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KR-33028, a potent inhibitor of the Na^+/H^+ Exchanger NHE1, suppresses metastatic potential of triple-negative breast cancer cells

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Abbreviations: HMA [5-(N, N-hexamethylene)-amiloride; NHE1, Na^+/H^+ exchanger isoform 1; pH_i, intracellular pH; TNBC, triple negative breast cancer;

Abstract

Hyper-activation of the Na^+/H^+ exchanger NHE1 occurs at the onset of oncogenic transformation and plays a critical role in breast cancer carcinogenesis. Dysregulation of NHE1 activity results in intracellular alkalinization and the acidification of the extracellular tumor microenvironment that promotes metastasis. Hence, the use of chemical inhibitors of NHE1 as chemotherapeutic agents is an alluring prospect. We previously demonstrated that two structurally different NHE1 inhibitors, EMD87580 [(2-methyl-4,5-di-(methylsulfonyl)-benzoyl)-guanidine], and HMA [5-(N, N-hexamethylene)-amiloride], were effective as co-adjuvants to potentiate paclitaxel-mediated cytotoxic chemotherapy in triple-negative breast cancer (TNBC) cells. Both these drugs, however, had reduced or minimal anti-cancer effects when used alone. Here, we tested KR-33028 (4-cyano (benzo[*b*]thiophene-2-carbonyl)guanidine), a potent and selective inhibitor of NHE1, to determine its efficacy in inhibition of metastatic potential of TNBC cells. In highly invasive MDA-MB-231, moderately invasive MDA-MB-468, and lowly invasive Hs578T TNBC cells, KR-33028 considerably reduced rates of cell migration and anchorage-independent colony growth. Invasion of MDA-MB-231 and MDA-MB-468 cells through extracellular matrix was also dramatically decreased in response to KR-33028. We further tested the effect of KR-33028 on MDA-MB-231 cells lacking NHE1 expression (231koNHE1); no differences were observed between untreated control and KR-33028-treated 231koNHE1 cells. Taken together, our results highlight the *in vitro* efficacy of KR-33028-mediated NHE1 inhibition on limiting cellular functions that are predictive of metastasis *in vivo*. We suggest that targeting NHE1 in the development of novel chemotherapeutics could be highly effective in combatting triple-negative breast cancer and that KR-33028 is potentially useful in prevention of metastasis.

Keywords: NHE1, triple-negative breast cancer, metastasis, KR-33028.

1. Introduction

At the onset of oncogenic transformation, several cellular processes are dysregulated. The dysregulation of pH homeostasis, in particular, is key in the establishment of the acidic extracellular tumor microenvironment that maintains the transformed phenotype and facilitates metastasis. This is chiefly due to the aberrant behavior of the major pH regulatory protein, the Na^+/H^+ exchanger NHE1 [1, 2]. NHE1 is an integral membrane protein 815 amino acids in length that is ubiquitously expressed in mammalian cells [3]. It comprises an N-terminal trans-membrane domain through which ion exchange occurs, and a cytosolic C-terminal domain that regulates exchanger activity via phosphorylation by kinases and association with lipid and protein binding partners [4, 5]. NHE1 plays a critical role in cancer cells, becoming hyperactive in cells undergoing neoplastic transformation. This hyper-activation results in a reversal of the proton gradient and intracellular alkalinization, and causes acidification of extracellular interstitial spaces between tumor cells. The hypoxic and serum-depleted tumor microenvironment further promotes NHE1 hyper-activation [6]. In metastatic cancer cells, NHE1 localizes to leading edges of cells, accumulating in invasive structures like lamellipodia and invadopodia, where high proton extrusion provides the optimal acidic pH for extracellular matrix digestion by proteolytic enzymes [7, 8]. Indeed, when NHE1 is knocked out in highly metastatic triple-negative MDA-MB-231 breast cancer cells, xenograft tumor growth in athymic nude mice is almost abolished [9]. Furthermore, mutations to regulatory sites on the NHE1 C-terminal tail significantly modulate the behavior of metastatic triple-negative breast cancer cells [10]. Consequently, there is a growing body of evidence supporting the potential for pharmacological inhibition of NHE1 as a novel chemotherapeutic strategy [6, 11].

There are two major classes of NHE1 inhibitors based on their chemical structure: amiloride and its more potent analogues (e.g. 5-(N, N-hexamethylene)-amiloride; HMA;

pyrazinoyl guanidines), and the highly selective acylguanidines (e.g. cariporide, benzoyl guanidine). However, neither type of NHE1 inhibitor has been developed as successful anti-cancer agents in humans. The use of amiloride in cancer therapy in animal models and clinical trials was recently reviewed [12], as was cariporide and other novel NHE1 inhibitors [13]. In this study, we focused on a new, potent and selective small molecule NHE1 inhibitor, KR-33028 (4-cyano (benzo[*b*]thiophene-2-carbonyl)guanidine) [14, 15]. KR-33028 was previously evaluated for cardioprotective effects [16] and was shown to reduce myocardial infarction due to ischemia-reperfusion injury in rats and dogs [17]. Similarly, KR-33028 was also found to be protective against cell death due to glutamate toxicity in cultured neuronal cells and significantly reduced the size of cerebral infarcts due to ischemic injury in rats [18]. We evaluated the potential chemotherapeutic effects of KR-33028 against triple-negative breast cancer (TNBC) for which targeted therapies do not currently exist. We found that KR-33028 significantly limits metastatic potential in highly invasive MDA-MB-231 and moderately invasive MDA-MB-468 cells, dramatically decreasing rates of migration and invasion, and anchorage-independent colony growth. Even in lowly invasive Hs578T TNBC cells, rates of migration and anchorage-independent growth were reduced. KR-33028 inhibition resulted in a cell phenotype comparable to cells where NHE1 was knocked out. Taken together, our data strongly suggest the potential for KR-33028 as a novel chemotherapeutic agent in the treatment of triple-negative breast cancer.

2. Materials and Methods

2.1. Chemicals and Reagents

KR-33028 [4-cyano (benzo[*b*]thiophene-2-carbonyl)guanidine was from Peacock Pharma, Germany; PubChem CID: 11515637]. Amiloride hydrochloride hydrate [N-Amidino-3,5-diamino-6-chloropyrazinecarboxamide hydrochloride hydrate; PubChem CID: 16230] and HMA [5-(N, N-hexamethylene)-amiloride; PubChem CID: 1794] were from Sigma-Aldrich, United States. EMD87580 [(2-methyl-4,5-di-(methylsulfonyl)-benzoyl)-guanidine was a gift from Merck, Germany].

2.2. Cell lines and culture conditions

MDA-MB-231 and MDA-MB-468 cells were cultured in high-glucose modified DMEM (HyClone) supplemented with 10% fetal calf serum (HyClone), 10 mM HEPES, and 1000 units/ml penicillin/streptomycin (Gibco) under standard culture conditions (5% CO_2, 37°C and high humidity). An NHE1-knockout version of the parental MDA-MB-231 cells was generated using CompoZr® Zinc Finger Nucleases (Sigma-Aldrich) specifically designed for the excision the human NHE1 gene, *SLC9A1*, as previously reported [9]. MDA-MB-231 cells with NHE1 knocked out (231koNHE1 cells) were cultured under the same conditions. Hs578T cells were grown in similar culture media but without penicillin/streptomycin and additionally supplemented with 0.01 mg/ml bovine insulin. Serum-deprived (stimulated conditions) media contained 0.2% serum but was otherwise identical in composition for each respective cell type; in unstimulated conditions, media contained 10% serum. All cell lines were authenticated by DNA analysis (DDC Medical, Ohio) and showed >95% homology to the ATCC DNA-STR profile.

2.3. Measurement of intracellular pH

Cells were seeded on rectangular glass coverslips in 35 mm culture dishes, grown to approximately 80% confluence, and either stimulated by serum deprivation (STIM, 0.2% serum) or left unstimulated (UNSTIM, 10% serum) overnight. The rate of recovery of pH_i after acidification by ammonium chloride was assessed using BCECF-AM (2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester) [9]. Cells were loaded with 3 μg/ml BCECF-AM for 20 min. at 37°C. The ratio of de-esterified BCECF fluorescence (excitation at 440 nm and 502 nm, and emission at 528 nm) was quantified using a PTI Deltascan spectrofluorometer (Photon Technology International, New Jersey, USA) as described earlier [9]. NHE1 activity due to KR-33028 was a measure of changes in intracellular pH (pH_i) in response to acute acid load using a two-pulse acidification assay as previously described [19]. In the first pulse, acute acid load was induced by treatment with NH_4Cl (50 mM for 3 min.) followed by incubation in Na^+-free (and NH_4Cl free) buffer and recovery in Na^+-containing buffer. A second NH_4Cl-induced acidification and recovery was induced immediately after the first recovery and was the same except that the second recovery was in the presence of KR-33028 at the indicated concentration. NHE1 activity (rate of recovery post-acid load) was expressed as ΔpH/sec and calculated from the slope of the first 20 sec. of recovery from acidification. The ratio of the rate of recovery of the second pulse *versus* the first pulse was used to evaluate effects of KR-33028. Controls were treated with equivalent amounts of DMSO in either stimulated and unstimulated cells and data were normalized for comparison with KR-33028-treated cells in the same culture conditions. In experiments to determine the IC_{50} of KR-33028 in MDA-MB-231 cells, varying concentrations of KR-33028 were used as indicated and the percent NHE1 activity was plotted and calculated using SigmaPlot (Systat Software, CA, US). For comparison, IC_{50} values for NHE1 inhibitors amiloride-HCl, hexamethylene amiloride (HMA), and EMD87580 were also determined.

2.4. Western Blot Analysis

Expression of NHE1 protein was confirmed by western blot analysis. Confluent cells were lysed and total protein was resolved on 10% SDS polyacrylamide gels prior to transferring to nitrocellulose membranes. Blots were incubated overnight with either anti-NHE1 antibody (BD Biosciences) or anti-β-tubulin (Sigma) antibodies. Protein was detected using chemiluminescence. The levels of the NHE1 immunoreactive protein present on the western blots were estimated using Image J 1.35 software (National Institutes of Health, Bethesda, Maryland, USA) and corrected for levels of β-tubulin.

2.5. Cytotoxicity and Proliferation

Cytotoxicity of KR-33028 and its effect on cell proliferation were evaluated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma] colorimetric assays as described earlier [9]. Briefly, 1×10^4 cells per well were seeded in a 96-well plates and allowed to adhere overnight. Cells were then treated with KR-33028 or DMSO (controls) in phenol red-free DMEM for a further 24 hr. in standard culture conditions before adding MTT at a final concentration of 0.5 mg/mL. For cytotoxicity assays, serial dilutions of KR-33028 were used at the indicated concentrations; for proliferation assays, 5 μM KR-33028 was used. Conversion of MTT from soluble tetrazolium (yellow) to insoluble formazan (purple) is dependent on cellular metabolic activity and is indicative of cell death (loss of viability) and net growth (rate of proliferation). Intracellular formazan was solubilized with DMSO and absorbance at 570 nm was read with a BioTek Synergy MX microplate reader (BioTek Instruments Inc.), with a background

subtraction at a reference wavelength of 630 nm. Data were analyzed with BioTek Gen5 software, and presented as the rate of proliferation over time, or percent viability (cytotoxicity).

2.6. Migration

The rate of cell migration was evaluated using wound-healing assays [9]. Cells were seeded in 6-well plates in equal numbers and grown to a confluent monolayer prior to inducing a scratch (wounding) with a pipette tip. Rate of gap closure was monitored over 24 hr. in the presence of 2.5 μM KR-33028 or DMSO (control). Images were acquired at 0 and 24 hr. and measurements of gap closure were quantified with Image Pro Plus software using a Leica DM IRB microscope (at 10X magnification). Three images per well and five measurements per image were acquired for each treatment; all treatments were done in triplicate. Data show mean percent gap closure at 24 hr. compared to 0 hr. over multiple independent experiments.

2.7. Invasion

Invasiveness of cells in response to treatment with KR-33028 was assessed using transwell invasion assays. Transwell inserts (24-well, Corning) with 8 μm pores were coated with Matrigel™ extracellular matrix (BD Biosciences). 1×10^5 cells were added to Matrigel™-coated inserts in serum-depleted media (0.2% serum) in the presence of 5 μM KR-33028 or DMSO (control) and allowed to invade for 24 hr.; bottom reservoir media contained 5 μM KR-33028 or DMSO plus 10% serum to create a chemotactic gradient for movement of cells. Cells adhered to bottom of inserts after 24 hr. invasion were fixed in 3.7% paraformaldehyde and permeabilized in 100% methanol prior to staining with Giemsa. Cells were visualized with Image Pro Plus

software using a Leica DM IRB microscope at a magnification of 10X. Data shown are a mean of 5 fields per insert, and triplicate inserts per experiment, over multiple independent experiments.

2.8. Soft Agar Colony Growth

Anchorage-independent colony growth was assessed by colony formation in soft agar as previously described [10, 20, 21]. Briefly, 6-well plates were coated with 0.5 mL of 0.5% DNA-grade agar (Thermo Scientific) dissolved in complete growth medium and allowed to solidify at room temperature. 1×10^5 cells per well were suspended in a top layer of 0.5 mL 0.7% agar made in complete medium. This high agar concentration limits the growth of non-tumorigenic cells [21]. After the top agar layer solidified, complete growth media supplemented with 20% fetal calf serum containing 2.5 μM KR-33028 or DMSO (control) was added to wells. Media was changed every 3 to 5 days for 4 weeks. Images were taken using bright-field microscopy at 2.5X magnification. There were an estimated 15 fields per well with a seeding density of approximately 6,500 cells per field. Colonies >1 mm diameter (arbitrarily >100 cells per colony) were counted. Data are a representation of the mean number of colonies from multiple independent experiments done in triplicate.

2.9. Statistical Analysis

Data are expressed as mean ± SD of three or more independent experiments with multiple replicates. Data were plotted using KaleidaGraph 4.1 (Synergy Software, PA, US), unless otherwise indicated. Statistical analysis was done using GraphPad Prism 5.0 (GraphPad Software Inc., CA, US); two-way analysis of variance (ANOVA) was used to compare between multiple

cell types, and one-way ANOVA was used to compare within treatment groups. Post hoc comparisons of data used Bonferroni's (for two-way ANOVA) and Dunnett's (for one-way ANOVA) multiple-comparison tests.

3. Results

3.1. KR-33028 inhibits Na^+/H^+ exchanger activity in triple-negative breast cancer cells.

Prior to testing the inhibitory effect of KR-33028 on NHE1 activity in triple-negative MDA-MB-231, MDA-MB-468, and Hs578T breast cancer cells, expression of NHE1 protein was confirmed by western blotting. In breast cancer cells, NHE1 becomes hyperactive with serum withdrawal (stimulated cells); in serum-supplemented culture conditions (unstimulated cells), a basal (resting) rate of NHE1 activity is observed [9]. In both stimulated (0.2% serum, +) and unstimulated (10% serum, −) conditions, NHE1 protein expression was detected in all cells except in NHE1-knockout MDA-MB-231 cells (231koNHE1; N=3; Fig. 1a,b).

We also determined the IC_{50} values for KR-33028 in unstimulated MDA-MB-231 cells to establish a benchmark for the drug concentration used in subsequent experiments. The concentration of KR-33028 that resulted in 50% inhibition of NHE1 activity was 2.6 μM. For comparison, we determined the IC_{50} values for NHE1 inhibitors amiloride-HCl, hexamethylene amiloride (HMA), and EMD87580 to be 6.5, 0.5, and 0.6 μM respectively (N=6; Fig. 2a). We confirmed that KR-33028 was an effective NHE1 inhibitor in several cell types (MDA-MB-231, MDA-MB-468 and Hs578T) in both stimulated and unstimulated conditions. Data showed that 10 μM KR-33028 decreased NHE1 activity by over 80% in both stimulated and unstimulated conditions in all TNBC cell types (*P<0.001, N=6; Fig. 2b).

3.2. Proliferation of triple-negative breast cancer cells is not affected by KR-33028.

To investigate the cytotoxic effects of KR-33028 on triple-negative MDA-MB-231, MDA-MB-468, Hs578T and 231koNHE1 breast cancer cells, we studied cell viability in response to varying drug concentrations. There was no cytotoxic effect of KR-33028 at experimental concentrations of up to 100 μM. A significant loss of viability was only observed with KR-33028 treatment at the highest concentrations, at greater than or beginning at 1000 μM (*$P<0.001$, N=4; Fig. 3a).

We tested the effect of inhibition of NHE1 with 5 μM KR-33028, approximately twice the IC_{50} concentration, on the three TNBC cell lines and on the NHE1 KO cells (231koNHE1). There was no significant effect on cell proliferation at either 24 or 48 hr. with any of the cell lines (N=4; Fig. 3b). The effect of KR-33028 on the proliferation (net growth rate) of triple-negative breast cancer cells was not different from the effect of KR-33028 on 231koNHE1 cells which lack NHE1.

3.3. KR-33028 inhibits in vitro *metastatic potential of triple-negative breast cancer cells expressing NHE1.*

We next examined the effect of inhibiting NHE1 activity with KR-33028 on the metastatic potential of triple-negative breast cancer cells. Metastatic potential can be evaluated *in vitro* by assessing rates of cell migration, invasion through Matrigel™ matrix, and anchorage-independent colony growth in soft agar. Cell migration was measured as the rate of gap closure over 24 hr. in wound-healing assays. A pictorial representation of the results is shown in Fig. 4a. When MDA-MB-231, MDA-MB-468 and Hs578T TNBC cells were treated with 2.5 μM KR-33028, rates of

cell migration were greatly reduced ($^{\#}P<0.05$, $^{+}P>0.01$, *P<0.001, N=3; Fig. 4b). MDA-MB-231 cells with NHE1 deleted (231koNHE1) have reduced rates of migration similar to our earlier results [9]. KR-33028 inhibition did not further reduce migration of 231koNHE1 cells.

Cell invasion was determined as the number of cells traversing Matrigel™ matrix-coated transwell inserts down a chemotactic gradient, from 0.2% serum-depleted media in inserts into 10% serum-supplemented media in reservoir wells over 24 hr. Pictorial representations of numbers of invading cells adhered to the underside of inserts are shown in Fig. 5a. In the highly invasive MDA-MB-231 cells, 5 μM KR-33028 decreased the number of invading cells by approximately 60%. The same concentration of KR-33028 almost completely abrogated the invasiveness of the moderately invasive MDA-MB-468 cells, where the number of invading cells was reduced by greater than 95% (*P<0.001, N=4; Fig. 5b). There was minimal invasion by Hs578T cells similar to what we reported earlier [10] and there was little change with KR-33028 treatment. 231koNHE1 cells were approximately as invasive as MDA-MB-231 cells that were inhibited with KR-33028. Addition of KR-33028 to 231koNHE1 cells did not further reduce their invasive capacity (Fig. 5b).

We also studied the effects of KR-33028-mediated NHE1 inhibition on anchorage-independent colony growth in MDA-MB-231, MDA-MB-468, Hs578T and 231koNHE1 TNBC cells in 0.7% semisolid agar medium. Cifone and Fidler [21] described anchorage-independent colony growth in semisolid medium as a key characteristic of tumorigenic cells that distinguished them from normal or non-tumorigenic cells. They found that colony growth of non-tumorigenic cells was significantly negated in semisolid media containing greater than 0.6% agar (Insert Ref.) Thus, the ability of tumorigenic cells to form new metastases *in vivo* can be predicted from their ability to establish anchorage-independent colony growth from single cells in soft agar *in vitro*. Figure 6a is a pictorial representation of colony growth in soft agar at 2.5X magnification. In

MDA-MB-231 and Hs578T cells, treatment with KR-33028 was dramatically effective, preventing anchorage-independent colony growth in soft agar by 85% and 80% respectively (Fig. 6b). In MDA-MB-468 cells, anchorage-independent colony growth was only reduced by 35% as a result of KR-33028 treatment (*P<0.001, N=5; Fig. 6b), which was significant, but not as large an effect as in the other cell types. For comparison, in the above experiments, we also examined the effects of KR-33028 on 231koNHE1 cells, where NHE1 was knocked out. We previously reported that deletion of NHE1 from MDA-MB-231 cells drastically reduces their invasive potential [9]. Here, we observed similarly reduced anchorage-independent colony growth of 231koNHE1 cells in soft agar compared to parental cells expressing endogenous NHE1. This colony growth was unaffected by the treatment with KR-33028.

4. Discussion

KR-33028, a potent and selective inhibitor of NHE1 has previously been evaluated for its protective effects against ischemic injury in myocardial and cerebral infarction [18, 22]. However, it has not been assessed for chemotherapeutic effects in a cancer model. In the present study, we investigated the effect of KR-33028 in TNBC. Currently, no therapies specifically targeted to combat TNBC exist, and cytotoxic chemotherapy in some combination with radiation and surgery is the mainstay of treatment. Hence, treatment regimens can greatly benefit from the development of novel targeted therapies. NHE1 is one such target. Na^+/H^+ ion exchange mediated by the aberrant hyper-activation of NHE1 in tumor cells is primarily responsible for tumor cell alkalinization and the establishment of the acidic tumor microenvironment. Combined with serum deprivation and hypoxia, this acidic milieu drives the selection of a metastatic tumor cell phenotype, facilitating invasion and dissemination of cells out of the primary tumor [6]. In breast cancer, it is metastasis that is the primary cause of fatality [23]. It stands to reason that

modulating the pH of the tumor microenvironment with pharmacological inhibition of NHE1 activity could either prevent or hinder metastasis and is therefore an important area of investigation.

In our *in vitro* cell models of TNBC, KR-33028 showed a marked inhibition of metastatic potential. We tested KR-33028 on three different TNBC cell models: highly invasive, tumorigenic MDA-MB-231 cells; moderately invasive, tumorigenic MDA-MB-468 cells; and lowly invasive, non-tumorigenic Hs578T cells. MDA-MB-231 and Hs578T cells can further be categorized as mesenchymal stem-like and MDA-MB-468 cells as basal-like subtypes of TNBC [24]. To confirm that effects of KR-33028 were specific to inhibition of NHE1, we also studied its effect on MDA-MB-231 cells where NHE1 was knocked out (231koNHE1). As expected, KR-33028 at a dose 4 times the IC_{50} concentration was able to reduce Na^+/H^+ exchange activity to less than 20% of what was seen in untreated controls regardless of stimulation of NHE1 by serum deprivation. At the IC_{50} dose (~2.5 μM), a marked decrease in the rate of cell migration and anchorage-independent colony growth of TNBC cells was observed that was not seen in 231koNHE1 cells. At twice this concentration, KR-33028 was also remarkably efficient in decreasing the invasive capacity of tumorigenic MDA-MB-231 and MDA-MB-468 cells, to the extent that almost no invasion was detected with treatment in the latter cell type. Migration of cells through Matrigel™ matrix mimics *in vivo* invasion of cells out of primary tumors and assesses their ability to digest a path through the extracellular matrix, a critical step in metastasis. Our data therefore clearly demonstrate a strong inhibitory effect of KR-33028 on the invasiveness of tumorigenic TNBC cells that was not observed in cells lacking NHE1 expression.

In vitro anchorage-independent colony growth embedded in soft agar is an important assessment of tumorigenicity [21], and is considered to be predictive of the ability of tumor cells to form new metastases *in vivo* [25], so inhibition of this growth by KR-33028, even in the non-

tumorigenic Hs578T cells, is noteworthy. However, despite the efficacy of KR-33028 in limiting colony growth in TNBC cells, the drug did not affect cell proliferation at non-cytotoxic concentrations. This could be due to differences in the way cells were grown in the two experiments: adherent cell growth in proliferation assays *versus* non-adherent 3-dimensional growth of cells in semisolid agar media. Additionally, MTT assays provide an assessment of cell proliferation as a measure of metabolic activity and cell viability; it is more a net growth assay. Thus, the exact reason for this discrepancy is not currently known, but previous work in our laboratory suggests that proliferation in these TNBC cells may not be mediated by the activity of NHE1 [9, 10].

It is interesting that there was little effect of KR-33028 on invasion through Matrigel™ matrix in the lowly invasive Hs578T cells though there were effects on migration. These cells clearly have less of a dependency on NHE1 for invasion, which may account for their poor invasive properties relative to MDA-MB-231 cells and MDA-MB-468 cells. It may be that different types of TNBC have varying susceptibility to inhibition of NHE1 and that this may be due, at least in part, to the activity of NHE1 which promotes metastasis. More invasive TNBC cells may have greater NHE1 activity and a greater susceptibility to prevention of metastasis by NHE1inhibition. We have previously shown that estrogen and progesterone receptor-positive MCF7 cells of the luminal breast cancer subtype express higher levels of NHE1 protein than both MDA-MB-231 and MDA-MB-468 cells [26]. Though MCF7 cells are tumorigenic, they have typically low rates of migration and invasion, and so were not included in this study. However, it could be presumed that expression of NHE1 protein alone cannot be used as a measure of metastatic potential of these cells. Indeed, our recent work suggests that it is the underlying regulatory mechanisms controlling the activity of the exchanger that may be a key determinant of metastatic potential [27]. Further experiments are necessary to confirm this hypothesis.

Because of its multifaceted role in promoting carcinogenesis, from transformation to metastasis, NHE1 presents an attractive chemotherapeutic target. However, while effective against tumor growth in several animal studies [12], pharmacological inhibitors of NHE1 in the clinical setting have shown only limited success with several more challenges yet to be overcome [6]. Chief amongst these is the issue of drug delivery directly to tumors [28, 29]. This is particularly true in terms of targeting NHE1, where delivery of NHE1 inhibitors directly into tumors could change the acidic pH of the tumor microenvironment sufficiently enough to hinder tumorigenesis or, alternatively, to increase the efficacy of chemotherapy drugs with minimal off-target effects. An attractive aspect of KR-33028 in this regard is its small molecular weight of approximately 340 daltons, and its low number of hydrogen bond donors and acceptors which might mean that it is orally active in humans and easily absorbed. Kim et al. [30] have shown rapid absorption upon oral administration in rats.

We have previously shown that *in vitro* treatment of MDA-MB-231 and MDA-MB-468 cells with low doses of two different classes of NHE1 inhibitors, HMA and EMD87580, considerably potentiated the anti-metastatic effects of low-dose paclitaxel chemotherapy. The mechanism of the potentiation is not yet known but could be that NHE1 inhibition mediates prevention of proton extrusion which blocks NHE1 enhancement of mitosis, and this may accentuate paclitaxel anti-mitotic effects. Alternatively, inhibition of NHE1 may block reversal of the proton gradient, preventing extracellular acidification which could neutralize paclitaxel efficacy or uptake [5]. Interestingly, the co-adjuvant effect of EMD87580 or HMA with paclitaxel was not observed in hormone receptor-positive, luminal MCF-7 cells, or MDA-MB-231 cells lacking NHE1 expression [9]. In our hands, when MDA-MB-231 cells were treated with EMD87580 alone, even at high concentrations (≥ 100 μM), we did not see significant effects

on metastatic potential, while HMA alone was cytotoxic at concentrations ≥ 10 μM, so any significant effect on migration and invasion could not be properly assessed (unpublished observations). In contrast, KR-33028 was not cytotoxic at the concentrations used in this study, had a demonstrable inhibition of metastatic potential in these cells, and shows promise as a potential anti-cancer agent. We suggest that a treatment strategy mediated by pharmacological inhibition of NHE1 could be specifically exploited for the treatment of triple-negative breast cancer and prevention of metastasis upon first detection of tumors. Future *in vivo* studies will be instrumental in supporting this hypothesis.

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6. Disclosure:

The authors declare no potential conflicts of interest.

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Figure Legends:

Figure 1: Expression of NHE1 in triple-negative breast cancer cells.

NHE1 expression was examined in the triple-negative breast cancer cells MDA-MB-231, MDA-MB-231 cells where endogenous NHE1 was knocked out (231koNHE1), MDA-MB-468, and Hs578T cells. Western blot analysis was of total protein of cell lysates from cells grown in culture in either serum supplemented (−, 10% serum, UNSTIM) or serum deprived (+, 0.2% serum, STIM) conditions for 24 hr. NHE1 is visualized as two bands representing the mature glycosylated form at ~100-110 kDa, and a non or partially-glycosylated precursor protein at ~90 kDa. ***a,*** One representative western blot showing anti-NHE1 and anti-β-tubulin antibodies is

shown and is typical of three experiments. ***b***, Summary of NHE1 protein expression. Expression levels are in comparison to MDA-MB-231 cells (unstimulated, mean ± SD; N=3]

Figure 2: Determination of IC_{50} and inhibition of NHE1 activity by KR-33028.

NHE1 activity was measured using a two-pulse acidification assay as described in the Materials and Methods. The indicated concentrations of the reagent were added to the second pulse which was compared to the recovery in the first pulse in the presence of vehicle. ***a,*** Determination of IC_{50} of KR-33028 and other NHE1 inhibitors in MDA-MB-231 cells. ***b,*** Inhibition of NHE1 activity by 10 μM KR-33028 in MDA-MB-231, MDA-MB-468 and Hs578T cells in stimulated (STIM, 0.2% serum) and unstimulated (UNSTIM, 10% serum) cells [*P<0.001; N=6].

Figure 3: Effect of KR-33028 on proliferation of triple-negative MDA-MB-231, 231koNHE1, MDA-MB-468 and Hs578T breast cancer cells.

Cytotoxicity of KR-33028 and its effect on cell proliferation was assessed using the MTT colorimetric assay. ***a,*** Determination of cytotoxicity of KR-33028 across a large concentration range. Cells were treated for 24 hours [*P<0.001; N=4]. ***b,*** Effect of KR-33028 (KR) on cell proliferation. Net growth of cells in the presence of 5 μM KR-33028 was evaluated at 24 and 48 hr. time-points [P>0.05; N=4].

Figure 4: Effect of KR-33028 on migration of triple-negative breast cancer cells.

Rate of cell migration of triple-negative MDA-MB-231, 231koNHE1, MDA-MB-468 and Hs578T breast cancer cells in response to 2.5 μM KR-33028. Cell migration was evaluated using a wound-healing assay. ***a,*** Example of cell migration in TNBC cells over 24 hr. (bright-field microscopy at 10X magnification). ***b,*** Graphical representation of the rate of gap closure (wound

healing) at 24 hr. in cells cultured in complete growth media with either KR-33028 or DMSO (control) [$^{+}$P<0.01, *P<0.001; N=3].

Figure 5: Effect of KR-33028 on invasion of triple-negative breast cancer cells.

Rate of cell invasion of triple-negative MDA-MB-231, 231koNHE1, MDA-MB-468 and Hs578T breast cancer cells treated with 5 μM KR-33028 or DMSO (control). Cell invasion was a measure of the number of cells traversing through porous Matrigel™-coated transwell inserts over 24 hr. and adhering to the underside of inserts. ***a,*** Illustration of invading cells (bright-field microscopy at 10X magnification) stained with Giemsa. ***b,*** Graphical representation of the number of invading TNBC cells per field, in the presence of KR-33028 or DMSO, compared to 231koNHE1 cells [*P<0.001; N=4].

Figure 6: Effect of KR-33028 on anchorage-independent colony growth of triple-negative breast cancer cells.

In vitro cell colonization was evaluated using anchorage-independent colony forming assays in soft agar. Anchorage-independent colony formation in response to 2.5 μM KR-33028 or DMSO (control) was determined in MDA-MB-231, 231koNHE1, MDA-MB-468 and Hs578T cells over 4 weeks as described in the Materials and Methods. ***a.*** Illustration of the total number of colonies per well (bright-field microscopy at 2.5X magnification). ***b.*** Graphical representation of anchorage-independent colony formation, in response to either KR-33028 or vehicle (DMSO), in TNBC [*P<0.001; N=5].