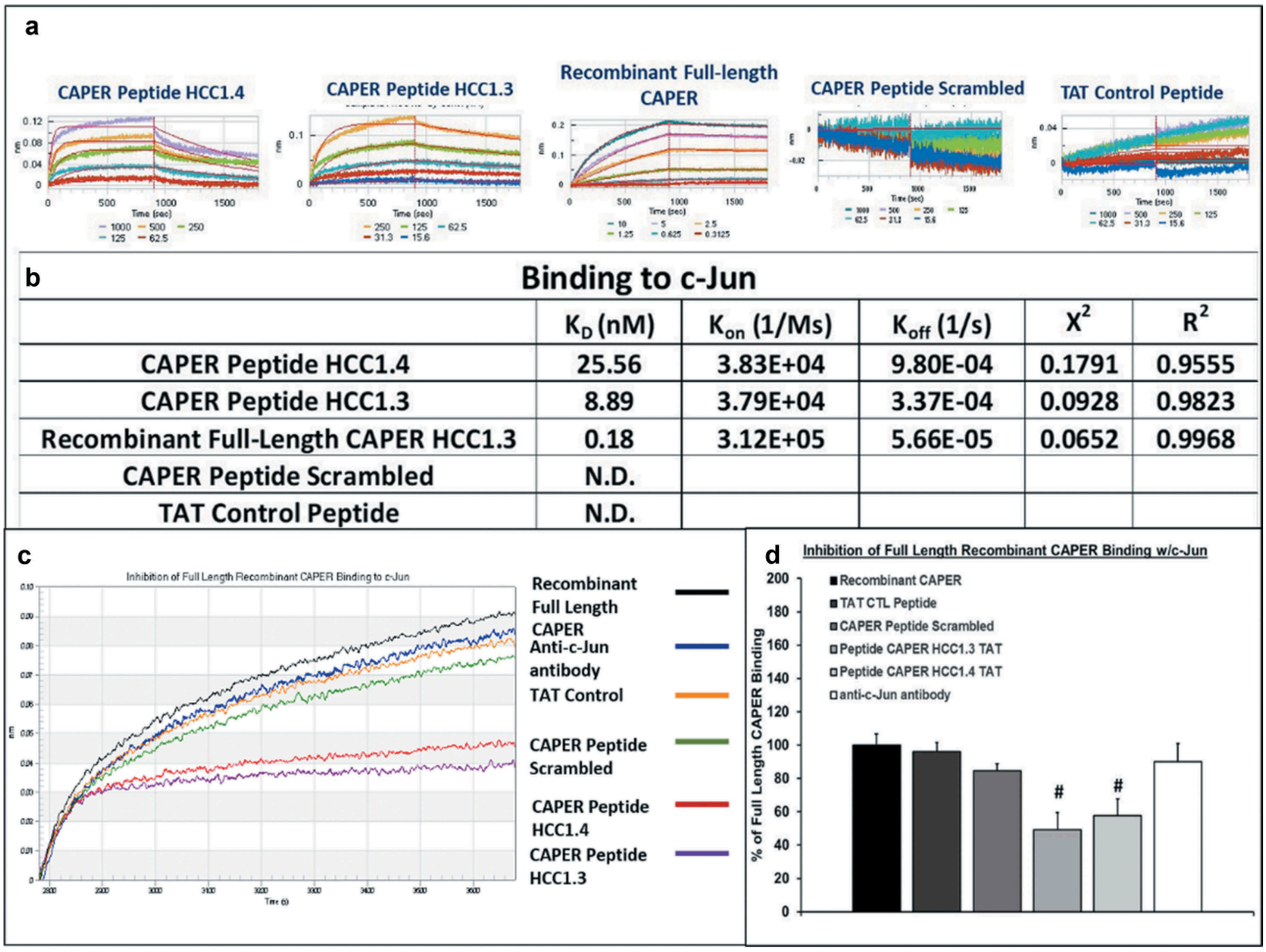
Results

CAPER peptides bind to c-Jun with nm affinity and alter binding of recombinant full-length CAPER with c-Jun

Binding kinetics of the peptides were determined, and the association and dissociation constants of each were calculated. The K_D of CAPER peptide HCC1.4 and CAPER peptide HCC1.3 were determined to be 25.56 and 8.89 nM, respectively, whereas the K_D of the CAPER scrambled peptide and TAT only control could not be determined (Figure 1a and b). To test if the CAPER peptides might prevent full-length recombinant CAPER from binding to c-Jun, a competition assay was conducted. Results show that when c-Jun was saturated with CAPER peptides HCC1.3 and HCC1.4, full-length recombinant CAPER binding was inhibited by 50.7% and 42.2%, respectively. The TAT control, CAPER scrambled peptide and the anti-c-Jun antibody showed no significant effect on CAPER binding to c-Jun (Figure 1c).

Peptides efficiently enter TNBC cells and MCF 10A cells

MDA-MB-231, BT-549 and MCF 10A cells were treated with 10 μM of CAPER peptide HCC1.3, CAPER peptide HCC1.4 and the CAPER scrambled peptide for 1 hr. Immunofluorescent staining of the cells shows the peptides effectively enter the cells and travel to the nucleus after 1 hr of treatment (Figure 2a–c). To confirm the results seen with immunofluorescence, fractionation was performed on the cell lines after treatment with the peptides to obtain cytosolic and nuclear proteins. Analysis of these lysates via Western blotting shows similar results as the immunofluorescence



	K_D (nM)	K_{on} (1/Ms)	K_{off} (1/s)	X^2	R^2
CAPER Peptide HCC1.4	25.56	3.83E+04	9.80E-04	0.1791	0.9555
CAPER Peptide HCC1.3	8.89	3.79E+04	3.37E-04	0.0928	0.9823
Recombinant Full-Length CAPER HCC1.3	0.18	3.12E+05	5.66E-05	0.0652	0.9968
CAPER Peptide Scrambled	N.D.				
TAT Control Peptide	N.D.				

Figure 1. CAPER peptides HCC1.3 and HCC1.4 bind to c-Jun with nM affinity and alter the binding of full-length recombinant CAPER. Panel A. Binding curves tested with CAPER peptides, full-length CAPER and peptide controls. Concentrations listed in nM. Panel B. Binding kinetics of the peptides with c-Jun, N.D. = not determined. Panel C. Competition binding curves using BLI. Amine-reactive tips were conjugated with an anti-His tag antibody. His-tagged c-Jun was then bound to the tips. Tips were then saturated with the CAPER peptides or controls. Panel D. Signals generated from the competition experiments using CAPER peptides were compared to full-length recombinant CAPER binding to c-Jun without the peptides present. n = 3, $p < 0.05$.

staining, confirming that the peptides are entering the cells and traveling to the nucleus (Figure 2d).

Treatment of TNBC cell lines with CAPER peptides shows a decrease in cell number and an increase in apoptotic cells with no effect on cell cycle

In order to assess a concentration response to the CAPER peptides, both TNBC cell lines were treated for 7 days with DMSO (vehicle), TAT only control, CAPER peptide HCC1.3 and CAPER peptide HCC1.4. A series of 2-fold dilutions of the peptides were tested ranging from 20 μM to 1.25 μM and results were compared to the DMSO (vehicle) control. Results shown in Figure 3a show an IC_{50} of approximately 6.7 and 6.0 μM for both CAPER peptide HCC1.3 and HCC1.4, respectively, in MDA-MB-231 cells. For the BT-549 cells, the approximate IC_{50} is 4.4 μM for CAPER peptide HCC1.3 and 10.5 μM for CAPER peptide HCC1.4. The TAT only control showed no decrease in cell number at any of the concentrations tested.

After 7 days of treatment with either CAPER peptide HCC1.3 or CAPER peptide HCC1.4 at concentration of 20 μM, both MDA-MB-231 and BT-549 cells showed a significant decrease in cell number (Figure 3b). MDA-MB-231 cells resulted in an approximate 3.7-fold and 4.5-fold reduction in cell count for CAPER peptides HCC1.3 and HCC1.4, respectively, when

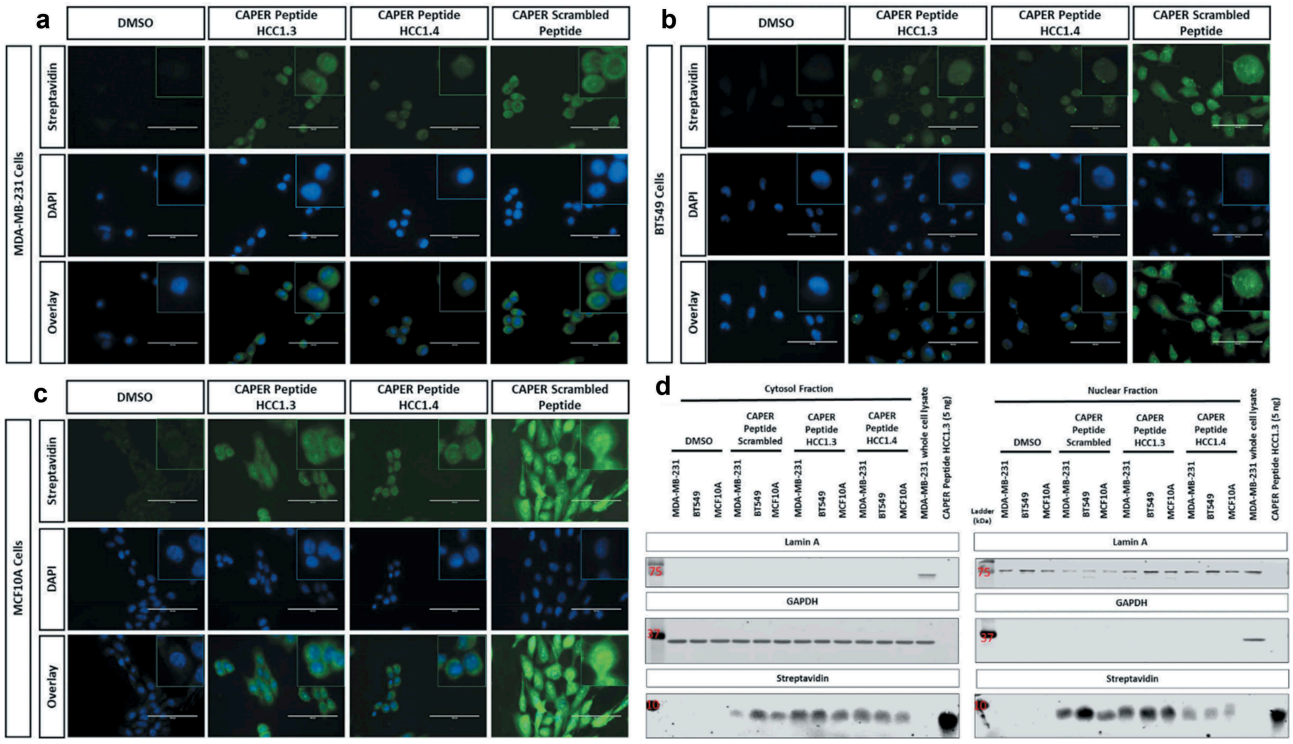


Figure 2. CAPER peptides enter cells and the nucleus. Panel A-C. MDA-MB-231, BT-549 and MCF 10A cells were treated with DMSO (vehicle), CAPER peptide HCC1.3, CAPER peptide HCC1.4 and CAPER scrambled peptide at a concentration of 10 µM for 1 hr. Cells were then stained with Alexa Fluor-conjugated streptavidin to visualize the biotinylated peptides. Cells were also stained with DAPI DNA dye. Cells were imaged at 10X magnification using DAPI and GFP fluorescent cubes on an EVOS cell imager, scale bar = 100 µm. Images shown in the upper corner were zoomed in 5X. Panel D. MDA-MB-231, BT-549 and MCF 10A cells were treated with DMSO (vehicle), CAPER peptide HCC1.3, CAPER peptide HCC1.4 and CAPER scrambled peptide at a concentration of 20 µM for 1 hr and fractionation was performed to obtain proteins from the cytosolic and nuclear fractions. Western blotting was then performed using streptavidin to identify the biotinylated peptides. Loading controls used were GAPDH (cytosol) and Lamin A (nuclear).

compared to DMSO (vehicle) and TAT controls. Cell line BT-549 resulted in an approximate 7-fold and 2-fold reduction in cell count for CAPER peptides HCC1.3 and HCC1.4, respectively, when compared to DMSO (vehicle) and TAT controls. When both cell lines were treated with CAPER scrambled peptide for 7 days, no significant change in cell count was observed when compared to the DMSO (vehicle) and TAT controls (Figure 3b). Additionally, when apoptotic cells were investigated, both TNBC cells lines treated with CAPER peptide HCC1.3 showed a significant decrease in live cells and a significant increase in early apoptotic and late apoptotic/dead cells which was observed in the Annexin V assay. CAPER peptide HCC1.4 showed the same effect in cell line MDA-MB-231, however did not show a significant increase in early apoptotic cells in the BT-549 cell line. No significant effect was seen in either cell line using the CAPER scrambled peptide. (Figure 4). Interestingly, TNBC cells treated with the CAPER peptides show no effect on cell cycle (Figure 5)

Treatment of non-tumorigenic breast epithelial cell line MCF 10A with CAPER peptides results in no effect on cell number or apoptosis

The non-tumorigenic breast epithelial cell line MCF 10A was treated with either CAPER peptide HCC1.3 or CAPER peptide HCC1.4 at a concentration of 20 µM for 7 days. After the 7-day treatment period, the MCF 10A cell line showed no significant change in cell count (Figure 6a) or apoptotic cells (Figure 6b) when compared to both DMSO (vehicle) and TAT controls.

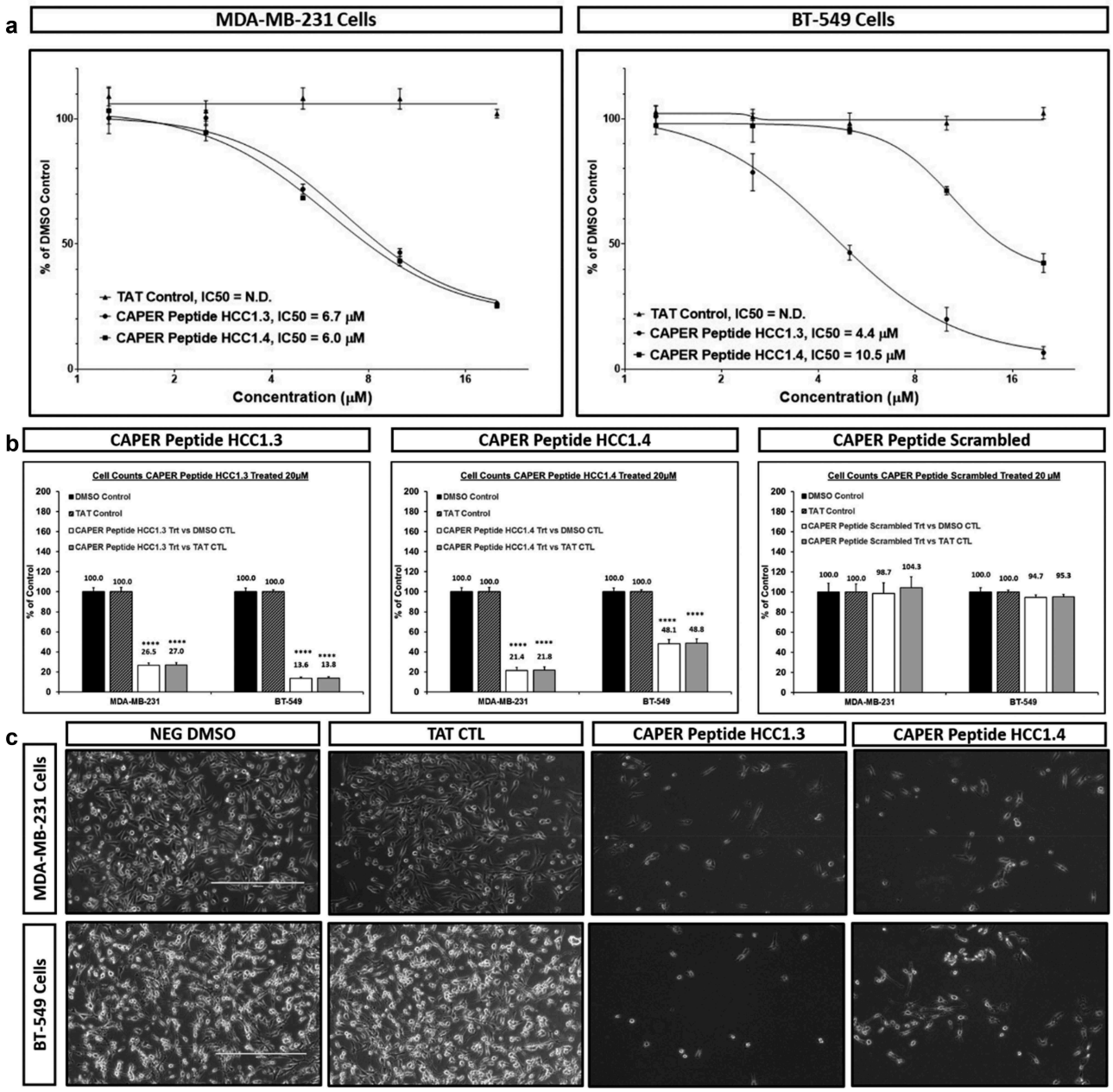


Figure 3. Treatment of TNBC cell lines with CAPER peptides results in lower cell number. Panel A. Cell counts for MDA-MB-231 and BT-549 cells treated for 7 days with DMSO (vehicle) control, TAT control, CAPER peptide HCC1.3 and CAPER peptide HCC1.4. The TAT control, CAPER peptide HCC1.3 and CAPER peptide HCC1.4 were tested at concentrations ranging from 1.25 to 20 μM and compared as a percentage of the mean DMSO (vehicle) control, n = 3. Panel B. Cell counts for MDA-MB-231 and BT-549 cells treated for 7 days with 20 μM of CAPER peptide HCC1.3, CAPER peptide HCC1.4, and CAPER scrambled peptide compared to DMSO (vehicle) and TAT only controls. ****$p < 0.0001$, n = 3 to 5 for each group. Panel C. Representative images of MDA-MB-231 and BT-549 cells treated with DMSO (vehicle), TAT only control, CAPER peptide HCC1.3, and CAPER peptide HCC1.4 at 20 μM, 10X magnification using an EVOS cell imager.

Treatment of TNBC cell line MDA-MB-231 with CAPER peptides decreases phosphorylated c-Jun and pro-survival protein Bcl-2 with no effect on cell cycle regulator cyclin D1

Since both CAPER peptides bind to c-Jun, and since c-Jun activation has been shown to be enhanced upon phosphorylation [13,14] total c-Jun and two phosphorylation events of c-Jun (Ser 73 and Ser 63) levels were investigated after treating MDA-MB-231 cells with the CAPER peptides using Western blotting. The results shown in Figure 7a show that treatment with either peptide does not alter the level of total c-Jun but does

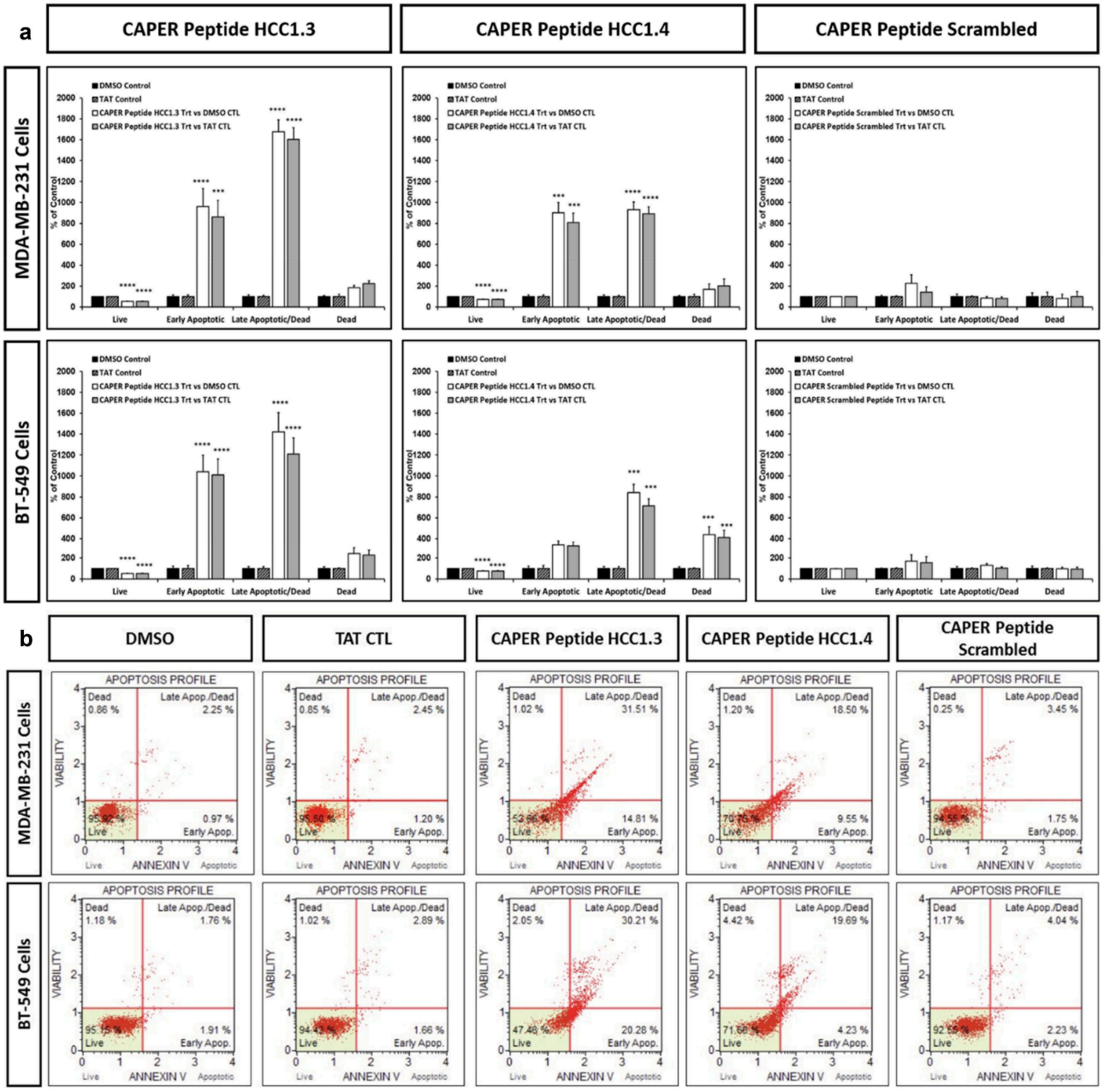


Figure 4. Treatment of TNBC cell lines with CAPER peptides increases apoptosis. Panel A. Results from Annexin V assay for MDA-MB-231 and BT-549 cells treated for 7 days with 20 μM of CAPER peptide HCC1.3, CAPER peptide HCC1.4 and CAPER scrambled peptide compared to DMSO (vehicle) and TAT only controls. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.005$, #$p < 0.05$, $n = 3$ to 5 for each group. Panel B. Results from Annexin V assay showing live, early apoptotic, late apoptotic/dead, and dead populations after treatment with DMSO (vehicle), TAT control, CAPER Peptide HCC1.3, CAPER peptide HCC1.4 and the CAPER scrambled peptide.

significantly decrease the levels of both c-Jun phosphorylation events. Additionally, we investigated the level of pro-survival protein Bcl-2, and the results showed a significant decrease after peptide treatment, indicating a shift toward the pro-apoptotic state. Since c-Jun can affect cell cycle progression primarily through the regulation of cyclin D1, we investigated these levels which shows no significant change confirming the results seen in the cell cycle assay [15].

Treatment of TNBC cell line BT-549 with CAPER peptide HCC1.3 increases phosphorylated c-Jun and decreases p21 with no effect on cell cycle regulator cyclin D1

Treatment of TNBC cell line BT-549 with CAPER peptide HCC1.3 shows an opposite effect on both phosphorylation events of c-Jun where a significant increase is seen (Figure 7a). Additionally, there is also

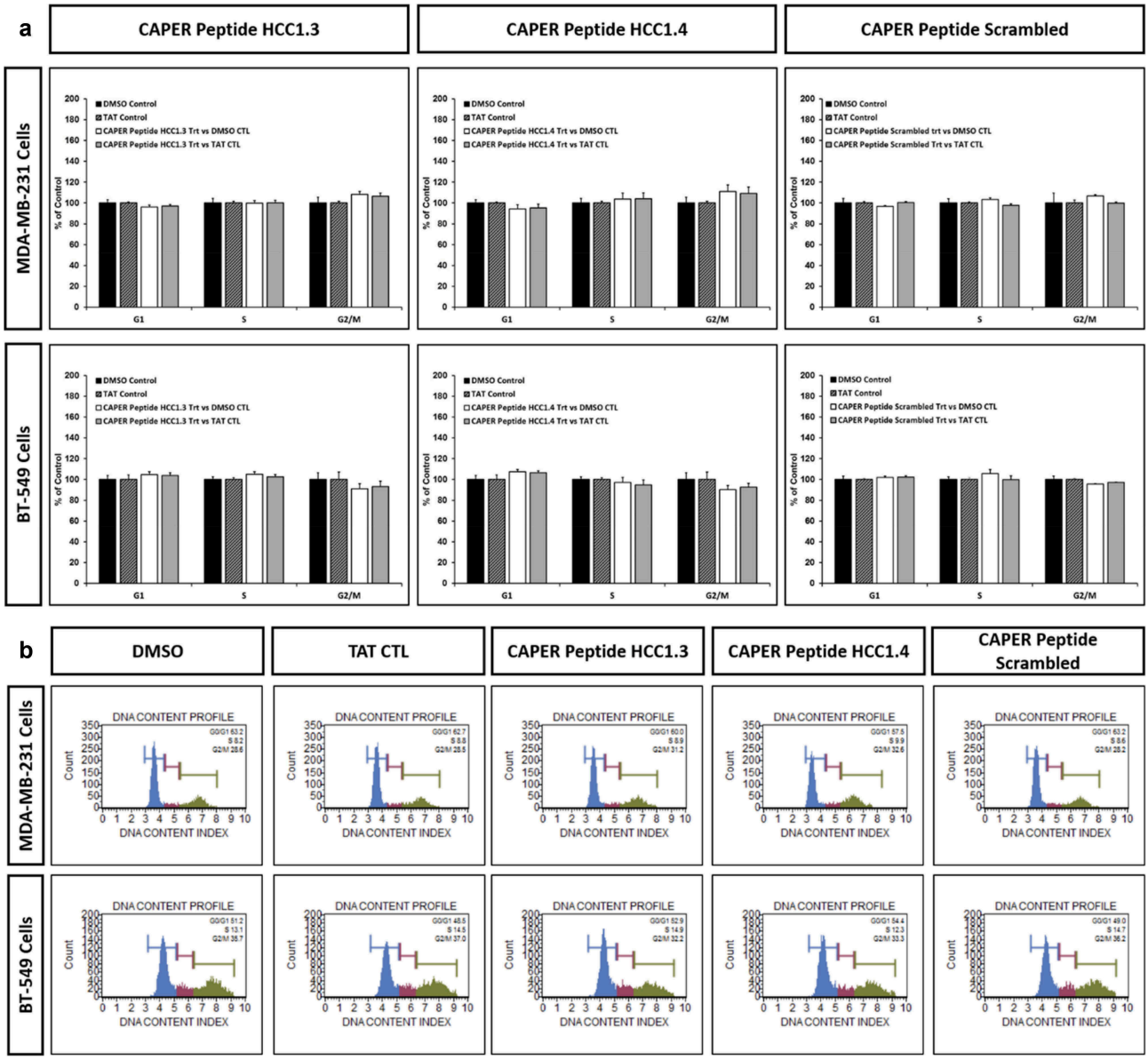


Figure 5. Treatment of MDA-MB-231 and BT-549 cells with CAPER peptides shows no effect on cell cycle. Panel A. Results from the Cell Cycle assay for nocodazole synchronized MDA-MB-231 and BT-549 cells treated for 7 days with 20 μM of CAPER peptide HCC1.3, CAPER peptide HCC1.4 and CAPER scrambled peptide compared to DMSO (vehicle) and TAT only controls, n = 3 to 5 for each group. Panel B. Results from the Cell Cycle assay showing populations in G0/G1, S and G2/M phases of cell cycle after treatment with DMSO (vehicle), TAT control, CAPER Peptide HCC1.3, CAPER peptide HCC1.4 and the CAPER scrambled peptide.

an increase in total c-Jun and Bcl-2. It has been reported that the chemotherapy drug Vinblastine exerts its activity by increasing phosphorylated c-Jun with a decrease in p21 [16]. Upon probing for p21, our results show similar results with a significant decrease in p21 upon treatment with both CAPER peptides. As seen in the MDA-MB-231 cell line, there is no effect on Cyclin D1. The only significant changes seen in the CAPER peptide HCC1.4 treated cells were an increase in Bcl-2 and a decrease in p21.

Treatment of TNBC cell line MDA-MB-231 with CAPER peptides induces phosphorylation of histone H2AX (γ-H2AX) while decreasing DNA repair proteins c-Abl and RAD51

Recent data utilizing lentiviral knockdown of CAPER in TNBC cells showed activation of the DNA damage marker, phospho-H2AX (γ-H2AX) and a decrease in DNA repair proteins RAD51 and c-Abl [7]. We investigated these markers to see if the CAPER peptides had

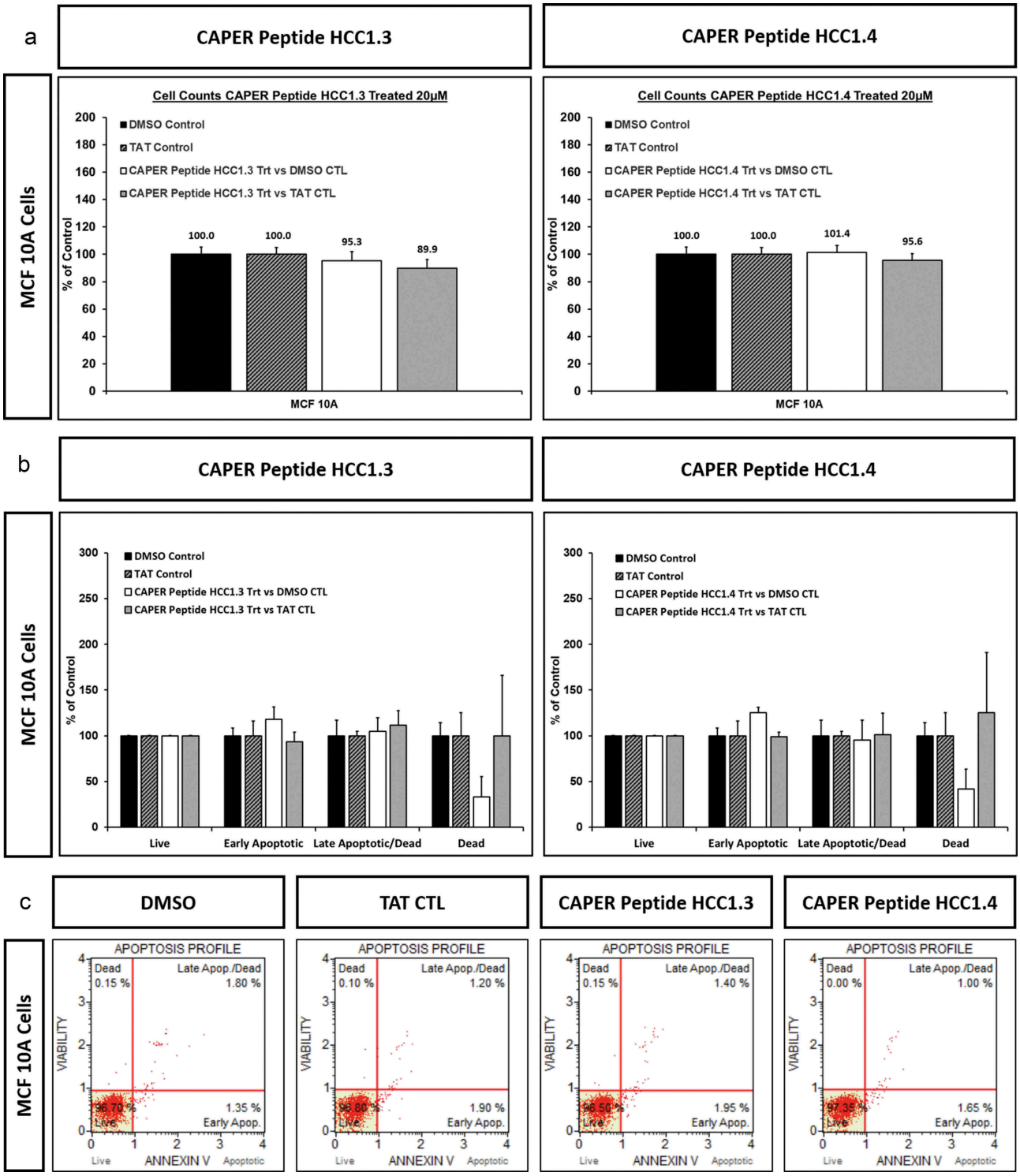


Figure 6. Treatment of non-tumorigenic breast epithelial cell line MCF 10A with CAPER peptides shows no change in cell count or apoptosis. Panel A. Cell counts for MCF 10A cells treated for 7 days with 20 μM of CAPER peptide HCC1.3 and CAPER peptide HCC1.4 compared to DMSO (vehicle) and TAT treated controls, p = not significant, n = 3. Panel B. Results from the Annexin V assay for MCF 10A cells treated for 7 days with 20 μM of CAPER peptide HCC1.3 and CAPER peptide HCC1.4 compared to DMSO (vehicle) and TAT only controls. p = not significant, n = 3 for each group. Panel C. Results from Annexin V assay showing live, early apoptotic, late apoptotic/dead, and dead populations after treatment with DMSO (vehicle), TAT control, CAPER peptide HCC1.3 and CAPER peptide HCC1.4.

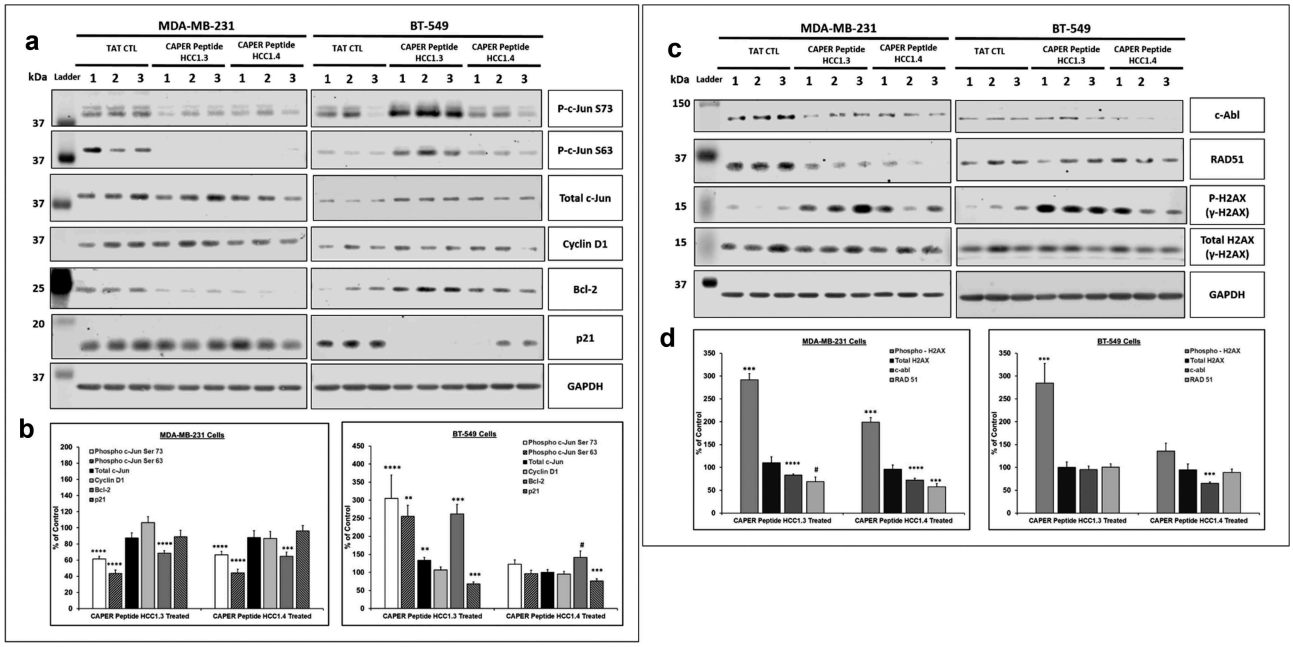


Figure 7. Western blot analysis after treatment of TNBC cell lines with CAPER peptides. Panels A and C. Western blotting results from MDA-MB-231 and BT549 cells treated with CAPER peptide HCC1.3, CAPER peptide HCC1.4 and TAT control for 7 days. Panels B and D. Quantitative analysis from Western blotting using LICOR Image Studio Version 5.2. For all quantitative analysis, each protein was divided by the GAPDH loading control and then normalized to TAT control treated which was normalized to 100%. Data represents the mean from a minimum of three independent Western blots. ****p < 0.0001, ***p < 0.001, **p < 0.005, *p < 0.01, # p < 0.05.

the same effect. The results for cell line MDA-MB-231 (Figure 7c) showed a significant increase in phospho-H2AX (γ-H2AX) indicating DNA damage, and a decrease in both proteins involved in DNA repair (RAD51, c-Abl) for both CAPER peptide HCC1.3 and HCC1.4. This data confirms what has been reported previously in this cell line using lentiviral knockdown of CAPER [7].

Treatment of TNBC cell line BT-549 with CAPER peptide HCC1.3 induces phosphorylation of histone H2AX (γ-H2AX)

In cell line BT-549 (Figure 7c) an increase in phospho-H2AX (γ-H2AX) is only seen with CAPER peptide HCC1.3. CAPER peptide HCC1.4 showed a trend in this direction, though it was not significant. Neither of the CAPER peptides showed a significant decrease in RAD51 in this cell line. A significant decrease in c-Abl was only observed with CAPER peptide HCC1.4.

Discussion

TNBC patients have a limited range of therapeutics available, and thus other options are urgently needed. Since CAPER has recently been implicated in the growth of TNBC cell lines, targeting the activity of the c-Jun component of AP-1 with CAPER-derived peptides could provide therapeutic benefit to TNBC patients. In the data presented here, we show for the first time the *in vitro* effect upon treating TNBC cell lines with CAPER peptides.

Our results show that CAPER peptides have good binding to c-Jun in the nM range. The treatment of TNBC MDA-MB-231 and BT-549 cell lines with either of the CAPER peptides results in a decrease in cell number and an increase in apoptotic cells. Additionally, the competition experiments show altered binding of recombinant full-length CAPER when the peptides are bound to c-Jun. We hypothesize that the peptides may be working to deliver pro-apoptotic effects via interaction with c-Jun by the two following mechanisms: 1) CAPER peptides bind to c-Jun and inhibit or alter the binding of endogenous CAPER thus modifying its coactivator activity

and/or pre-mRNA splicing function and 2) the peptides bind to c-Jun and induce a conformational change which modulates phosphorylation and/or the recruitment of corepressors, thus altering c-Jun's function. The data from our competition experiments support altered binding of full-length recombinant CAPER to c-Jun, though we cannot conclude the outcome of this binding event based on the current results alone. Additionally, while the peptides bind to c-Jun, we cannot conclude that the peptides are solely acting through this target. Future work needs to be conducted to fully understand the mechanism and to confirm the target via target engagement assays. Interestingly, the CAPER peptides showed no effect on either cell counts or apoptosis in the non-tumorigenic breast epithelial cell line MCF 10A. This might be due, at least in part, to a lower expression of c-Jun in MCF 10A but future studies are warranted to precisely determine why such peptides have no effect on non-tumorigenic breast epithelial cells.

Western blotting results show different *in vitro* modes of action upon treatment with CAPER peptides depending on the cell line used. Since our initial hypothesis was that the CAPER peptides were interacting with c-Jun, the first markers investigated were those for the two main phosphorylation events. The results obtained via Western blotting showed a significant decrease in both events in cell line MDA-MB-231 when treated with either CAPER peptide with a decrease in pro-survival protein Bcl-2. However, when we probed for these markers in cell line BT-549 we observed a significant increase in both phosphorylation events with CAPER peptide HCC1.3 and no significant change when treated with CAPER peptide HCC1.4. It has been reported that c-Jun can act as both a pro-survival and pro-apoptotic protein. In fact, the chemotherapy drug vinblastine has been shown to cause apoptosis via activation of c-Jun which causes downregulation of p21 as it has been reported that c-Jun can serve as a negative regulator of p21 [16]. Accordingly, our results demonstrate a significant decrease in p21 in BT-549 cells treated with both CAPER peptides, indicating a similar mode of action in this cell line. The mechanisms by which p21 can inhibit apoptosis under certain conditions include blocking activation of procaspase 3, caspase 8, caspase 9 and caspase 10 [17–19]. Therefore, we hypothesize the decrease in p21 in the BT-549 cells observed after CAPER peptides treatment is resulting in the apoptotic state described here. Interestingly, BT-549 cells showed an increase in Bcl-2 levels upon treatment with both CAPER peptides. Since it has been reported that c-Jun can control Bcl-2 at the transcriptional level [20], it is logical that as c-Jun is activated by phosphorylation, as in the case of the BT-549 cells, this would result in higher levels of Bcl-2, and vice versa in the MDA-MB-231 cells. It has been shown that c-Jun induced apoptosis can be slowed by overexpression of Bcl-2 however it is not enough to stop apoptosis [21]. Given the complexity of the cell environment and the other factors being altered by the CAPER peptides, these events may override this increase in pro-survival Bcl-2.

The CAPER peptides have shown no effect on the cell cycle of TNBC cells in either the cell cycle assay or by changes in cyclin D1 levels. c-Jun is known to affect cell cycle progression primarily through cyclin D1 [15]. However, it has been shown that c-Jun's cell cycle and anti-apoptotic effects occur via different mechanisms and that the cell cycle effect does not require c-Jun phosphorylation to occur [15]. Furthermore, it has also been reported that c-Jun's cell cycle effect may also be dependent on the presence of p53 [22]. Since both TNBC cell lines used in our studies contain p53 mutations, our data is in agreement with the literature. Therefore, the CAPER peptides may alter c-Jun's apoptotic function without affecting its cell cycle effect.

The second mode of action which is observed in MDA-MB-231 cells is related to CAPER's role in DNA repair. A recent publication by Campbell et al. showed a decrease in DNA repair proteins RAD51 and c-Abl, and an increase in the DNA damage marker phospho-H2AX (γ-H2AX) with lentiviral knockdown of CAPER in MDA-MB-231 cells. Interestingly, when MDA-MB-231 cells are treated with the CAPER peptides, an increase in phospho-H2AX (γ-H2AX) and decreases in RAD51 and c-Abl was also observed. However, when we investigated RAD51 and c-Abl protein expression in BT-549 cells, the only significant decrease observed was with c-Abl using CAPER peptide HCC1.4. However, an increase in

phospho-H2AX(γ-H2AX) was observed with peptide HCC1.3. Historically, H2AX(γ-H2AX) has been used as a marker of DNA damage however it should be noted that its phosphorylation can also be the result of the apoptotic process [23]. Further experimentation via cellular localization of H2AX (γ-H2AX) upon treatment with the CAPER peptides would be required to fully understand the mechanisms presented here.

TNBC is a highly heterogeneous disease and differences in cell line responses is not unexpected, as the molecular profile of the tumor can alter the cells response to different therapeutic agents [24,25]. While both cell lines originate from invasive ductal carcinoma, they have significant differences that demonstrate the complexity of TNBC. BT-549 and MDA-MB-231 cells are classified as mesenchymal (M) and mesenchymal like (ML) TNBC cells, respectively. Additionally, BT-549 cells were derived from a primary tumor, whereas MDA-MB-231 cells were derived from a pleural effusion [26]. It should be noted that BT-549 cells contain a homologous deletion for PTEN, which has been shown to play a role in the control of DNA repair, specifically having transcriptional control of RAD51 [27]. In fact, a publication by Zhao et al., showed that the RAD51 inhibitor, RI-1, had little to no effect on the efficiency of double stranded DNA break repair seen in BT-549 cells, therefore suggesting that this cell line is not as reliant on RAD51 for DNA repair [27]. Interestingly, CAPER peptide HCC1.3 still showed an increase in phospho-H2AX (γ-H2AX) in BT-549 cells, without a decrease in RAD51 or c-Abl. This result may be indicative of another unknown role of CAPER within the DNA repair pathway.

When BT-549 cells were treated with CAPER peptide HCC1.3, no decrease in c-Abl was observed. However, this decrease was observed when treating this cell line with CAPER peptide HCC1.4. c-Abl is a tyrosine kinase that has been implicated in many aspects of cancer from survival, proliferation, angiogenesis, and invasion, additionally playing a role in DNA repair [28]. The differences seen with c-Abl between the two cell lines, as well as between the two CAPER peptides in the BT-549 cells may be indicative of 1) a different role of c-Abl in BT-549 cells vs. MDA-MB-231 cells and 2) isoform differences between the CAPER peptides which may be cell line dependent. A publication by Chevalier et al. (2015) showed that treatment of these same cell lines with c-Abl inhibitors, showed opposite effects; with the MDA-MB-231 cells having a decrease in invasiveness, whereas the BT-549 cells showed an increase [29]. This was confirmed with shRNA knockdown of c-Abl [29]. If we consider cell line specific roles of c-Abl together with the fact that CAPER peptide HCC1.4 showed lesser efficacy in the BT-549 cell line, one might hypothesize that the different isoform activity of CAPER peptide HCC1.4 observed in BT-549 cells, which resulted in a decrease in c-Abl, is an unwanted effect in this cell line. This mechanism may be in opposition to the mechanism seen with the modulation of c-Jun and the decrease in p21, resulting in the decreased efficacy observed. Little is known about the functional differences of CAPER isoforms in cancer, though it has been reported that overexpression of full-length CAPER isoform HCC1.4 did indeed cause lung cancer cells to proliferate at a much higher rate as compared to CAPER isoform HCC1.3 [9]. Therefore, further investigation into the function of the different CAPER isoforms within different cancer cell lines is warranted.

The mechanisms by which CAPER peptides are decreasing proteins involved in DNA repair in MDA-MB-231 cells is unknown. However, a theory presented by Campbell et al. (2018) suggests that RAD51, c-Abl, and CAPER are forming a complex which facilitates repair of DNA double-stranded breaks. If CAPER is missing, this complex cannot form and therefore RAD51 and c-Abl levels may be decreased as the uncomplexed forms may represent an unstable conformation favorable for ubiquitination and ultimately degradation. Alternatively, these results may be due to CAPER's alternate splicing function causing a decrease in these proteins via alternative splicing defects [11,30]. Although our studies confirm the results observed by Campbell et al., further studies are warranted to fully understand the role that CAPER in the DNA repair process.

These findings show promise for further development of CAPER peptides for the treatment of TNBC. The role of CAPER as a possible target for cancer treatment is only beginning to be understood. It would be interesting to further investigate its possible role in other aspects of cancer, such as invasion and migration or epithelial to mesenchymal transition. While targeting transcription factors has historically been challenging, recent publications have demonstrated that peptides can be useful in disrupting the activity of transcription factors which were once considered to be undruggable [31]. Future exploration of CAPER peptides can include encapsulation in liposomes or nanoparticles to facilitate *in vivo* studies, or the structure of the peptide can be used for small molecule prediction screening thus helping to facilitate the transition to a molecule that would be more clinically relevant. In summary, the data presented here show for the first time the use of CAPER peptides as a promising mechanism for the treatment of patients with TNBC.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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