Antitumor activity of NRC-AN-019 in a pre-clinical breast cancer model

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Abstract. Breast cancer is the second most frequently diagnosed tumor in women. Overexpression of human epidermal growth factor receptors (EGFRs) represents a biological subclass of breast cancer with distinct molecular alterations, clinical behavior and response to systemic therapy. In this study, we describe a novel compound (NRC-AN-019), which has better antitumor activity than Lapatinib. Here, we demonstrate that NRC-AN-019 is more effective in inhibiting angiogenic potential and proliferation of both MDAMB231 and HTB20/BT474 cells. FACS analysis shows that NRC-AN-019 treatment caused the accumulation of MDAMB231 and BT474 cells in the sub G0/G1 phase in a dose-dependent manner and was accompanied by increased PARP cleavage, which is indicative of apoptosis. In addition, we observed inhibition of EGFR phosphorylation in both MDAMB231 and BT474 cells. From our animal studies using SCID mice implanted with BT474 cells, we observed dose-dependent inhibition of tumor growth in NRC-AN-019-treated animals compared to controls or Lapatinib-treated mice at comparable concentrations. The dose-dependent inhibition of EGFR phosphorylation was confirmed by immunohistochemical analysis of tumor sections. In vitro results demonstrate that NRC-AN-019 is superior to Lapatinib in EGFR-overexpressing cells and has strong anti-angiogenic, anti-proliferative and pro-apoptotic properties in an EGFR-overexpressing background (BT474). In vivo studies demonstrate that the antitumor activity of NRC-AN-019 is better over Lapatinib. These results suggest that NRC-AN-019 has greater therapeutic potential in the treatment of Her-2-positive breast cancer.

Introduction

Recent studies have shown that breast cancer incidence in women in the United States is 1 in 8 (~13%). In 2008, an estimated 182,460 new cases of invasive breast cancer were diagnosed in women in the US, along with 67,770 new cases of non-invasive (in situ) breast cancer. Recent world-wide statistics have determined that in 2010, 1.5 million people worldwide will be diagnosed with breast cancer (1).

Targeted therapies with compounds that inhibit a specific target molecule represent a new perspective in the treatment of breast cancer. The ErbB family of receptor (EGFR, also called HER-1, ErbB-1), HER-2 (neu, ErbB-2), HER-3 (ErbB-3) and HER-4 (ErbB-4). All four receptors are tyrosine kinases and consist of an extracellular ligand-binding domain, a single membrane-spanning region, and intracellular tyrosine kinase and regulatory domains. The progression of malignant tumors is a consequence of multiple alterations of the genome such as Her-2. It is a protein with extracellular and intracellular domain with tyrosine kinase activity involved in signal transduction of cell growth and development. Amplification of the HER-2 receptor tyrosine kinase has been implicated in the pathogenesis of 25% of invasive human breast cancers. The aberrant signaling of HER-2 also contributes to tumor initiation and disease progression (2). Inhibition of HER-2, dramatically enhances the anti-tumor activity of chemotherapy (3).

The agents that are currently approved for HER-2-positive breast cancer include trastuzumab and Lapatinib, though, trastuzumab treatment is widely used for the treatment of HER-2-positive breast cancer, only 15% response as monotherapy and 49% as combination therapy have been achieved. On the other hand, lapatinib, a small molecule tyrosine kinase inhibitor is approved for the treatment of advanced or metastatic HER-2 positive breast cancer in combination with capecitabine.

In this study, we demonstrate the antitumor activity of NRC-AN-019, a small molecule tyrosine kinase inhibitor with dual activity on HER-2 and pEGFR expression. We have also made and attempt to compare the efficacy of the NRC-AN-019 with Lapatinib in in vitro and in vivo models with vehicle as a negative control.
Materials and methods

Compounds. The test compound NRC-AN-019 was dissolved in DMSO (dimethyl sulfoxide) and used. DMSO was used as a vehicle control.

Cell and culture conditions. MDAMB231 and HTB20/ BT474 cells were obtained from ATCC. MDAMB231 cells were cultured in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum and cultured at 37°C in a 5% CO₂ humidified atmosphere. BT474 cells were cultured in ATCC complete 46-X Hybri-Care growth medium supplemented with 1.5 g/l sodium bicarbonate and 10% fetal bovine serum at 37°C in a 5% CO₂ humidified atmosphere.

TUNEL assay and determination of IC₅₀ values. We determined the induction of apoptosis in breast cancer cells after we treated breast cancer cell lines MDAMB231 and BT474 with Lapatinib (0-317.9 µM) or NRC-AN-019 (0-579.7 µM). TUNEL assay was carried out using the APO-BrdU™ TUNEL Assay Kit (Invitrogen) as per the manufacturer's instructions. Percent apoptosis was determined by flow cytometry. IC₅₀ values were determined according to a previously described protocol after seven days of drug treatment (4).

Angiogenic assay. We determined in vitro angiogenesis of MDAMB231 and BT474 breast cancer cells in the presence of specified concentrations of experimental compounds. Briefly, cells (2x10⁴/well) were seeded in 8-well chamber slides and treated with various concentrations of NRC-AN-019 and Lapatinib. After a 24-h incubation period, conditioned medium was removed and added to a 4x10⁴ human dermal endothelial cell monolayer in 8-well chamber slides, and the human dermal endothelial cells were allowed to grow for 72 h. Cells were then fixed in 3.7% formaldehyde and stained with H&E and photographed. Angiogenesis was quantified as percent of controls based on the formula modified from a previously described method:

\[
\frac{\sum (Bx \times Nx)}{\sum (Bc \times Nc)} \times 100
\]

where Bc is the number of branch points, Nc is the number of branches around a branch point of control, Bx is the number of branch points, Nx is the number of branches around a branch point of treatments, and a is the number of branch points counted, which is equal in both control and treated groups. Five replications were done to obtain statistically significant results where a=50 which represents the number of branch points counted (5).

MTT proliferation assay. We determined the change in cell proliferation of breast cancer cells after we treated breast cancer cell lines MDAMB231 and BT474 with Lapatinib (0-211.9 µM) or NRC-AN-019 (0-386.5 µM). MTT assay was performed in a 96-well plate as per standard protocol. Briefly, 10,000 cells in 200 µl media per well were plated in a 96-well plate and incubated overnight at 37°C in 5% CO₂ to allow for cell attachment. Vehicle or NRC-AN-019 (0-19.3 µM) or Lapatinib (0-10.5 µM) dissolved in DMSO were added to each plate (2 µl/well max) and placed on a shaking table at 150 rpm for 5 min for thorough mixing. The plate was further incubated at 37°C in 5% CO₂ for 72 h to allow the drug to take effect. A 5 mg/ml of MTT solution in PBS was made fresh, and 20 µl of MTT solution were added to each well and placed on a shaking table (150 rpm) for 5 min to thoroughly mix the MTT into the media. The plate was further incubated at 37°C in 5% CO₂ for 1 h to allow the MTT to be metabolized after which excess media were discarded and the formazan precipitate resuspended in 200 µl DMSO. The precipitate was allowed to dissolve in DMSO completely and optical density at 560 nm was measured and subtracted from background at 670 nm.

Antibodies. Antibodies were obtained from Abcam (Cambridge, MA). Cleaved PARP antibody (cat. no. ab4830) specifically recognizes the 85-kDa fragment of cleaved PARP. Ki-67 is a cell cycle-related nuclear protein expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2 and M phase) but is absent in G0 cells, and hence is an indicator of proliferation (cat. no. ab15580). EGFR (cat. no. ab24340) and pEGFR (cat. no. ab40815) overexpression is indicative of proliferating tumor cells and poor prognosis. GAPDH antibody was used to verify loading controls (cat. no. ab8245).

Western blot analysis. We carried out Western blot analysis of MDAMB231 and BT474 breast cancer cells in the presence of specified concentrations of compounds as per standard protocols. Cells were treated with vehicle or NRC-AN-019 or Lapatinib at the specified concentrations. Twenty-four hours after treatment, cells were collected and cell lysates extracted. Equal quantities of proteins (100 µg) were fractionated by SDS-PAGE. The fractionated proteins were blotted onto nylon membranes and immunoprobed for cleaved PARP, Ki-67, EGFR and pEGFR as per standard protocols. The membranes were stripped and levels of GAPDH were determined; GAPDH served as a loading control.

Cell cycle analysis. To determine the change in cell cycle progression of breast cancer cells when treated with NRC-AN-019 or Lapatinib, we treated breast cancer cell lines MDAMB231 and BT474 with Lapatinib (0-105.9 µM) or NRC-AN-019 (0-193.2 µM) and cultured for 12 h. Higher molar concentrations of NRC-AN-019 were used to maintain equal weight concentrations used. Cells were then trypsinized and treated with 50 µg/ml propidium iodide + 0.001% RNase-A solution as per standard protocols and sorted on a fluorescence-activated cell sorter (10,000 cells sorted).

Quantification of pEGFR expression by ELISA. We performed ELISA as per manufacturer's instructions (Cell Signaling Pathscan ELISA kit no. 7250, Danvers, MA) to determine total pEGFR levels. Briefly, cells were lysed in 1X cell lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM ethylene diamine tetraacetate (EDTA), 1 mM ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetate (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₂VO₃, 1 µg/ml leupeptin] followed by sonication. Each diluted cell lysate (100 µl) was added to the appropriate well
and incubated for 2 h at 37°C. Then, the detection antibody was added for 1 h at 37°C followed by HRP-conjugated secondary antibody for 30 min at 37°C. Detection was done by the addition of 100 µl of TMB substrate to each well followed by the addition of 100 µl of STOP solution. Absorbance was read at 450 nm within 30 min of STOP solution addition.

Animal studies. For this study, SCID mice were used (homozygous genotype: NOD.CB17-Prkdc^scid/NCrHsd, NOD.CB17/JHliHsd-Prkdc^scid). We used 8 mice per group (4 female and 4 male). Animals were subcutaneously implanted with 2x10^6 BT474 cells. After tumors developed (2-3 mm diameter), drug treatment was initiated. For Lapatinib: 30 and 100 mg/kg (two times a day) for 21 days by oral gavage. For NRC-AN-019: 10, 20 and 40 mg/kg once daily for 30 days by oral gavage. Periodic tumor volumes were determined. Thirty days after initiation of drug treatment, mice were sacrificed and tumors harvested. Tumor volumes were measured using the formula,

\[
tumor \text{ volume} = \frac{\pi}{6} (l \times s^2)
\]

where \(l\) = large diameter and \(s\) = small diameter of tumor. After termination of the experiment, subcutaneous tumors were harvested and processed for paraffin sectioning followed by immunohistochemistry for pEGFR expression. All animal procedures were approved by the University of Illinois College of Medicine at Peoria, Instructional Animal Care Committee.

Immunohistochemistry. Tumors resected from SCID mice treated with NRC-AN-019, Lapatinib or vehicle controls were processed for paraffin sectioning as per standard protocols. Sections were deparaffinized as per standard protocol followed by blocking in 1% BSA in PBS for 1 h, and the sections were subsequently transferred to primary antibody pEGFR diluted in 1% BSA in PBS (1:500). Sections were allowed to incubate in the primary antibody solution for 2 h at 4°C in a humidified chamber, followed by washing in 1% BSA in PBS (three 5-min washes), and further followed by the addition of appropriate HRP-conjugated secondary antibody in 1% BSA in PBS. After secondary antibody addition and subsequent washing, the sections were allowed to incubate in HRP DAB substrate as per standard protocols to develop color. Transmitted light images were obtained after counterstaining with hematoxylin as per standard protocol to visualize the morphology of the sections. A control study was performed using a normal rabbit immunoglobulin fraction as the primary antibody, which served as the negative control. The expression levels of pEGFR were quantified by measuring DAB pixel density per unit area (DAB pixels per 10 fields using a 1024x1024 image) and graphically represented as percent expression.

Results

NRC-AN-019 induces apoptosis in Lapatinib-resistant BT474 breast cancer cells. It was observed that NRC-AN-019 efficiently induced apoptosis at concentrations as low as 1.9 µM in MDAMB231 cells, and 96.6 µM in BT474 cells. From the quantitative analysis of apoptotic induction in MDAMB231 and BT474 cells, we observed that apoptotic induction by NRC-AN-019 in MDAMB231 was more effective than Lapatinib treatment in a dose-dependent manner (Fig. 1A). Similar response was noted in BT474 also.

IC_{50} values were determined by graphical extrapolation on semi-log graphs, where we observed that at least 0.016 µM of NRC-AN-019 was required for 50% apoptotic induction in BT474 cells when compared to 0.026 µM of Lapatinib. In MDAMB231-treated cells, IC_{50} value of NRC-AN-019 was 0.085 µM compared to Lapatinib, which was 7.01 µM (Fig. 1B).

NRC-AN-019 is more efficient in retarding the angiogenic potential of MDAMB231 and BT474 cells than Lapatinib. To determine the inhibition of angiogenic potential by NRC-AN-019, MDAMB231 and BT474 cells were treated with Lapatinib (0-105.9 µM) or NRC-AN-019 (0-193.25 µM) followed by human dermal endothelial cells (HMEC) grown in the presence of conditioned media from these cells. We observed that under control conditions, HMEC cultured with MDAMB231 or BT474-conditioned media formed a network-like structure, which is indicative of angiogenesis. MDAMB231 cells treated with NRC-AN-019 or Lapatinib showed a dose-dependent response of angiogenic inhibition with NRC-AN-019 showing more angiogenic inhibition than Lapatinib. BT474 cells treated with Lapatinib showed little angiogenic inhibition as compared to NRC-AN-019 treatment (Fig. 2A). Quantitative analysis...
revealed that MDAMB231 cells had a similar decrease in angiogenic potential with either NRC-AN-019 or Lapatinib treatment. However, NRC-AN-019 showed better reduction in angiogenesis than Lapatinib in MBAMB231 cells at comparable concentrations (Fig. 2B). Similar reduction in angiogenic potential was also seen in BT474 cells (Fig. 2C).

NRC-AN-019 retards the proliferative potential of MDAMB231 and BT474 cells. We further determined the inhibition of proliferation in BT474 and MDAMB231 cells treated with various concentrations of NRC-AN-019 or Lapatinib using standard MTT assay. Quantitative analysis of percent proliferation by MDAMB231 breast cancer cells treated with various concentrations of NRC-AN-019 or Lapatinib revealed that both NRC-AN-019 and Lapatinib treatments had a dose-dependent effect in decreasing proliferation (Fig. 3A). Similarly, quantitative analysis of percent proliferation by BT474 cells treated with various concentrations of NRC-AN-019 or Lapatinib revealed that Lapatinib treatment did not result in a dose-dependent decrease in proliferation when compared to NRC-AN-019 treatment (Fig. 3B).

To confirm the decrease in proliferation in MDAMB231 and BT474 cells by NRC-AN-019, we determined the expression levels of Ki-67 using Western blot analysis. Expression levels of Ki-67 in MDAMB231 cells decreased in a dose-dependent manner in cells treated with both Lapatinib and NRC-AN-019. The decrease of Ki-67 in NRC-AN-019-treated MDAMB231 cells was more potent than in cells treated with Lapatinib at comparable concentrations. BT474 cells had the least decrease in Ki-67 expression levels after Lapatinib treatment whereas NRC-AN-019 treatment resulted in a dose-dependent decrease in Ki-67 expression (Fig. 3C). GAPDH expression levels served as loading controls.

NRC-AN-019 causes the accumulation of Lapatinib-resistant BT474 cells in the G0/1 phase and induces PARP cleavage in a dose-dependent manner. To further determine the effect of NRC-AN-019 on the cell cycle, we carried out FACS analysis as per standard protocols. Dose-dependent accumulation of MDAMB231 in sub G0/1 phase was observed when treated with increasing concentrations of Lapatinib and NRC-AN-019. However, greater accumulation was seen in NRC-AN-019-treated cells (Fig. 4). In contrast to MDAMB231 cells, BT474 cells behaved differently when treated with Lapatinib and exhibited no dose-dependent accumulation of cells in the sub G0/1 phase. However, like the MDAMB231 cells, BT474 cells treated with NRC-AN-019 showed a dose-dependent increase of cells in the sub G0/1 phase (Fig. 4A). Quantitative analysis revealed that NRC-AN-019 more efficiently causes accumulation of both MDAMB231 and BT474 cells in the sub G0/1 phase when compared to Lapatinib (Fig. 4B).

To further confirm the accumulation of cells in the sub G0/1 phase, Western blot analysis for cleaved PARP was carried out. We observed that both NRC-AN-019 and Lapatinib caused an increase in PARP cleavage in a dose-dependent manner in MDAMB231 cells, with NRC-AN-019 showing increased cleavage of PARP when compared to Lapatinib at comparable doses. In BT474 cells, little or no PARP cleavage was observed.
after Lapatinib treatment whereas NRC-AN-019 treatment caused a dose-dependent increase in the cleavage of PARP, which is indicative of nuclear fragmentation (Fig. 4C).

**NRC-AN-019 retards Lapatinib-resistant subcutaneous BT474 tumor growth in a dose-dependent manner in nude mice.** To further validate whether NRC-AN-019 could have an anti-tumor effect in vivo, we subcutaneously implanted BT474 cells in SCID mice and treated the mice with 10, 20 or 40 mg/kg once daily with NRC-AN-019 or 30 or 100 mg/kg of Lapatinib twice daily. We observed that in the controls, tumors developed uniformly and were of comparable size in both male and female mice. Mice treated with 10 mg/kg of NRC-AN-019 showed a decrease in tumor size when compared to the controls with no discernable variation between male and female mice. A further decrease in tumor size was observed in mice treated with 20 mg/kg of NRC-AN-019. Mice treated with 40 mg/kg of NRC-AN-019 showed the most significant decrease in tumor size with female mice showing the most decrease. Lapatinib-treated mice at 30 mg/kg were similar to controls while we observed a dose-dependent decrease in tumor size in mice treated with 100 mg/kg of Lapatinib. Of note, the decrease in tumor size in mice treated with 10 mg/kg of NRC-AN-019 was similar to mice treated with 100 mg/kg of Lapatinib (Fig. 6A). Quantification of tumor volumes revealed that mice treated with 10 mg/kg of NRC-AN-019 had a tumor volume of 4.3±0.2 mm³, mice treated with 20 mg/kg had a tumor volume of 4.1±0.1 mm³, and mice treated with 40 mg/kg had a tumor volume of 2.4±0.2 mm³. Mice treated with 30 mg/kg of Lapatinib had a tumor volume of 11±1.0 mm³, and mice treated with 100 mg/kg had a tumor volume of 4.8±1.2 mm³; control animals had a tumor volume of 13.2±1.0 mm³ (Fig. 6B). (p>0.002)

To validate the in vitro results for pEGFR inhibition, we carried out immunohistochemical analysis for pEGFR expression. Control tumors showed intense staining of pEGFR as observed from DAB reaction for HRP. A dose-dependent decrease in the expression levels of pEGFR was observed in tumor sections of mice treated with NRC-AN-019. Tumor sections from mice treated with NRC-AN-019 at high doses also showed a decrease in pEGFR expression.
sections from mice treated with Lapatinib also showed a dose-dependent decrease in pEGFR expression but the decrease was not as significant as those observed with NRC-AN-019 treatment (Fig. 6C). Quantitative analysis of pEGFR expression normalized to controls indicated that NRC-AN-019-treated mice had a pEGFR expression of 40±11% (10 mg/kg), 22±7% (20 mg/kg) and 7.5±2% (40 mg/kg). In contrast, mice treated with Lapatinib had a pEGFR expression of 76±5% (30 mg/kg) and 61±2% (100 mg/kg) (Fig. 6C).

Discussion

Preclinical experiments, clinical experience with the use of trastuzumab beyond progression, and a phase III clinical trial with Lapatinib (a dual EGFR/HER-2 tyrosine kinase inhibitor) demonstrate that the HER-2 signaling axis remains an important therapeutic target (6). Trastuzumab is a monoclonal antibody targeted against the human epidermal growth factor receptor (HER-2), which is overexpressed in ~25% of invasive breast cancers. However, the majority of patients with metastatic breast cancer who initially respond to trastuzumab demonstrate disease progression within one year of treatment initiation (7).

In the present study, we demonstrate that NRC-AN-019 shows greater anti-tumor activity than Lapatinib. From our TUNEL assay results, it is evident that NRC-AN-019 shows good activity in BT474 cells even at doses as low as 19.3 µM and also has a significantly low IC₅₀ value when compared to Lapatinib.
support angiogenesis in preclinical models, suggesting that angiogenesis precedes transformation of mammary hyperplasia to malignancy. Transfection of tumor cells with angiogenic stimulatory peptides has been shown to increase tumor growth, invasiveness and metastasis (8,11-14). Conversely, transfection of tumor cells with inhibitors of angiogenesis decreases growth and metastasis (15).

Several strategies have been developed to target the angiogenic aspect of breast cancers but have met with little success (16). Hence, targeting angiogenesis is of critical importance at controlling metastatic breast cancers. NRC-AN-019 is a promising candidate for use as an anti-angiogenic agent. Our in vitro studies demonstrate that NRC-AN-019 has a significant anti-angiogenic effect.

In addition to significant anti-angiogenic activity, NRC-AN-019 had a potent anti-proliferative effect. Although not considered an obligatory marker, Ki-67 is also frequently measured both as a static marker of proliferative activity and as a possible dynamic intermediate or surrogate marker of treatment efficacy. The expression of Ki-67 varies in intensity throughout the cell cycle, and this has raised concern that it could lead to a misclassification of cycling cells as resting ones (17). Overall, evidence indicates that levels of Ki-67 are low during G1- and early S-phase and progressively increase to reach a maximum during mitosis, hence making it a good indicator and the expression of Ki-67 has often been referred to as Ki-67 index for proliferative index (18-20).

In our study, the determination of Ki-67 levels indicates that treatment of both MDAMB231 and BT474 cells with NRC-AN-019 did decrease the expression of Ki-67 and confirms the results from the MTT proliferation assay. These results demonstrate that NRC-AN-019 has the potential to retard the proliferation of Lapatinib-resistant breast cancer cells in a dose-dependent manner and may have significant therapeutic potential as an anti-proliferative and anti-angiogenic agent. Targeting these types of cells with relevant contributing genetic modifications is necessary. Our cell cycle studies show that even at relatively low concentrations (19.3 µM), NRC-AN-019 caused significant disruption of the cell cycle in BT474 cells. This experiment was only carried out for 12 h; an extended experiment may produce a greater response at lower concentrations.

The apoptotic effect of NRC-AN-019 was confirmed by measuring the levels of cleaved PARP, which is indicative of DNA damage (21). The broad anti-kinase activity of NRC-AN-019 was evident from ELISA and Western blot analysis for the expression of pEGFR. We observed that pEGFR levels were significantly lower in NRC-AN-019-treated cells than in Lapatinib-treated cells at comparable concentrations.

A number of EGFR inhibitors have been tested in breast cancer clinical trials but have had limited effect (22), thereby...
making the development of new chemotherapeutic agents all the more relevant. Our in vivo studies clearly demonstrate that at comparable concentrations, NRC-AN-019 is more effective than Lapatinib in retarding BT474-induced tumors. NRC-AN-019 even showed significant inhibition of pEGFR in vivo when compared to Lapatinib.

In conclusion, our study demonstrates that NRC-AN-019 has a lower IC$_{50}$ than Lapatinib with strong anti-angiogenic, anti-proliferative, and pro-apoptotic properties in EGFR-overexpressing cells. The in vivo studies also demonstrated that NRC-AN-019 has a better anti-tumor activity than Lapatinib. These results suggest the therapeutic potential of NRC-AN-019 as an anticancer agent for the treatment of breast cancer.

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