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Targeting Estrogen Receptor Negative Breast Cancer Cells Using Diarylthiourea

Analogs of SHetA2

By

Hongye Zou

A culminating thesis submitted to the faculty of Dominican University of California in
partial fulfillment of the requirements for the degree of Master of Science in Biology

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Abstract

Breast cancer is one of the most common cancers that occur in women in the United States. Depending on the expression of estrogen receptor α (ER α), breast cancer can be classified as ER-positive or negative. ER-negative (ER-) breast cancers are often more aggressive and have poorer prognosis compared with the ER-positive (ER+) subtype. Consequently, there is a great need to develop more targeted therapeutic options for ER- breast cancer. One such compound is SHetA2, a flexible heteroarotinoid (flex-hets) that has been shown to inhibit growth of multiple cancer types including breast cancer. However, the clinical utility of SHetA2 is limited by its high lipophilicity (LogP). The goal of this study is to screen eleven 2nd generation analogs of SHetA2 with lower LogP values— SL1-22, 24, 27, 29, 30, 32, 36, 37, 38, 39, and 40— on different breast cancer cell lines to identify potential lead compounds that show the highest anti-growth activities against ER- breast cancer cells and further delineate the mechanism by which these compounds affect breast cancer cell growth. Results from this study demonstrate that SL1-38 (1-(3-chloro-4-methylphenyl)-3-(4-nitrophenyl)thiourea) and SL1-39 (1-(4-chloro-3-methylphenyl)-3-(4-nitrophenyl)thiourea), displayed the most potent effects and inhibit ER-negative breast cancer cells effectively at micromolar concentrations. Our results show that these two analogs reduce the expression of cyclin A, cyclin B, cyclin D1, cyclin E and cdk2 and block S-phase progression. Results from this study demonstrate for the first time that a flexible heteroarotinoid analog—SL1-39— inhibits breast cancer cell growth by rapidly down-regulating the protein expression levels of total HER2 and pHER2, and consequently, the phosphorylation of its downstream effectors, MAPK and Akt. While future studies are necessary to further elucidate the mechanism of

action of SL1-39, these findings strongly support further development of SL1-39 as a therapeutic option for HER2+ breast cancer in particular and underscores the importance of studying the effects of flexible heteroarotinoids, like SL1-39, on cancer in general.

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Introduction

Breast cancer is the most common female cancer worldwide and comprises 25% of malignancies in women [1]. In 2012, more than 1.6 million breast cancer cases were diagnosed worldwide, with about 0.5 million deaths [2, 3]. Specifically in the United States, breast cancer is the most common non-skin cancer that occurs in women and has the second highest cancer-associated mortality rate (0.0215%) second only to lung cancer [4, 5]. Indeed, one in eight woman in the US will develop invasive breast cancer sometime during her life [5], and according to the American Cancer Society, by the close of 2016 there will be 246,660 new cases of invasive breast cancer diagnosed in women and over 2600 in men with an estimated 40,890 deaths [2, 6].

Multiple environmental and biological risk factors contribute to the development of breast cancer. Environmental risk factors include diet, alcohol, lack of physical exercise, and exposure to certain chemicals including ionizing radiation and metalloestrogens such as cadmium and nickel. Biological risk factors include genetics, density of mammary gland tissue, age, ages at menarche and menopause, age at first full-term pregnancy, gender, and life-time exposure to hormones like estrogen and progesterone [5].

Breast cancers can be classified as either luminal, which are tumors derived from the luminal or inner lining of the mammary gland ducts, or basal-like, which are derived from the basal or outer cells of the duct [7, 8]. Gene expression profiling further classifies the tumors into several sub-types according to the presence of certain breast cancer biomarkers, including estrogen receptor (ER), progesterone receptor (PR) and human

epithelial receptor 2 (HER2) (Table 1) [7-9]. These subtypes have different prognoses and should be considered in clinical management of the disease.

Table 1. Subtype of breast cancer

Subtype	Characteristic	Prognosis
Luminal A	ER ⁺ and/or PR ⁺ HER ⁻	Better prognosis Higher survival rate and lower recurrence
Luminal B	ER ⁺ and/or PR ⁺ HER ⁻	Poorer prognosis than Luminal A High survival rate
HER2+	ER ⁻ and PR ⁻ HER ⁺	Poor prognosis Early and frequent recurrence
Basal-like triple negative	ER ⁻ and PR ⁻ HER2 ⁺	Poor prognosis More aggressive

The estrogen receptors mediate the biological effects of estrogen, which is the most prevalent endogenous steroid hormone in females that regulates the growth and development of breast cancer [10, 11]. Major subtypes of estrogen receptors include estrogen receptor α , β (ER α and ER β , respectively), and G protein-coupled receptor 30 (GPR30) [12-14]. While ER α and ER β are nuclear receptors, GPR30 is a receptor found on the plasma membrane [14]. The binding of estrogen to its receptor results in receptor dimerization (Fig. 1). ER dimers can then bind to the estrogen receptor element (ERE) located within the promoter region of target genes [15-17]. Binding of the ER to the ERE leads to transcriptional activation of the associated genes via recruitment of co-activators including steroid receptor coactivator-1 (SRC-1) and histone acetyl transferase [16, 18]. ER can also affect cell growth in a non-genomic pathway via plasma membrane-bound

estrogen receptors [14, 19]. Thus, estrogen mediates different signaling pathways depending on cell type and on the physiological status of the cell [20, 21].

Figure 1: Estrogen signaling pathway. 17β-estradiol diffuses into the cells where it binds to estrogen receptor and induces dimerization, conformational change and translocation into the nucleus where it binds to estrogen response element (ERE) located within the promoter regions of target genes. The expression of target genes can further modulate cell growth, differentiation and migration. Adapted from Estrogen signaling disruptors [irset.org, https://www.irset.org/page?id=213](https://www.irset.org/page?id=213).

ERα's role in breast cancer has been well established, while the exact function of ERβ still remains elusive and controversial [22, 23]. Many cell-based studies suggest that ERβ acts as a negative modulator of ERα action by inhibiting ERα transcriptional activity [24-26], while others show that in ERα/ERβ breast cancer cells, ERβ is associated with elevated levels of the proliferation markers Ki67 and Cyclin A, suggesting it also plays a role in cell proliferation [27, 28]. Studies have shown that breast cancer cells that express ERα account for approximately 70-80% of all breast cancer cases [29, 30], and are often referred as ER-positive (ER+). The presence of ERα serves as a valuable prognostic marker and therapeutic target. Although ER+ breast cancer includes luminal A and B

subtypes which differ in growth rate (Table 1), both are considered to be less aggressive and have better prognosis when ER+ is present [31-33]. The five-year overall survival rate of ER+ breast cancer patients is 15% higher compared to patients with other subtypes [34-36].

ER+ breast cancers are often treated with endocrine therapies that target estrogen synthesis and/or the estrogen receptor. These include selective estrogen receptor modulators (SERMs), selective estrogen receptor down-regulators (SERDs) and aromatase inhibitors (AIs) [32]. SERMs can act as either estrogen agonists or antagonists, depending on the target organ [31, 32, 37]. Tamoxifen, the most widely used SERM, binds to the estrogen receptor and recruits co-repressor proteins, blocking transcriptional activation and subsequently decreasing the expression of ER regulated genes [31, 37]. Five years of tamoxifen treatment can reduce the recurrence risk by 13% compared to no endocrine therapy [38]. Davies and colleagues further demonstrated that ten-year tamoxifen treatment can give a better reduction in recurrence and mortality compared with only five-year tamoxifen treatment [39].

On the other hand, SERDs are considered pure ER antagonists that block receptor dimerization and nuclear translocation after binding to ER [40]. The only SERD currently approved by the FDA for treatment of breast cancer is fulvestrant, which binds to ER to form a drug-ER complex that is unstable and is rapidly degraded to reduce ER protein levels and further block ER signaling pathway [31, 41, 42]. In general, fulvestrant has similar efficacy compared with other endocrine therapies [43].

Aromatase inhibitors (AIs) are compounds that inhibit the activity of the aromatase, an enzyme that catalyzes the conversion of androgens to estrogens, thus reducing endogenous estrogen levels [44]. Third generation AIs are considered better than SERMs and SERDs in controlling advanced disease, as the AIs anastrozole and letrozole have been shown to delay disease progression by 3-4 months when compared with tamoxifen treatment [45, 46]. But AIs are not effective in premenopausal women unless used in conjunction with other drugs or when the ovaries have been surgically removed. [47].

While there are many positive attributes associated with endocrine therapies, there are also problems linked to their usage. Specifically, prolonged use of tamoxifen is associated with a two- to four-fold increase in the risk of developing endometrial cancer [48]. In addition, prolonged treatment with tamoxifen often results in the development of tamoxifen resistance, which has been shown to be associated with altered or modified estrogen receptor signaling [49, 50]. As a result, tamoxifen resistant breast cancer cells can continue to proliferate even in the absence of estrogen [50]. AIs also have side effects, including elevated plasma lipid levels, increased bone turnover, and increased androgenic effects [51]. Fulvestrant has very low bioavailability and must be given by intramuscular injection [52]. Similar to tamoxifen, prolonged usage of AIs and fulvestrant are associated with the development of resistance [53-55].

About 20% of all breast cancer cells do not express ER α [29, 30], and these cancers are often referred to as ER-negative (ER-). Patients with ER-negative breast cancer often have poorer prognosis and more aggressive and highly recurrent cancers when compared with patients with ER-positive breast cancer (Table 1). In general, the

cancer-associated mortality of ER- cancers is three-fold higher within two years of diagnosis when compared with ER+ breast cancers [36]. ER- breast cancer patients have significantly lower survival rates [34], with a 76.5% 5-year survival rate versus 94.7% in ER+ breast cancer patients [56] and a 57% 10-year survival rate versus 69% in ER+ patients [34, 57].

Like ER, the progesterone receptor (PR) is considered a good prognostic factor [29, 58]. Since the expression of PR is regulated by ER, PR-positive breast cancer has long been considered to be associated with ER-positive breast cancer [59]. Although the existence of ER-/PR+ breast cancers has been reported to account for less than 4% of all breast cancer cases [60], the biological and clinical significance of the ER-/PR+ breast cancer subtype is controversial as many researchers argue that PR expression requires the presence of a functioning ER [59, 61]. However, PR gene expression can also be regulated by other transcription factors like c-fos and c-jun [62-64]. Whether these transcription factors mediate the expression in the absence of ER is unclear.

In tumor epithelial cells, high levels of PR expression can drive cell proliferation by both PR-dependent signaling and transcriptional activity [65, 66]. In general, PR-positive breast cancers have a higher 5-year overall survival rate compared with the PR-negative subtype (95.2% versus 82.0%) [56]. While several studies with rodent models have shown that inhibition of PR or its downstream pathway leads to significant reduction in mammary carcinogenesis [67-69], no anti-progesterone has been recommended for breast cancer treatment [70]. One of the reasons might be that PR is a phospho-protein whose regulation of gene expression is highly influenced by protein kinase cross-talk [71]. Unfortunately, selective modulators of PR can act both as

antagonists in the mammary gland and agonists in the ovaries, thereby increasing the risk of developing ovarian cancer [72]. Furthermore, some of anti-progestins have anti-glucocorticoid effects, which can impact the inflammatory response and thus contribute to the progression of the disease [73]. On the other hand, recently published data suggest that the expression of PR can directly modulate ER function and thus potentially improve the tumor response to ER antagonists [74].

Breast cancers that express human epidermal growth factor 2 (HER2) make up another subtype of breast cancer often referred to as HER2+ breast cancers. Approximately 20% of all breast cancers over express HER2 [75-78]. Although 10-14% of breast cancers are HER2+ and ER α +, ER-/HER2+ breast cancers account for 6- 10% of all breast cancer cases[56]. HER2 is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases that act through the EGF signaling cascade (Fig. 2) [79, 80]. The dysregulation of HER2/EGFR pathway often results in uncontrolled growth that can contribute to cancer progression [81, 82]. The activation of the EGFR pathway first requires either homo- or hetero-dimerization of receptors in the EGFR family, which includes EGFR (HER1), HER2, HER3 and HER4 (Fig. 2). The binding of ligands causes dimerization of the receptors which results in auto-phosphorylation of the dimers and subsequent activation of several downstream pathways, including the PI3K (phosphatidylinositol-3-kinase)/ Akt (protein kinase B) pathway and the Ras-Raf (rapidly accelerated fibrosarcoma)-MAPK (mitogen-activated protein kinases) pathway [80, 83]. These two important pathways can further regulate gene expression and affect cell proliferation, migration, angiogenesis, invasion and apoptosis [84, 85]. However, unlike other EGFR members, HER2 does not have a specific ligand binding domain and thus

can easily interact with other activated EGFR receptors, making HER2 the preferred dimerization partner for the other receptors [86-88].

Figure 1. HER2 pathway. Heterodimerization of HER2 with other receptors in EGFR family (EGFR, HER3 and HER4) results in autophosphorylation and subsequent activation of PI3K/Akt pathway and Ras/Raf/MAPK pathway. The activation of these cell signaling pathways further modulate cell proliferation, migration and apoptosis. Adapted from *Cardiotoxicity Associated With the Use of Trastuzumab in Breast Cancer Patients*, by Patricia Peri, December 2007, <http://www.medscape.com/viewarticle/560053>

The 5-year overall survival rate for all HER2-positive breast cancers is 85.6% compared with 92.2% for total HER2-negative subtype. For ER-/HER2+ cells, the 5-year overall survival rate lowers to 76.1% [56]. In addition to a lower 5-year overall survival rate, HER2+ breast cancers have a higher risk of recurrence, higher mortality rates in early stage disease and an increased incidence of metastases [89, 90]. Additionally,

HER2+ breast cancers are reported to be associated with a higher risk of brain metastases than HER2- breast cancers, with incidence ranging 30–53%, compared to approximately 10% in other breast cancers subtypes [91]. Once brain metastases have occurred, the 1-year survival rate is only around 20% [92].

One of the most widely used HER2-targeted therapies is trastuzumab. Trastuzumab is a monoclonal antibody, raised against human HER2 protein. Trastuzumab binds to HER2 to block dimerization and promote its degradation via endocytic destruction of the receptor. This in turn induces the accumulation of the cyclin-dependent kinase inhibitor (CKI), p27, and promotes cell cycle arrest [93]. Studies have demonstrated that trastuzumab improves overall survival rate in HER2+ breast cancer patients from 86.6% to 91.4% [94-96]. Although patients generally show a positive response to trastuzumab therapies, only 25% of patients with HER2+ metastatic breast cancers respond to trastuzumab monotherapy. These patients also exhibit a higher risk of loco-regional recurrence compared to other subtypes [97]. Additionally, the mechanism of action of trastuzumab and the clinical response to the drug depends on the patient's immune system [98]. As with other cancer therapies, the development of resistance to anti-HER2 therapy is another challenge [99, 100], with ~ 15% of patients relapsing after one-year of trastuzumab treatment, suggesting the existence of acquired resistance [83]. Iben et.al proposed several possible mechanisms that could explain this resistance development, including an altered interaction between receptor and antibody and activation of downstream pathways like the PI3K/Akt pathway via increased signaling from other receptors such as insulin-like growth factor receptor, vascular endothelial growth factor (VEGF), or other members of EGFR family [83].

Other than trastuzumab, several other therapeutics that target HER2 have been developed. These include: (1) lapatinib, which inhibits the intracellular kinase domain of HER2 and is the only directed HER2 therapy that can penetrate the blood–brain barrier to target brain metastases [91, 101]; (2) pertuzumab, which is a monoclonal antibody that blocks ligand-dependent pairing of HER2 with other HER receptors and increase the progression-free survival rate if used in combination with trastuzumab [102-104]; and (3) bevacizumab, which is a monoclonal antibody that targets VEGF and inhibits VEGF signaling pathways [101]. Although these therapies show some advantages over trastuzumab, they suffer from the same problem— the development of resistance [105, 106]. More importantly, in HER2+/ER+ breast cancer cells, lapatinib can up-regulate ER signaling by activation of other factors (including FOXO3a and caveolin-1) that facilitate the transcriptional activity of ER, and convert cells from HER2-dependence to HER2/ER-codependence [107]. Therefore, combination therapies are strongly recommended to produce a greater anti-proliferative effect and to overcome resistance.

Breast cancers that do not express any of the three key biomarkers—ER α , PR, and HER2— are referred to as triple-negative breast cancer (TNBC). TNBC accounts for 10% to 15% of all breast cancer cases [108, 109]. It occurs at higher prevalence among premenopausal and African-American women [5, 110]. TNBCs are typically characterized by a ductal histology and high grade with increased cell proliferation rates (Table 1). This subtype of breast cancer has fewer available therapies, poorer prognosis and a higher local recurrence rates [108, 111-113]. The primary therapeutic strategies are surgery, radiation and chemotherapy [114]. Conforti et al. showed that chemotherapy administered after surgery was associated with 46% decrease in death rates for patients

with TNBC [115]. Furthermore, patients with TNBC treated with chemotherapies before surgery also reported to have less residual invasive tumors left, indicating a good initial response [116]. However, only a minority of TNBC patients is responsive to chemotherapy [113]; and similar to other cancer subtypes, TNBC can also become resistant to conventional treatments. Sadly, relapse in TNBC patients is much more aggressive, which often results in lower progression-free survival and overall survival rates compared to other breast cancer subtypes [108, 117, 118]. In a study carried out by Liedtke et al., the researchers showed that TNBC had a significantly lower 3-year progression-free survival when compared to other breast cancer cases (63% vs 76%, respectively), and a lower 3-year overall survival (74% vs 89%, respectively) [108]. Similarly, Haffty et al. also reported that patients with TNBC had lower survival rates and lower distant metastasis-free rates in comparison to non-TNBC patients [119]. Consistent with these results, data from the California Cancer Registry also confirms that women with TNBC have decreased survival compared to those with other types of breast cancer [118].

The above studies underscore the need to develop new therapeutic drugs aimed at improving patient outcomes in ER- breast cancer. Additionally, more targeted therapy is necessary to reduce potential side effect. Amongst the compounds being developed to target cancer, flexible heteroarotinoids (flex-hets)— which are derived from retinoids— is a new class of drugs that have been shown to possess promising anti-cancer activity [120-123]. Retinoids can regulate growth, differentiation and apoptosis by acting through the nuclear retinoic acid receptor (RAR) and retinoic X receptor (RXR) [124-126]. Flex-hets have increased flexibility due to the substitution of the conventional two-atom linker

between two aryl rings with urea or thiourea linker, and this allows for more relevant conformations to interact with potential targets (Fig. 3) [123, 124]. Interestingly, flex-hets show anti-cancer activities without activating RAR or RXR [122, 123, 127].

SHetA2 was amongst the first flex-hets identified to have anti-cancer activities. Studies have shown that SHetA2 can block growth of the National Cancer Institute's panel of 60 human tumor cell lines, which include cervical, head and neck, and breast cancer cell lines [121, 123, 124]. More specifically, Liu et al. showed that SHetA2 induces apoptosis in the Caki-1 kidney cancer cells through reduction of the Bcl-2 protein and induction of PARP-1, while normal kidney epithelial cells were arrested in G1 cycle through the degradation of cyclin D1 [128]. SHetA2 also inhibits the growth of non-small-cell lung carcinoma (NSCLC) cells and kidney cancer cells by inducing differentiation and apoptosis via the activation of the nuclear factor-kappa B (NF- κ B) signaling pathway [129]. It has also been shown that SHetA2 can inhibit angiogenesis by blocking cytokine release from cancer cells [130]. Growth inhibition effects of SHetA2 had also been observed *in vivo*. The administration of SHetA2 at the high dose of 60 mg/kg/day in mouse xenograft models significantly reduced tumor size 13 days after treatment, with a maximal reduction of 36% 23 days after treatment [128]. Furthermore, long-term treatment in both rats and dogs resulted in no significant toxicity even at doses as high as 1500 mg/kg/day for 28 days [131] [132]. These results demonstrate that SHetA2 is a promising compound that can be further developed for cancer treatment.

However, SHetA2 also has some limitations due to its high lipophilicity and low yields associated with a difficult synthesis schema. The high lipophilicity of SHetA2 ($\log P = 7.09$) is higher than that of current drugs on the market [132, 133]. $\log P$ is the

logarithm of the octanol/water partition coefficient, and if the value of logP is higher than 5, it is predicted to have poor absorption and permeation ('Lipinski rule of 5'). The high lipophilicity can also increase a drug's non-specific interactions and liver toxicity [134]. Therefore, efforts have been made by Dr. Shengquan Liu (Touro University of California, College of Pharmacy, Vallejo, CA) to develop second generation flex-het compounds that retain the anti-cancer activity of SHetA2 but with reduced side effects associated with its higher lipophilicity. In the second generation drugs, the nitrophenyl group and the thiourea linker in the SHetA2 structure are retained while the thiochromane ring is replaced with other ring structures (Fig. 3).

The goal of this study was to screen eleven 2nd generation drugs— SL1-22, 24, 27, 29, 30, 32, 36, 37, 38, 39, and 40— on different breast cancer cell lines to identify potential lead compounds that have highest anti-growth activities against ER-negative breast cancer cells and further delineate the mechanism by which these compounds affect breast cancer cell growth. Results from this study demonstrate that SL1-38 (1-(3-chloro-4-methylphenyl)-3-(4-nitrophenyl)thiourea) and -39 (1-(4-chloro-3-methylphenyl)-3-(4-nitrophenyl)thiourea) inhibit ER-negative breast cancer cells effectively at micromolar concentrations and may target HER2/EGFR-positive breast cancer cells as well.

SHetA2

S13

Figure 1: Structure of SHet and the 1st generation of SL

Materials and methods

Cell Culture

Human breast cancer cell lines MCF-7, T47D, SKBR3, MDA-MB-453, BT474, MDA-MB-468, MDA-MB-231, HCC1806 and BT20, the normal breast cell line MCF-10A, and the human mammary epithelial cell line (HMEC,; isolated directly from adult female breast tissue) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). MCF-7, SKBR3, MDA-MB-453, BT474, MDA-MB-468 and MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gemini Bio Products, Sacramento, CA) and 1% penicillin and streptomycin (P/S; Life Technologies, Carlsbad, CA). T47D and HCC1806 cells were maintained in RPMI Medium 1640 supplemented with 10% FBS and 1% P/S. BT20 cells were maintained in Minimum Essential Medium supplemented with 10% FBS and 1% P/S. MCF-10A and HMEC cells were maintained in mammary epithelium basal medium (MEBM) supplemented with insulin, brown pituitary extract (BPE), epidermal growth factor (rhEGF), hydrocortisone, gentamicin sulfate (GA-1000) (Lonza Walkersville, Inc., Walkersville, MD), and cholera toxin (Sigma, St. Louis, MO). All cells were grown at 37°C, 5% CO₂, and cells were sub-cultured every three to four days.

Growth Assay

Approximately 2×10^3 cells per well were plated in 96-well plates in complete media. One day later, cells were either treated with the drug at different concentrations (0.5, 1.0, 5.0, 10.0, and 20.0 μ M) or mock treated with the vehicle dimethyl sulfoxide

(DMSO; Sigma-Aldrich, St. Louis, MO). Two days after treatment, cell proliferation was monitored by incubating cells with 0.5 mg/ml MTT (Life Technologies) for 1 hour at 37 °C. MTT crystals were dissolved in DMSO and absorbance at 595nm was determined using the iMark plate reader (Bio-Rad, Hercules, CA). Cells were also treated with 2.5 and 5 μ M of SL1-39, or mock treated with DMSO for two, three and four days, and cell growth was evaluated with MTT over four days without the addition of new drugs.

Cell viability and apoptosis assay

The Apolive-Glo™ Multiple Assay (Promega, Sunnyvale, CA) was used to evaluate cell viability and apoptosis. HMEC, SKBR3 and MDA-MB-453 cells were plated at 1000 cells per well in a 96-well half area assay plate (Corning, Corning, NY). One day later, HMEC cells were treated with 5 μ M SHetA2, SL1-38, SL1-39, and 0.5 μ M digoxin or mock treated; SKBR3 and MDA-MB-453 cells were treated with one of the following: 5 μ M SHetA2, SL1-38, SL1-39, 10 μ M digoxin, 100nM cabazitaxel or mock treated. Forty-eight hours after treatment, the assay was performed according to manufacturer's instructions to detect cell viability and apoptosis. The reaction product was detected using FLUOstar® Omega multimode microplate reader (BMG Lab Tech Inc, Cary, NC).

Bromodeoxyuridine (BrdU) incorporation assay

Approximately 2×10^5 SKBR3 and MDA-MB-453 cells were plated in 6-well plates containing glass coverslips. The cells were treated with 2.5 or 5 μ M SL1-39 or mock treated 24 hours later. One hour prior to harvesting the cells (12, 24 and 48 hours), cells were pulsed labeled with 10 μ M BrdU for one hour. Cells were fixed with 10%

formalin in phosphate buffer saline (PBS) and BrdU-positive cells were labeled using an anti-BrdU antibody (Roche, Pleasanton, CA) and detected using a secondary antibody conjugated to Alexa-488 (Life Technologies, Grand Island, NY). Cells were counter-stained with 4',6-diamidino-2-phenylindole (DAPI). Digital images were captured with a fluorescent microscope (Leica Microsystem, Inc., Deerfield, IL). Cells labeled with BrdU and DAPI were counted separately in at least five frames and percentage of cells in S-phase were calculated by dividing BrdU-positive cells by total cells.

Western Blot Analysis

Cells were lysed in buffer containing 0.05M HEPES, 1% Triton, 0.1% SDS, 0.002M EDTA, 1% deoxycholate, 0.002M EGTA, 0.15M NaCl, 0.01M NaF, and 1% Halt™ protease inhibitor cocktail (Thermo Scientific, Waltham, MA). Total protein concentrations were determined using the DC protein assay kit (Bio-Rad), and total protein was separated using 8%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After being transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad), protein expression was analyzed using protein-specific antibodies: β -actin (AC-15) (Sigma-Aldrich); cyclin A (H-432), cyclin D1 (A12), cyclin E (HE12), cdk2 (M2), PR (F-4) and HER2 (3B5) (Santa Cruz Biotechnology, Santa Cruz, CA); GAPDH (D16H11), ER α (D8H8), AR (D6F11), EGFR (D38B1), HER3 (D22C5), HER4 (111B2), pHER2 (6B12), MAPK (137F5), pMAPK (D13.14.4E), Akt (40D4) and pAkt (D9E) (Cell Signaling Technology, Danvers, MA).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

MDA-MB-453 cells were plated, treated as mentioned above and harvested 12, 24 and 48 hours after treatment. Total RNA was isolated via Direct-zol RNA kit according to the manufacturer's protocol (Zymo Research Corporation, Irvine, CA). Three micrograms (μg) of total RNA were used for the reverse transcription reaction with oligo-dT₁₈ primers and Moloney Murine Leukemia Reverse Transcriptase (MMLV RT; Promega). Gene expression was monitored using SYBR-green based quantitative RT-PCR (qRT-PCR) using the Viia-7 instrument from Applied Biosystems (Life Technologies). The GAPDH, cyclin A, cyclin B, cyclin D1, cyclin E, cdk2 and HER2 primers were synthesized by Integrated DNA Technologies, Inc. (IDT; San Diego, CA).

Table 1: Primer sequences for RT-PCR

Primer name	Primer sequences	$T_m(^{\circ}\text{C})$
GAPDH _F	GAAATCCCATCACCATCTTCC	55.6
GAPDH _R	ATGAGTCCTTCCACGATACCA	56.5
Cyclin A _F	CCCCCAGAAGTAGCAGAGTTTGTG	51.1
Cyclin A _R	GCTTTGTCCCGTGACTGTGTAGAG	51.1
Cyclin B _F	GTACCCTCCAGAAATTGGTGA	51.1
Cyclin B _R	GACTACATTCTTAGCCAGGTG	52.6
Cyclin D1 _F	AATGTGTGCAGAAGGAGGTC	55.0
Cyclin D1 _R	GAGGGCGGATTGGAAATGAA	55.1
Cyclin E _F	ATACAGACCCACAGAGACAG	53.1
Cyclin E _R	TGCCATCCACAGAAATACTT	51.1
cdk2 _F	TTTGCTGAGATGGTGACTCGC	51.6
cdk2 _R	CACTGGAGGAGAGGGTGAGATT	51.1
HER2 _F	CTGGTGTATGCAGATTGCCA	55.1
HER2 _R	GAGAATTCAGACACCAACTCC	55.3

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 6 (Graphpad Software Inc., California). Data were presented in the form of mean $\pm$ SD, and for growth proliferation, best-fit GI₅₀ curves were fitted to one phase decay or sigmoidal dose-response algorithms.

Result

Screening of 2nd generation SHetA2 drugs

Eleven 2nd generation heteroarotinoid compounds were screened to identify potential lead compounds that have the highest anti-growth activities against breast cancer cell lines, particularly those that are ER-negative. The diarylthiourea analogs SL1-22, 24, 27, 29, 30, 32, 36, 37, 38, 39, 40 were tested on five breast cancer cell lines-- MCF7 and T-47D (ER+), MDA-MB-231, MDA-MB-453 and MDA-MB-468 (ER-) (Table 3). While the majority of the compounds showed some inhibitory effects on cell proliferation in at least one breast cancer cell line, only SL1-22, 29, 30, 32, 38 and 39 reduced the growth of all five breast cancer cell lines. Amongst these 6 analogs, SL1-38 and SL1-39 exhibited the most potent effects with GI₅₀ values of $6.39 \pm 1.20 \mu\text{M}$, $3.89 \pm 0.51 \mu\text{M}$, $7.82 \pm 2.99 \mu\text{M}$, $2.16 \pm 0.61 \mu\text{M}$ and $4.77 \pm 0.95 \mu\text{M}$ for SL1-38, and $5.53 \pm 1.11 \mu\text{M}$, $3.06 \pm 1.40 \mu\text{M}$, $5.47 \pm 1.15 \mu\text{M}$, $2.20 \pm 1.13 \mu\text{M}$ and $3.47 \pm 0.33 \mu\text{M}$ for SL1-39 in MCF7, T-47D, MDA-MB-231, MDA-MB-453 and MDA-MB-468, respectively. Interestingly, while SL1-38 and SL1-39 are stereoisomers, no significant differences in growth inhibitory activity were observed between the two compounds in MCF7, T-47D, MDA-MB-231, and MDA-MB-453 ($p > 0.2$), with the exception of MDA-MB-468 cells ($p = 0.009$). However SL1-39 was slightly more effective in decreasing the growth of most of the breast cancer cell lines (Table 3).

After confirming the growth inhibitory activities in three ER- breast cancer cell lines (MDA-MB-231, MDA-MB-453 and MDA-MB-468), the anti-cancer effects of SL1-38 and SL1-39 on additional ER- cell lines— SKBR3, HCC1806 and BT20— were further investigated. Results in table 4 demonstrate that both SL1-38 and SL1-39

effectively decreased the growth of these additional ER-negative breast cancer cells. Amongst the ER-negative breast cancer cell lines tested, MDA-MB-453 and SKBR3 showed the greatest sensitivities towards both SL1-38 and SL1-39, with the GI_{50} values of $2.16 \pm 0.61 \mu\text{M}$ and $2.20 \pm 1.13 \mu\text{M}$ on MDA-MB-453 cells, and $3.53 \pm 1.03 \mu\text{M}$ and $2.91 \pm 0.91 \mu\text{M}$ on SKBR3 cells, respectively. These compounds were further tested on normal breast epithelial cells— MCF-10A and human mammary epithelial cells (HMECs) — and results show the growth inhibitory effects of SL1-38 and -39 on normal breast cancer cell proliferation was comparable to those observed in MDA-MB-453 and SKBR3 cells (Table 5). Interestingly, the parental compound, SHetA2 displayed a greater ability to block normal breast epithelial cell growth ($GI_{50} = 1.02 \pm 0.34 \mu\text{M}$ and $1.05 \pm 0.51 \mu\text{M}$ in MCF-10A and HMEC, respectively), suggesting that SL1-38 and SL1-39 have less of an effect on normal breast epithelial cells than SHetA2.

SL1-38 and -39 do not induce apoptosis

Given that these two analogs have some advantages over SHetA2, further evaluation was done to understand the mechanism of action by examining whether the effects of SL1-38 and SL1-39 on cell number were associated with decreased cell growth or increased apoptosis. HMEC cells were treated with either SHetA2, SL1-38, SL1-39, digoxin (positive control), or mock treated. Forty-eight hours later, the relative cell number (Fig. 4A) and caspase 3 and 7 activities (Fig. 4B) were determined in the same cell population using the Apo-live assay (Promega). Cells treated with SHetA2, SL1-38, SL1-39 and digoxin all showed a decrease in cell number (viability) in comparison to mock treated cells. More specifically, 2.3-fold decrease in viability was seen with digoxin, while SHetA2, SL1-38 and SL1-39 exhibited a 100, 3.1 and 2.6-fold reduction,

Table 3. Potency of SHetA2 analogs in different breast cancer cell lines

Potency (IC₅₀ values) in μM (mean $\pm$ SD)

Name and chemical structure		ER-positive cell lines		ER-negative cell lines		
		MCF7	T47D	MDA-MB-231	MDA-MB-453	MDA-MB-468
SL1-22	10.55±0.53 ^a	7.16±2.11 ^b	18.02±0.58 ^a	10.51±0.23 ^b	10.55±0.35 ^a
SL1-24	ND	13.01±3.38 ^b	ND	ND	ND
SL1-27	ND	7.19±1.92 ^b	ND	12.23±1.15 ^b	10.83±0.44 ^a
SL1-29	8.46±0.98 ^b	12.78±2.72 ^b	10.05±0.37 ^a	5.34±0.48 ^b	5.40±0.24 ^a
SL1-30	5.51±0.89 ^b	4.56±1.17 ^b	6.45±1.90 ^b	3.03±0.86 ^b	3.04±0.86 ^b
SL1-32	10.96±0.44 ^a	4.84±0.45 ^b	14.11±3.84 ^a	12.07±2.91 ^b	11.02±0.61 ^a
SL1-36	ND	5.72±2.94 ^b	ND	9.41±0.78 ^b	ND
SL1-37	ND	6.46±2.08 ^b	ND	10.75±0.40 ^b	ND
SL1-38	6.39±1.20 ^b	3.89±0.51 ^b	7.82±2.99 ^b	2.16±0.61 ^b	4.77±0.95 ^b
SL1-39	5.53±1.11 ^b	3.06±1.40 ^b	5.47±1.15 ^b	2.20±1.13 ^b	3.47±0.33 ^b
SL1-40	ND	ND	ND	ND	ND
SHetA2	3.05±0.84 ^b	4.23±0.18 ^b	2.58±0.40 ^b	3.67±0.75 ^b	2.07±0.31 ^b

^a Based on sigmoidal dose-response model^b Based on one phase decay modelND: not determined or GI₅₀ $\geq$ 20 μM

Table 4. Potency of SL1-38 and SL1-39 in different ER-negative breast cancer cell lines

Potency (IC50 values) in μM (mean $\pm$ SD)			
ER- breast cancer cell lines	Cell type	SL1-38	SL1-39
SKBR3	Luminal-type	3.53 $\pm$ 1.03	2.91 $\pm$ 0.91
MDA-MB-453	Luminal-type	2.16 $\pm$ 0.61	2.20 $\pm$ 1.13
MDA-MB-468	Basal-type	4.77 $\pm$ 0.95	3.47 $\pm$ 0.33
MDA-MB-231	Basal-type	7.82 $\pm$ 2.99	5.47 $\pm$ 1.15
HCC1806	Basal-type	8.38 $\pm$ 1.95	5.37 $\pm$ 0.30
BT20	Basal-type	4.09 $\pm$ 1.11	3.41 $\pm$ 1.26

Table 5. Potency of SHetA2, SL1-38 and SL1-39 in normal breast cell lines

Potency (IC50 values) in μM (mean $\pm$ SD)			
normal breast cell lines	ShetA2	SL1-38	SL1-39
MCF-10A	1.02 $\pm$ 0.34	2.67 $\pm$ 0.16	2.66 $\pm$ 0.12
HMEC	1.05 $\pm$ 0.51	3.05 $\pm$ 1.13	2.79 $\pm$ 0.64

respectively, in cell growth two days after treatment. In the same population of cells, the caspase 3 and 7 activities were also measured. As expected, digoxin— known to induce apoptosis— showed high caspase 3 and 7 activities, while the three heteroarotinoid analogs showed very low levels of caspase 3 and 7 activities (Fig. 4A), suggesting that SHetA2 and its analogs do not induce apoptosis in normal breast epithelial cells.

Previous studies have indicated that while SHetA2 does not promote apoptosis in normal cells, it does induce apoptosis in some cancer cell types, including head and neck, ovarian, and lung cancer [121, 123, 124]. To determine whether SL1-38 and 39 induce apoptosis in ER- breast cancer cells, MDA-MB-453 and SKBR3 cells were plated and treated with SHetA2, SL1-38, and SL1-39 or mock treated. Cells treated with digoxin or cabazitaxel, a derivative of taxol, served as positive controls. Similar to the HMEC cells, SL1-38 and SL1-39 reduced MDA-MB-453 cell growth by 2.2 and 2.5-fold, and SKBR3 cell growth by 2.3 and 2.6-fold, respectively (Fig. 4C and E). Cells treated with digoxin and cabazitaxel also displayed a decrease in cell viability (3.3 and 3.5-fold reduction in MDA-MB-453 cells, and 12.5 and 3.7-fold reduction in SKBR3 cells) (Fig. 4C and E). Concomitantly, digoxin and cabazitaxel increased caspase 3 and 7 activities by 2.1 and 3.9-fold in MDA-MB-453 cells, and 2.9 and 4.7-fold in SKBR3 cells, respectively (Fig. 4D and 4F). Interestingly, breast cancer cells treated with SHetA2 and its two analogs showed low caspase 3 and 7 activities in comparison to digoxin and cabazitaxel, suggesting that SL1-38 and SL1-39-induced growth inhibition of ER- breast cancer cells is associated with decreased cell proliferation and not increased apoptosis.

Figure 4: SHeA2, SL1-38 and 39 reduced cell viability but did not induce apoptosis in both normal breast cell line and breast cancer cell lines. HMEC(A), MDA-MB-453(B) and SKBR3(C) cells were plated in 96-wells half plate and treated with different drugs for 48 hours. Cell viability and caspase activities were analyzed with the Caspase-Glo®3/7 assay kit (Promega). Assays were repeated at least three times and average values (with SD) are presented from one representative experiment (***, $P < 0.001$).

SL1-39 reduces ER-negative breast cancer cell proliferation by preventing S phase progression

Since SL1-38 and SL1-39 are stereoisomers and have displayed little difference in their effects on growth and apoptosis, the two compounds likely share a similar mechanism of action. Thus, further experiments were carried out on just SL1-39. To determine whether the growth inhibitory effects of SL1-39 were sustained beyond two days, MDA-MB-453 cells were plated and treated with 2.5 or 5 μ M of SL1-39 or mock treated, and cell growth was evaluated two, three, and four days after treatment (Fig. 5A). Consistent with the drug screen (Table 3), SL1-39 inhibited SKBR3 and MDA-MB-453 cells at 2.5 and 5 μ M starting at two days ($P < 0.001$), and this growth inhibitory effect was sustained for four days without addition of new drugs. To further elucidate how SL1-39 blocks cell growth, a BrdU incorporation assay was used to evaluate SL1-39's effects on S-phase progression. Breast cancer cells were treated with 2.5 and 5 μ M of SL1-39 or mock treated and collected 12, 24, and 48 hours after treatment. Cells treated with 2.5 μ M and 5 μ M of the analog for 24 hours showed not only a decrease in total cell number but also a decrease in the percentage of cells entering S-phase, as noted by the reduction in BrdU-positive cells (Fig. 5C and D). Quantitative analysis confirmed that even as early as 12 hours, the number of SKBR3 cells entering S-phase decreased from 33.8% in mock treated cells to 21.9% and 17.2% in cells treated with 2.5 μ M ($P < 0.05$) and 5 μ M ($P < 0.01$) of SL1-39, respectively (Fig. 5C). Forty-eight hours after 5 μ M SL1-39 treatment, less than 2% of the SKBR3 cells were in S-phase ($P < 0.001$). Similar results were observed in MDA-MB-453 cells, with the percentage of cells in S-phase decreasing from 43.2% in mock-treated cells to 25.7% and 15.4% in cells treated for 12 hours with 2.5 μ M and 5 μ M

Figure 5. SL1-39 reduced ER-negative breast cancer cell proliferation up to 4 days by preventing cells into S phase. SKBR3(A) and MDA-MB-453(B) cells were plated in 96-wells plate and treated with 2.5μM, 5μM SL1-39 or mock treated. Relative cell numbers were determined using MTT 2, 3, 4 days after treatment (***, $P < 0.001$). SKBR3(C) and MDA-MB-453(D) cells were seeded on coverslips in 6-wells plate and treated with 2.5μM, 5μM SL1-39 or mock treated. After 12, 24 and 48 hours, cells were stained with BrdU, fixed and then analyzed on a fluorescent microscope. Nuclei were visualized by DAPI staining (in blue). Cells undergoing DNA synthesis appeared in green (BrdU). Columns represent the percentage of cells in S phase with SD (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Experiments were repeated at least three times.

of SL1-39, respectively ($P<0.001$) (Fig. 5D). After 48 hours of 5 μ M SL1-39 treatment, only 10% of the cells were in S-phase ($P<0.001$).

SL1-39 decreases the expression of cell cycle regulators

Since SL1-39 appears to block S-phase progression, the expression levels of key cell cycle regulators were analyzed in MDA-MB-453 cells using western blot analysis. Results in figure 6 show that 5 μ M of SL1-39 was sufficient to reduce the expression of cyclin A ($P<0.01$), cyclin B, cyclin D1, cyclin E ($P<0.001$) within 12 hours of treatment and cdk2 ($P<0.01$) by 24 hours (Fig. 6A), and this decrease is also observed 48 hours after treatment. Amongst the cell cycle proteins analyzed, cyclin D1 was the most significantly impacted, with expression levels dropping to only 5% in cells treated with 5 μ M of SL1-39 for 48 hours in comparison to vehicle treated cells (Fig. 6B).

To determine if the decrease observed at the protein level is also observed at the mRNA level, semi-quantitative PCR and quantitative PCR (qPCR) were used to analyze the transcript levels of cell cycle genes in MDA-MB-453 cells treated as previously mentioned. Cells treated with SL1-39 showed lower mRNA levels of cyclin A, cyclin E and cdk2 12 hours after treatment, while cyclin B and cyclin D1 levels did not decrease until 48 hours after treatment (Fig. 7A). Consistent with this, qPCR data confirm that MDA-MB-453 cells treated with 5 μ M of SL1-39 expressed significantly less cyclin A, B, D1, E, and cdk2 12 hours after treatment (32%, 73%, 31%, 83% and 35%, respectively; $P<0.001$; Fig. 7B). Furthermore, the mRNA levels of cyclin B, cyclin D1 and cyclin E were decreased with only 2.5 μ M of SL1-39 12 hours after SL1-39 treatment ($P<0.001$). Coupled with the growth data, these data strongly demonstrate that SL1-39 inhibits breast cancer cell growth by decreasing the expression of key cell cycle regulators at both the

Figure 6. SL1-39 reduced the expression of cell cycle proteins. MDA-MB-453 cells were plated in 6-wells plate and treated with 2.5μM, 5μM SL1-39 or mock treated for 12, 24 or 48 hours. (A) Cyclin A, Cyclin B, CyclinD1, Cyclin E and CDK2 protein expressions in cell lysates were analyzed with western blot. Actin served as control. (B) Quantification of western blot, column represent average protein expression level (with SD) of at least three independent experiments (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Figure 7. SL1-39 decreased mRNA expression of cell cycle genes. MDA-MB-453 cells were plated in 6-wells plate and treated with 2.5 μ M, 5 μ M SL1-39 or mock treated for 12, 24 or 48 hours. (A) RT-PCR analysis of Cyclin A, Cyclin B, CyclinD1, Cyclin E and CDK2 in total RNA extracted from cell lysates. GAPDH served as control. (B) Real-time PCR analysis of Cyclin A, Cyclin B, CyclinD1, Cyclin E and CDK2 in total RNA extracted from cell lysates. GAPDH served as control. (***, $P < 0.001$).

protein and mRNA levels.

SL1-39 reduces HER2 expression and inhibits the EGFR signaling pathways

Having demonstrated SL1-39 inhibits breast cancer cell growth, we wanted to investigate its mechanism of action in ER- breast cancer cells. To this end, the similarities amongst the breast cancer cell lines that exhibited a good response to SL1-39 were evaluated. Western blot analysis was used to determine the expression of important breast cancer biomarkers— ER α , PR, AR, EGFR (HER1), HER2, HER3 and HER4— in all the breast cancer cell lines previously analyzed (Fig. 8). According to the western blot analysis, MCF7, T47D and BT474 are ER+, while T47D, SKBR3, MDA-MB-453 and BT474 are HER2+, and MDA-MB-468, MDA-MB-231, HCC1806 and BT20 are ER α -, PR- or HER2- and are considered as TNBC cell lines. The expression status of each biomarker was compared to drug response using the GI₅₀ values (Table 6). Interestingly, cells expressing HER2 collectively exhibited a greater sensitivity towards SL1-39. T-47D, SKBR3, MDA-MB-453 and BT474 all express HER2, and the GI₅₀ values of these cells were all below 5 μ M (3.06 $\pm$ 1.40 μ M, 2.91 $\pm$ 0.91 μ M, 2.20 $\pm$ 1.13 μ M and 2.39 $\pm$ 0.28 μ M, respectively). Additionally, cells with moderate to high levels of EGFR (MDA-MB-468 and BT20) also displayed higher sensitivity (3.47 $\pm$ 0.33 μ M and 3.16 $\pm$ 1.22 μ M) to SL1-39, while cells with low EGFR expression (MDA-MB-231 and HCC1806) exhibited a moderate response with GI₅₀ values in the 5 μ M range. The correlation of HER2/EGFR expression to GI₅₀ suggests that SL1-39-mediated growth inhibition may involve modulating the HER2/EGFR signaling pathway.

Figure 8. Receptor expression (ER α , PR, AR, EGFR, HER2, HER3, HER4) in total cell lysates (MCF7, T47D, SKBR3, MDA-MB-453, BT474, MDA-MB-468, MDA-MB-231, HCC1806 and BT20) were analyzed with western blot. Actin served as control.

Table 6. Breast cancer cell lines and SL1-39 response.

breast cancer cell lines	Cell type	Characteristic	IC50	ER α	PR	AR	EGFR	HER2	HER3	HER4
MCF7	Luminal-type	ER+	5.53 ± 1.11	++	-	+++	-	-	++	+
T47D	Luminal-type	ER+/PR+/HER2+	3.06 ± 1.40	++	++	++	+	+	++	++++
SKBR3	Luminal-type	HER2+	2.91 ± 0.91	-	-	-	+	++++	++	-
MDA-MB-453	Luminal-type	HER2+	2.20 ± 1.13	-	-	+++	-	++	++	-
BT474	Luminal-type	ER+/HER2+	2.39 ± 0.28	++	-	+	+	++++	+++	-
MDA-MB-468	Basal-type	TNBC	3.47 ± 0.33	-	-	-	++++	-	+	-
MDA-MB-231	Basal-type	TNBC	5.47 ± 1.15	-	-	-	++	-	-	-
HCC1806	Basal-type	TNBC	5.37 ± 0.30	-	-	-	+	-	+	-
BT20	Basal-type	TNBC	3.16 ± 1.22	-	-	-	+++	-	-	-

To determine whether the HER2/EGFR pathway plays a role in mediating the effects of SL1-39, we analyzed the expression status of HER2 and its downstream effectors. Western blot analysis was used to determine the expression of HER2 and EGFR in MDA-MB-453 cells treated with SL1-39. Results in figure 9A and B show a 36% decrease in HER2 protein expression within 12 hours of 5 μ M SL1-39 treatment; and after 48 hours of treatment, the expression dropped further to 13.6% in comparison to mock-treated cells. Consistent with this observation, the levels of phosphorylated MAPK and Akt (pMAPK and pAkt), two downstream effectors of the HER2/EGFR signaling pathway also showed significant reduction at 12 hours, while the total MAPK and Akt levels did not decrease until 48 hours.

After confirming that SL1-39 reduces MAPK phosphorylation in MDA-MB-453 cells, we questioned whether other HER2/EGFR positive breast cancer cells (SKBR3, T-47D and MDA-MB-468) have a similar response to SL1-39. Cells were plated in 6-wells plates and treated as previously mentioned. Consistent with the results seen in MDA-MB-453 cells, the two other HER2-positive cell lines, SKBR3 and T-47D, also exhibited lower expression of pMAPK after 12 hours of 2.5 μ M SL1-39 treatment, while the total MAPK did not show any significant change until 48 hours with 5 μ M of SL1-39 (Fig. 9C). Quantification of western blots also confirm that after 48 hours of SL1-39 treatment at 5 μ M, the relative ratio of pMAPK to total MAPK was reduced by 87.6%, 62.3% and 65.8% in MDA-MB-453, SKBR3 and T-47D ($P < 0.001$, Fig. 9D), respectively. In HER2-/EGFR+ MDA-MB-468 cells, the relative ratio of pMAPK to total MAPK was decreased by 75.5% when treated with 5 μ M of SL1-39 for only 12 hours ($P < 0.001$), although this reduction recovered after 48 hours of treatment.

Figure 9. SL1-39 decreased HER2 expression and phosphorylation of HER2 downstream effectors. Cells were plated in 6-wells plate and treated with 2.5 μM, 5 μM SL1-39 or mock treated for 12, 24 or 48 hours. (A) HER2, P-MAPK, MAPK, P-Akt, Akt protein expressions in MDA-MB-453 cells were analyzed with western blots. Actin served as control. (B) Quantification of HER2 expression of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (C) P-MAPK and MAPK protein expressions in MDA-MB-453, SKBR3, T47D and MDA-MB-468 cell lysates were analyzed with western blot. (D) Quantification of western blot, in which column represent average ratio of p-MAPK/total MAPK (with SD) of at least three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

To determine whether the decrease in HER2 and pMAPK occurred earlier than 12 hours, MDA-MB-453 cells were treated with 5 μ M SL1-39 and collected at 2, 4, 8, 12 and 24 hours after treatment. Results in figure 10A show a dramatic reduction in HER2 expression as early as 2 hours after treatment. While the total expression of MAPK did not significantly change, levels of pMAPK were dramatically reduced by 80.1% two hours after treatment ($P < 0.001$, Fig. 10B). The effects of SL1-39 on Akt were also analyzed and results indicate that total Akt levels did not show a significant decrease with the exception of a transient decrease two hours after treatment. Consistent with pMAPK, pAkt levels were also decreased by 94.7% by two hours and 83.5% four hours after treatment ($P < 0.001$, Fig 10B).

To determine whether the changes in HER2 expression were due to alterations at the transcriptional level, qPCR was used to evaluate the transcript levels of HER2 in MDA-MB-453 cells treated as previously mentioned. Results show that there are no significant changes in HER2 mRNA levels even after 24 hours of treatment with 5 μ M of SL1-39 (Fig. 11). Taken together, the early decrease in both HER2 and its activated downstream effectors strongly suggest that SL1-39 likely mediates its growth inhibitory effects on breast cancer cells at least in part by directly targeting the proteins in the HER2/EGFR pathway, post-transcriptionally and/or post-translationally.

A

B

Figure 10. SL1-39 induced early decrease in HER2, pMAPK and pAkt expression. (A) MDA-MB-453 cells were plated in 6-wells plates and treated with 5μM SL1-39 for 2, 4, 8, 12, 24 hours. HER2, p-MAPK, MAPK, p-Akt, Akt protein expressions were analyzed with western blots. Actin served as control. (B) Quantification of western blot, column represent average protein expression level (with SD; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 11. The reduction of HER2 expression is not due to the decrease in transcription level. MDA-MB-453 cells were plated in 6-wells plate and treated with 2.5 μ M, 5 μ M SL1-39 or mock treated for 12 or 24 hours. Real-time PCR analysis of HER2 in total RNA extracted from cell lysates. GAPDH served as control. ($P>0.05$).

Discussion

As one of the original flexible heteroarotinoids (flex-hets), SHetA2 exhibits promising growth inhibitory effects on kidney, lung, head and neck, breast and prostate cancer cells [121, 123, 124, 135]. However, the utility of SHetA2 is limited by its high lipophilicity (logP), which can lead to non-specific interactions and an increased potential for liver toxicity [134]. Thus, a 2nd generation of SHetA2 analogs was designed and synthesized by Dr. Shengguan Liu at Touro University of California, College of Pharmacy (Vallejo, CA). The 2nd generation analogs were focused on increasing the logP value by modifying the structure of the thiochromane ring while retaining the nitrophenyl group and the thiourea linker [135]. Based on the structure-activity relationship established by previous studies [122, 136], the nitrophenyl group and the thiourea linker in the SHetA2 structure are crucial for its anti-cancer activity. In this study, we screened eleven 2nd generation analogs for anti-growth effects against a panel of breast cancer cells including both ER+ breast cancer cells (MCF7, T47D) and ER- breast cancer cells (MDA-MB-231, MDA-MB-453 and MDA-MB-468; Table 3). Consistent with previous studies on similar SHetA2 analogs that retained the nitrophenyl group and the thiourea linker, the majority of the eleven new analogs tested in this study exhibited growth inhibitory effects [135].

Amongst the eleven compounds tested, SL1-38 (1-(3-chloro-4-methylphenyl)-3-(4-nitrophenyl)thiourea) and SL1-39 (1-(4-chloro-3-methylphenyl)-3-(4-nitrophenyl)thiourea), which are structural isomers, displayed efficacies comparable to SHetA2 in breast cancer cells (2-7 μ M and 2-5 μ M range for SL1-38 and SL1-39, respectively, versus 2-4 μ M range for SHetA2). While SL1-38 and -39 can effectively

inhibit cell growth in both ER+ and ER- breast cancer cell lines, our current study was focused on ER- breast cancer cells, as this subtype is known to be more aggressive with poorer prognosis and fewer therapeutic options in comparison with the ER+ subtype [34, 36, 56, 57]. We evaluated the effects of SL1-38 and SL1-39 activities on the three ER- breast cancer cell lines SKBR3, HCC1806 and BT20, and consistent with the other ER- cell lines tested (MDA-MB-231, MDA-MB-453 and MDA-MB-468), these analogs inhibited cell growth at micromolar concentrations (2-9 μ M for SL1-38 and 2-6 μ M for SL1-39; Table 4). However, when tested on normal breast epithelial cells (MCF-10A and HMEC), the GI₅₀ values of SL1-38 and SL1-39 were significantly higher ($p < 0.01$) in comparison to SHetA2 (Table 5), suggesting the two analogs have retained the anti-growth activity while reducing their effects on normal breast cells.

To understand whether the decrease in cell growth was associated with either inhibition of cell proliferation or apoptosis, we also evaluated the ability of SL1-38 and -39 to induce apoptosis on the two cell lines MDA-MB-453 and SKBR3 that exhibited the highest sensitivity to these two compounds. Our data strongly suggest that the ability of SL1-38 and -39 to inhibit breast cancer cell growth is due to inhibiting cell cycle progression and not due to induction of apoptosis (Fig. 4). This differs from what has been observed with SHetA2, which induces apoptosis in head and neck, ovarian, and lung cancer cells [121, 123, 124]. Interestingly, SHetA2 did not induce apoptosis in the breast cancer cells MDA-MB-453 and SKBR3 (Fig. 4). This difference may be associated with cell and cancer type differences. Furthermore, earlier studies that showed induction of apoptosis within 48 hours was observed at 10 μ M SHetA2 treatment [121, 123, 124, 128], a concentration that is 5 fold higher than its GI₅₀ for the ovarian and kidney cancer cells

tested. Guruswamy, S., et al. demonstrated that SHetA2 was able to induce apoptosis at 1 μ M of SHetA2 in ovarian cancer cells cultured in an organotypic model, but only after two weeks of treatment [137]. Neither SHetA2 nor its 2nd generation analogs—SL1-38 and SL1-39— induced apoptosis in normal breast epithelial cells (HMEC and MCF-10A), even when tested at 5 μ M, almost 5-fold of the GI₅₀ value for these normal breast epithelial cells (Fig. 4) and this is consistent with results from previous studies [124, 128]. Because SL1-38 and SL1-39 are structural isomers and have similar potencies on ER- breast cancer cells and normal breast cells, we chose to focus on just one of the two, SL1-39.

Since our results strongly suggest that the effects of SHetA2 and its analogs on breast cancer cell growth is not due to increased apoptosis, but likely a result of cell cycle arrest. Indeed, the results in figures 5C and D demonstrate that both MDA-MB-453 and SKBR3 cells treated with SL1-39 showed not only a decrease in total cell number, but also a decrease in the percentage of cells undergoing DNA synthesis in S-phase. Furthermore, several cell cycle regulators —cyclin A, cyclin B, cyclin D1, cyclin E and cdk2— were decreased at both the protein and mRNA levels (Fig. 6 and 7). More specifically, the expression of cyclin D1 was blocked as early as 12 hours after SL1-39 treatment, suggesting that cyclin D1 is likely one of the earlier targets of the drug and may play an important role in mediating the growth inhibitory effects of SL1-39. Cyclin D1 has been shown to be an essential player in regulating G1/S transition [138, 139], and the down regulation of cyclin D1 has been studied as a potential mechanism for controlling cancer cell growth [140, 141]. In support of this, several studies on ovarian and kidney cancer cells have shown that SHetA2 induced a down-regulation of cyclin

D1, which was due to inhibition of protein synthesis and increased degradation via the proteasome, and this subsequently prevented cells from entering S-phase [128, 142, 143].

To further delineate the mechanism by which SL1-39 blocks ER- breast cancer growth, the expression levels of ER, PR, AR, HER2 and EGFR were evaluated.

Interestingly, our results show that breast cancer cells expressing HER2 and high levels of EGFR tend to display a higher sensitivity towards SL1-39 (Fig. 8 and Table 6), which indicates that SL1-39 may modulate the HER2/EGFR signaling pathway, a pathway known to play an important role in the development and progression of breast cancer [144-147].

To understand the role of the HER2/EGFR pathway in SL1-39 mediated growth inhibition, the expression of HER2 family members and their downstream regulators were analyzed in response to SL1-39 treatment. In support of our hypothesis, the expression of total HER2 was significantly reduced in MDA-MB-453, SKBR3 and T47D cells. Similarly, total EGFR expression was also decreased in SKBR3 and MDA-MB-468 cells (Fig. 9A and data not shown). As expected, the expression of phosphorylated-HER2 (pHER2) was also decreased with SL1-39 treatment (data not shown). Since the HER2 is known to directly regulate the phosphorylation of Akt and MAPK, it is not surprising that reductions in total HER2 and pHER2 would decrease the levels of pAkt and pMAPK in MDA-MB-453 cells (Fig. 9A), while no significant changes in the levels of total Akt and MAPK were observed until 24 hours. Similarly, other HER2 expressing cells, SKBR3 and T47D, also exhibited a significant reduction in pMAPK levels with SL1-39 treatment (Fig. 9B and C), while the EGFR expressing cell line MDA-MB-468 exhibited a transient decrease in pMAPK at 12 hours. Further analysis revealed decreases in HER2, pMAPK

and pAkt levels occurred as early as 2 hours after treatment of MDA-MB-453 cells with SL1-39 (Fig. 10). Since MAPK is known to regulate the expression of multiple cell cycle regulators (i.e. cyclin D1, cyclin E, cdk2) and consequently the progression of the cell cycle [148, 149], the decrease in pMAPK at 2 hours is consistent with the cell cycle effects observed at 12 hours after SL1-39 treatment (Fig. 5C and D).

To further elucidate how SL1-39 alters HER2 expression, we analyzed the expression of HER2 at the mRNA level. Interestingly, the HER2 transcript levels stayed largely unchanged until 24 hours after treatment, suggesting that the earlier decrease in protein at two hours is unlikely due to changes at the transcriptional level (Fig. 11). Additional experiments also suggest that SL1-39 does not induce the degradation of HER2 via the proteasome since the treatment with multiple proteasome inhibitors (MG132, ALLN and epoxomicin as single agents or in combination) did not restore HER2 expression to normal levels (data not shown). Based on these results, we speculate that the down-regulation of HER2 may be associated with either lysosomal degradation or alterations of protein translation.

Collectively, our data strongly suggest that S1-39 inhibits ER- breast cancer cell growth by decreasing the expression of HER2 and thus interrupting the HER2/EGFR signaling pathway. While other anti-HER2 agents have been developed to target HER2+ breast cancers, including trastuzumab, lapatinib and pertuzumab, this is the first study to show that a flexible heteroarotinoid analog can target the HER2 signaling pathway. Trastuzumab [93, 150] and lapatinib [91, 101] are monoclonal antibody-based drugs that bind selectively to the extracellular and intracellular domains of HER2 with high affinity, whereas pertuzumab (another monoclonal antibody) directly blocks ligand-dependent

dimerization of HER2 with other HER receptors [102-104]. Interestingly, SL1-39 promotes a down-regulation of total HER2 in a relatively short time (2 hours), whereas trastuzumab, the most frequently used drug for HER2+ breast cancer, does not affect HER2 expression until 24 hours after treatment [151-154]. However, the GI₅₀ value for trastuzumab in breast cancer cells (SKBR3 and BT-474) is around 1nM [155, 156], while the GI₅₀ for SL1-39 is significantly higher. Unfortunately, many of the anti-HER2 agents like trastuzumab can eventually result in the development of resistance [99, 100, 157], and therefore, developing alternative drugs to target HER2+ breast cancer can provide more therapeutic options to patients who have developed resistance to trastuzumab, lapatinib and/or pertuzumab. Furthermore, these three drugs are monoclonal antibodies whose synthesis and isolation requires sophisticated eukaryotic system and extensive purification steps that in terms of both time and money are quite costly [158, 159]. The relatively rapid action of SL1-39 coupled with its relatively lower synthesis costs make SL1-39 a promising compound that should be further developed to target the HER2+ breast cancer subtype, particularly those that are also ER-negative.

Conclusion

This study evaluated the anti-cancer activities of eleven 2nd generation analogs of SHetA2, amongst which SL1-38 and -39 displayed the most potent effects toward ER-breast cancer cells. Both analogs exhibited similar effects in reducing cell growth by blocking S-phase progression and reducing the expressions of key cell cycle regulators. Results from this study demonstrate for the first time that a flexible heteroarotinoid analog—SL1-39— inhibits breast cancer cell growth by rapidly decreasing the protein expression levels of total HER2 and pHER2, and consequently, the phosphorylation of its

downstream effectors, MAPK and Akt. While future studies are necessary to determine if SL1-39 binds directly to HER2 to mediate down-regulation or if it works through alternative pathways, these findings strongly support further development of SL1-39 as a therapeutic option for HER2+ breast cancer in particular and underscores the importance of studying the effects of flexible heteroarotinoids, like SL1-39, on cancer in general.

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