Drug Screening of Potential Multiple Target Inhibitors for Estrogen Receptor- α -positive Breast Cancer

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Abstract. *Background/Aim:* Estrogen receptor α (ER α) antagonist is the most common treatment for ER α -positive breast cancer. However, compensatory signaling contributes to resistance to ER α antagonists. Thus, to explore the potential agents for targeting compensatory signaling, we screened multiple target inhibitors for breast cancer treatment. *Materials and Methods:* We attempted to build a structure-based virtual screening model that can find potential compounds and assay the anticancer ability of these drugs by overall cell survival assay. The downstream compensatory phosphorylated signaling was measured by immunoblotting. *Results:* Hamamelitannin and glucocheirolin were hits for ER α , phosphoinositide 3-kinase (PI3K), and KRAS proto-oncogene, GTPase (KRAS), which were active against estrogen and epidermal growth factor-triggered proliferation.

Additionally, we select aminopterin as a hit for ER α , PI3K, KRAS, and SRC proto-oncogene, non-receptor tyrosine kinase (SRC) with inhibitory activities toward AKT serine/threonine kinase 1 (AKT) and mitogen-activated protein kinase kinase (MEK) signaling. Conclusion: Our structure-based virtual screening model selected hamamelitannin, glucocheirolin, aminopterin, and pemetrexed as compounds that may act as potential inhibitors for improving endocrine therapies for breast cancer.

Breast cancer is the most common cancer in the world and is the second leading cause of death in women (1). Endocrine therapies are considered the standard-of-care for ER α -positive breast cancer treatment (2). However, disease in almost 30% of such patients eventually becomes resistance to endocrine therapies (3, 4). This resistance may be due to the induction of compensatory signaling that negates the effects of anti-estrogens (5-11). Given these compensatory effects, recent studies found beneficial effects from the combination of inhibitors of compensatory signaling such as phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) or cyclin-dependent kinases (12-16).

Virtual screening is increasingly being used for large-scale screening. Furthermore, virtual screening provides intuitive and effective model for rational drug discovery at a relatively low cost (17-19). Structure-based virtual screening is a kind of virtual screening that was developed following the completion of the Human Genome Project based on advances in structural biology, X-ray crystallography, and nuclear magnetic resonance spectroscopy, which in turn

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revealed druggable target proteins and structure-related detail of their interactions with small molecular agents (20, 21).

The activation of receptor tyrosine kinase (RTK) signaling has been associated with the induction of resistance to antiestrogens. To date, targeting the downstream targets of RTKs with specific small molecules had demonstrated benefits in treatment for patients suffering from breast cancer (22). PI3K-AKT serine/threonine kinase 1 (AKT) signaling plays an important role in regulating cell survival and proliferation in ER α -positive breast cancer, and inhibition of downstream target mTOR has been validated as potential drug combinations for advanced breast cancer (23). Apart from PI3K-AKT-mTOR signaling, RAS-mediated mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) signaling is critical for RTK activation (22), and accumulating evidence has revealed the important role of RAS-MEK-ERK activated mutation in breast cancer (24-26). Although specific inhibitors of PI3K-AKT-mTOR or RAS-MEK-ERK signaling have been developed, multiple targets remain unexplored. Additionally, the proto-oncogene SRC plays a role in the resistance to endocrine therapies (27, 28), implying the value of a combinational approach targeting of ER α and SRC. Unfortunately, saracatinib, a dual kinase inhibitor of SRC and BCR-ABL, plus aromatase inhibitor did not improve the outcome in patients with advanced breast cancer (29). Similar results were also found for the combination of RTK inhibitors and aromatase inhibitor. Co-blockade of human epidermal growth factor receptors 1, 2, and 3 with RTK inhibitor AZD8931 and anastrozole did not show benefits for patients with advanced breast cancer (30). On the other hand, several inhibitors of downstream signaling nodes such as mTOR and CDK4/6 significantly improved on the combination of endocrine therapies in breast cancer (15, 31, 32). It is intriguing to explore potential anticancer agents with inhibitory effects on PI3K-AKT-mTOR and RAS-MEK-ERK signaling.

In this study, we applied structure-based virtual screening to explore multiple inhibitors from an available compound library targeting ER α , PI3K, KRAS, and SRC proto-oncogene, non-receptor tyrosine kinase (SRC).

Materials and Methods

Database. Data regarding a total of 49,752 compounds were downloaded from the natural product databases of the Ambinter (n=41,588), Selleck (n=146), Natural products database of Universidad Estadual de Faria de Santana (UEFS) (n=499), SPECS (n=701), and Traditional Chinese Medicine database Taiwan (n=6,818) and retrieved from the ZINC database (<https://zinc.docking.org/>, accessed on: 19 Jan 2012) (33) for virtual screening.

Preparation of protein structures. The structures of ER α (PDB ID: 3ERT) (34), PI3K (PDB ID: 3HHM) (35), KRAS (PDB ID: 3GFT)

(36), and SRC (PDB ID: 2H8H) (37) were obtained from the Protein Data Bank (<https://www.rcsb.org/>) (38). The co-crystal water molecules, cofactors, and ligands included in the protein structures were all removed using DS 3.5 Visualizer software (39). After removing these co-crystal substances, the atomic hydrogens and charges of each protein's structure were modified according to the CHARMM force field with the partial charge Momany-Rone method using DS 3.5 Visualizer software.

Structure-based virtual screening. To find multiple inhibitors of the target proteins ER α , PI3K, KRAS, and SRC, we performed structure-based virtual screening through molecular docking calculations using GOLD 5.1 software (40-42). The binding site definition was set as follows. The binding site radius was set to 10 Å (default), and the centroid was defined as the center of the co-crystal ligand binding site (active site) of the protein. Subsequently, cavity detection calculations with the Ligsite algorithm (43) were performed to refine the docking space and expand the setting size to include associated residues, with the (x, y, z) coordinates of the cavity centers of ER α , PI3K, KRAS, and SRC being set at (31.41, -0.22, 22.75), (60.78, 63.69, 110.87), (70.72, 97.03, 52.77), and (21.01, 21.28, 59.31), respectively. In addition, the radius of the cavity of ER α , PI3K, KRAS, and SRC was set at 14.379 Å, 15.829 Å, 13.795 Å, and 12.770 Å, respectively. Moreover, search efficiency of 30% (virtual screening parameter) with auto settings and the scoring function GoldScore were set by GOLD 5.1 software. The binding affinities of the inhibitors 4-hydroxytamoxifen (a known ER α inhibitor) (44), NVP-BEZ235 (a known PI3K inhibitor) (45), salirasib (a known KRAS inhibitor) (46), and saracatinib (a known SRC inhibitor) (47) were also estimated by molecular docking calculations using GOLD 5.1 software in order to set one of the filters (that is, the binding affinities of the inhibitors) for the virtual screening. These were used to filter out the compounds with scores lower than the scores of the inhibitors. To do so, the structures of these inhibitors were prepared using ChemBioDraw Ultra 11.0 software (PerkinElmer Informatics, Waltham, MA, USA), with the 3D arrangements of the structures being constructed and optimized with MMFF94 force field minimization using ChemBio3D Ultra 11.0 software (PerkinElmer Informatics). The definitions of the binding sites and docking parameters were set as the same as setting in carrying out virtual screening. Subsequently, to intersect the docking results for ER α , PI3K, KRAS, and SRC, and in order to clearly show the docking results of multiple targets through the virtual screening, a 3D point chart was generated with a 3D point plot module using DS 3.5 Visualizer software.

Cell lines and reagents. Cell lines, except MCF-7 R, were purchased from the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan, ROC), MCF-7 R were kindly provided by Dr. Yao-Tsung Yeh (48). MCF-7 and MCF-7 R cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 (Life Technologies Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (Life Technologies Gibco). T47D and BT474 cells were cultured in RPMI1640 (Gibco) with 10% FBS. MDAMB-361 cells were cultured in L-15 (Sigma-Aldrich) with 20% FBS. All of the culture media contained 1% penicillin-streptomycin (P/S; Gibco). Cells were incubated at 37°C in 5% CO₂. The following compounds were purchased from Sigma-Aldrich and were used: Alexidine dihydrochloride, ($\pm$)-amethopterin, aminopterin, atranorin,

Figure 1. The flow chart of virtual screening targeting estrogen receptor alpha (ER α), phosphoinositide 3-kinase (PI3K) and KRAS proto-oncogene, GTPase (KRAS).

benfotiamine, benzthiazide, bucladesine, cefamandole sodium, cefmetazole sodium, cephalosporin C sodium, chlorhexidine dihydrochloride, curcumin, cytidine 5'-diphosphocholine sodium, 1,3-dicaffeoylquinic acid, fumarprotocetraric acid, folic acid, glipizide, glucocheirolin, hamamelitannin, hyperoside, isochlorogenic acid C, kaempferol 3-*O*-glucuronide, methotrexate, methyl prednisolone sodium succinate, mitoxantrone hydrochloride, nicardipine hydrochloride, pemetrexed, phthalylsulfathiazole, pyritinol, riboflavin 5-phosphate sodium, rottlerin, and sulfapyrazone. The compounds were dissolved in dimethyl sulfoxide to a final concentration of 100 mM and stored at -20°C .

Survival assays. Assessments of cell viability were performed as follows. Cells were seeded in 96-well plates so that the control cells would reach approximately 80% confluency at the end of the assay. On the following day, the cells were treated for 5 days with increasing concentrations (0, 6.25, 12.5, 25, 50 and 100 μM) of the study drugs with/without stimulation for 30 min with 1 ng/ml epidermal growth factor (EGF) or 1 nM 17 beta-estradiol (E2). Treatment with each concentration was performed three times ($n=3$). Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent as previously described (49-51).

Immunoblotting. Cell lysates were prepared as previously described (52). Antibodies against p-AKT (Ser473), AKT, p-p42/44 ERK (Thr202/Tyr204), p42/44 ERK, MEK 1/2 and p-MEK1/2 (Ser217/221) were obtained from Cell Signaling Technology (Danvers, MA, USA). Equal amounts of protein were separated by 10% TrisGlycine Gel and then transferred onto polyvinylidene

difluoride (PerkinElmer, Boston, MA, USA) membrane using Wet/Tank Blotting Systems (BIO-RAD, Hercules, CA, USA). The membrane was blocked with 5% nonfat dried skimmed milk powder (Cell Signaling Technology) and incubated with primary antibody at a ratio of 1:1,000 overnight and washed with double-distilled water three times 10 min each. Then the membrane was incubated with goat anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and protein signals were developed with WesternBright ECL HRP substrate (Advansta, San Jose, CA, USA) then detected by ChemiDoc XRS+ (BIO-RAD).

Statistical analysis. The results are presented as the mean $\pm$ standard deviation (SD) of three independent experiments. The data were analyzed using GraphPad Prism version 5. (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant when the *p*-value was less than 0.05.

Results

Building a model for finding multi-target inhibitors by virtual screening. Because both PI3K and RAS signaling pathways have been reported to induce endocrine resistance in breast cancer (53, 54), we sought to identify small molecules for re-sensitizing breast cancer cells to endocrine therapies. To discover novel small molecules that inhibit ER α , PI3K, and KRAS, we first conducted virtual screening

Figure 2. The principal triple-targeted fitness score analysis of compounds selected from the ZINC database. The cyan spheres falling within the grey circles are compounds with a reasonable score for interaction with the targets estrogen receptor alpha (ER α), phosphoinositide 3-kinase (PI3K), and KRAS proto-oncogene, GTPase (KRAS). We utilized three well-established agents, 4-hydroxytamoxifen (4-OH; inhibitor of ER α), NVP-BEZ235 (inhibitor of PI3K) and salirasib (inhibitor of RAS), as references for scoring new compounds. The red spheres falling within the grey circle of at top right are novel triple-hit compounds which interact with all three targets.

using multiple natural product and drug libraries. We established a screening mode that combined the molecular docking of ER α , PI3K, and KRAS using GOLD5.1 software. Additionally, we used five compound libraries, namely, the Ambinter, Selleck, UEFS, SPECS, and TCM databases, with more than 40,000 small molecules being selected for inclusion in the screening mode (Figure 1). Well-known inhibitors of ER α (4-hydroxytamoxifen), PI3K (NVP-BEZ235), and KRAS (salirasib) were used as positive standard for evaluating the GoldScores. As show in Figure 1, the GoldScores of these inhibitors were 72.69, 65.40, and 68.73, respectively. We then selected candidate compounds with values higher than those of these inhibitors. Using this approach, we found 812 hits for ER α , 3,478 hits for PI3K, and 5,952 hits for KRAS. We further analyzed which of these compounds dual- or triple-inhibited ER α , PI3K, and KRAS, and identified 315 molecules that interacted with both ER α and PI3K, 382 molecules that interacted with both ER α and KRAS, 1645 molecules that interacted with both PI3K and KRAS, and 187 molecules that interacted with

ER α , PI3K, and KRAS at the binding sites of the three targeted proteins (Figure 2). There were 10 compounds, namely, nicardipine hydrochloride (GoldScores for ER, PI3K and KRAS of 80.38, 87.87 and 80.34, respectively), alexidine dihydrochloride (74.89, 68.46 and 76.52), ($\pm$)-amethopterin (75.35, 73.69 and 81.67), chlorhexidine dihydrochloride (84.5, 72.27 and 69.06), cytidine 5'-diphosphocholine sodium (89.89, 66.3 and 106.72), kaempferol 3-*O*-glucuronide (73.35, 69.45 and 84.22), hyperoside (84.79, 67.63 and 84.79), hamamelitannin (72.9, 75.92 and 76.99), glucocheirolin (73.35, 69.45 and 84.22), and 1,3-dicaffeoylquinic acid (74.46, 67.63 and 84.79), that were selected from the 187 triple-hit compounds using knowledge-based selection.

To examine whether these triple-hit compounds exhibited biological activity against cancer cells upon EGF or E₂ stimulation, we assessed their effects on cell viability. EGF or E₂ was deliberately added with the intention to increase ER α -mediated and EGFR-mediated cell growth signaling. T47D were treated with select compounds at one-fold their half-

Figure 3. Cell viability assay of T47D breast cancer cell line after treatment with triple-hit compounds. Cells were treated only with hamamelitannin and glucocheirolin at 1-fold half-maximal inhibitory concentration and in combination with stimulation by 1 nM 17 beta-estradiol (E₂) or 1 ng/ml epidermal growth factor (EGF), and then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to monitor viable cells at 570 nm. Data are the mean $\pm$ SD, n=3.

maximal inhibitory concentration (IC₅₀) combined with/without E₂ or EGF for 5 days, subsequently, the viability was assessed by MTT assay. As shown in Figure 3, we found that of the selected triple-hit compounds, hamamelitannin and glucocheirolin had inhibitory effects against E₂ and EGF-driven proliferation, respectively, in the T47D breast cancer cell line. Treatment with 1 nM E₂ or 1 ng/ml EGF significantly enhanced cell growth by up to 120% in comparison to non-stimulate treatment. Cells treated with hamamelitannin at the IC₅₀ concentration suppressed E₂- and EGF-stimulated cell growth. Notably, glucocheirolin showed similar activity against E₂ and EGF. Additionally, the mode of the compound-protein interactions showed that both hamamelitannin and glucocheirolin had a similar binding pattern even though these compounds had different types of chemical structure (Figure 4). Of particular note, both hamamelitannin and glucocheirolin interacted with Glu353 and Arg394 of ER α , which are critical to E₂-ER α interactions in the downstream signaling process (Figure 4). Moreover, hamamelitannin and glucocheirolin interacted with Tyr836 and Val851 of PI3K, which are AKT-binding sites (Figure 4).

Screening hits for multiple targets ER α , PI3K, KRAS, and SRC. Apart from the identification of triple inhibitors of ER α , PI3K and KRAS, HER2 is another target for breast cancer treatment. Recently, the importance of SRC in trastuzumab resistance has been emphasized (37-39), suggesting the urgent need for screening novel inhibitors for SRC inhibition. Thus, we further sought to screen triple-hits for inhibiting SRC, PI3K and KRAS. For this, we analyzed the GoldScores of the hit compounds shared between PI3K and KRAS (1,645 hits) for activity against SRC. The GoldScore of saracatinib, a positive standard for SRC protein, was 72.96. However, we extended the range of the search to all the docked compounds of SRC in order to

increase the rate of discovery of triple inhibitors of PI3K, KRAS and SRC. In doing so, we found that only 119 molecules were common for inhibition of SRC, PI3K, and KRAS. Ultimately, we selected four targets in total, namely, ER α , SRC, PI3K and KRAS, in order to discover new hit compounds. As shown in Figure 5, 187 hit compounds were shared between ER α , PI3K, and KRAS, and another 119 hits between SRC, PI3K, and KRAS. Therefore, in the knowledge-based selection, a total of 19 compounds, namely, glipizide (GoldScores for PI3K, KRAS and SRC of 71.14, 83.12 and 67.14, respectively), sulfapyrazone (69.46, 71.61 and 60.99), atranorin (69.22, 70.28 and 63.97), cefamandole sodium (67.19, 80.28 and 63.55), benfotiamine (72.34, 93.94 and 70.51), aminopterin (80.05, 79.49 and 73.43), mitoxantrone hydrochloride (72.88, 71.21 and 66.96), cefmetazole sodium (68.24, 72.04 and 62.44), bucladesine (66.4, 77.98 and 65.24), benzthiazide (69.85, 72.41 and 69.33), cephalosporin C sodium (65.72, 88.86 and 60.01), rottlerin (65.72, 88.86 and 67.97), methyl prednisolone sodium succinate (67.3, 73.35 and 30.3), fumarprotocetraric acid (66.66, 75.58 and 62.57), riboflavin 5-phosphate sodium (80.64, 80.88 and 65.54), isochlorogenic acid C (66.15, 71.15 and 74.92), phthalylsulfathiazole (70.61, 74.06 and 71.9), pyritinol (71.05, 77.9 and 68.18), and curcumin (66.75, 70.52 and 63.09) were selected.

In-vitro validation of verified screened inhibitory compounds.

Cell survival assay: After the verification of the screened compounds next, we examined whether the hit compounds of SRC, PI3K, and KRAS had the ability to inhibit cell viability upon EGF treatment. To assess their activities, cells were treated with 1 ng EGF and then were co-treated with the selected hit compounds. Eighteen hit compounds were subjected to MTT assays. As shown in Figures 6 and 7, we found that eight of the hit compounds exhibited effects against

Figure 4. Typical stick structure model showing docking orientation and interaction of triple-hit compounds hamamelitannin and glucocheirolin with different residues of estrogen receptor alpha (ERα), phosphoinositide 3-kinase (PI3K), and KRAS proto-oncogene, GTPase (KRAS). Hamamelitannin (green stick) and glucocheirolin (pink stick) successfully docked into the ligand binding sites of ERα (cyan), PI3K (white) and KRAS (yellow), respectively.

Figure 5. 3D Point chart representing the triple-targeted fitness score of docked compounds. The compounds (show as red spheres) from the database interact with three chosen target proteins phosphoinositide 3-kinase (PI3K), KRAS proto-oncogene (KRAS), and SRC proto-oncogene (SRC). The ligand docking scores were compared to those of three well-established agents NVP-BEZ235 (inhibitor of PI3K) salirasib (inhibitor of RAS), and saracatinib (inhibitor of SRC) to filter out other irrelevant compounds from the database.

EGF-stimulated cell proliferation. Specifically, atranorin, mitoxantrone, aminopterin, rottlerin, fumarprotocetraric acid, riboflavin 5-phosphate sodium, isochlorogenic acid C and curcumin reduced the viability of the cells by over 50%. Interestingly, we found that atranorin and fumarprotocetraric acid, which are metabolites of the acetyl-polymalonyl pathway, both exist in lichen and showed similar activity profiles.

Immunoblot analysis: In order to investigate the effects of the hit compounds on the pathways of downstream signaling, we analyzed the status of phosphorylation of AKT and ERK upon treatment with the hit compounds under EGF stimulation. Cells were treated with EGF and then co-treated with the hit compounds at 0.25-, 0.5-, 1- and 2-fold IC₅₀ values. As shown in Figures 8 and 9, treating the cells with EGF elevated their levels of p-AKT and p-ERK at 2 and 24

h, respectively. Our data indicated that atranorin and curcumin inhibited the phosphorylation of AKT at 0.25- to 0.5-fold IC₅₀ values, but the signals were restored at 1- to 2-fold IC₅₀ values in the T47D cell line. Riboflavin 5-phosphate inhibited the phosphorylation level of AKT and ERK at 24 h at more than 1-fold IC₅₀ values. Rottlerin inhibited the phosphorylation of AKT at low concentrations in the T47D and MCF-7 cells. Mitoxantrone not only inhibited the phosphorylation of AKT in both the MCF-7 and T47D cell lines but also inhibited the phosphorylation of ERK in MCF-7 cells at 2 h. Aminopterin inhibited AKT and ERK in T47D cells at 0.25- to 2-fold IC₅₀ values and inhibited the phosphorylation of AKT in MCF-7 cells upon EGF stimulation for 24 h.

As shown in Figures 8 and 9, aminopterin was found to exhibit dual inhibition of the phosphorylation of AKT and

Figure 6. Cell survival analysis of the MCF-7 cell line upon treatment with potential hit compounds and the effect of co-treatment with 1 ng/ml epidermal growth factor (EGF). MCF-7 cells were treated 1 ng/ml EGF combined with 0, 6.25, 12.5, 25, 50, or 100 μ M of compounds [atranorin, fumarprotocetraric acid (FPC), mitoxantrone, riboflavin 5-phosphate sodium (FMN-Na), aminopterin, isochlorogenic acid C, rottlerin, and curcumin] for 5 days and viability of the cells was analyzed by employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent. Data are the mean $\pm$ SD, n=3.

Figure 7. T47D breast cancer cell line was treated with candidate compounds (atranorin, FPC, mitoxantrone, FMN-Na, aminopterin, isochlorogenic acid C, rottlerin, and curcumin) at 0, 6.25, 12.5, 25, 50, 100 μM with and without 1 ng/ml epidermal growth factor (EGF) for 5 days, then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent was added. Viability of cells in each well was determined at 570 nm. Data are the mean $\pm$ SD, $n=3$.

Figure 8. Critical signaling proteins involved in target inhibition by hit compounds in MCF-7 cells. MCF-7 cells were pre-treated with potential hit compounds at different fold of half-maximal inhibitory concentrations for 2 or 24 h in phenol-free medium comprising 5% charcoal-stripped fetal bovine serum and co-treated with 1 ng/ml epidermal growth factor (EGF). Cells were then harvested and the cell lysate was used to determine the change in expression pattern of phosphorylated and non-phosphorylated target proteins in serine/threonine kinase 1 (AKT) and extracellular signal-regulated kinase (ERK).

Figure 9. Critical signaling proteins involved in target inhibition by hit compounds in T47D cells. T47D cells were pre-treated with potential hit compounds at different fold of half-maximal inhibitory concentrations for 2 or 24 h in phenol-free medium comprising 5% charcoal-stripped fetal bovine serum and stimulated with 1 ng/ml epidermal growth factor (EGF). After the incubation, cells were then harvested and the cell lysate was used to monitor the changes in the expression pattern of phosphorylated and non-phosphorylated target proteins in serine/threonine kinase 1 (AKT) and extracellular signal-regulated kinase (ERK).

Folic acid

Pemetrexed

Methotrexate

Aminopterin

Figure 10. The molecular structure of folic acid, pemetrexed, methotrexate and aminopterin.

ERK at a wide range of concentrations. We found that the structure of aminopterin is similar to that of folic acid. Moreover, pemetrexed and methotrexate have structures like that of folic acid and can be developed as anticancer drugs by acting as folic acid antagonists (Figure 10). To examine whether pemetrexed and methotrexate have the same function as aminopterin, we used several breast cancer cell lines, MCF-7, T47D, MDAMB-361, BT-474, and tamoxifen-resistant MCF-7 (MCF-7 R). For synchronizing all the cell lines at the same phosphorylation state at baseline, we starved these cell

lines overnight and then treated them with a given drug for 1.5 h and then stimulated them with 1 nM E_2 and 1 ng EGF for 30 min. Then, we determined the phosphorylation status of AKT and MEK, which are downstream signaling of PI3K and KRAS. In the BT474, T47D and MDAMB361 breast cancer cell lines, aminopterin inhibited the phosphorylation of AKT. Pemetrexed inhibited the phosphorylation of AKT and of MEK in the MDAMB-361 cell line (Figure 11). We used MCF-7 and the MCF-7 R cell lines to test these compounds. Our data indicate that pemetrexed inhibited the phosphorylation of AKT and MEK in the MCF-7 cell line. However, in the MCF-7 R cells, pemetrexed inhibited the phosphorylation of MEK, while aminopterin inhibited the phosphorylation of AKT (Figure 11).

Discussion

Structure-based virtual screening has proven to be a convenient and time-saving tool for lead-compound screening in drug development. In this study, we established a screening model that gave hits hamamelitannin and glucocheirolin, which we subsequently found effectively inhibited E_2 and EGF-stimulated proliferation of breast cancer cell lines (Figure 3). The docking data showed that hamamelitannin and glucocheirolin interacted with Glu353 on the E_2 -binding site of $ER\alpha$ and with Tyr836 and Val851 on the AKT-binding site of PI3K by forming hydrogen bonds. In previous studies, raloxifene and tamoxifen were also interactive with the same residue of Glu353 in $ER\alpha$ (34, 55). In addition, NVP-BEZ235 was found to interact with Val851 in PI3K (35, 56). On the other hand, some reports showing that Tyr836 in PI3K was a key binding residue for active inhibitors (56, 57). Hamamelitannin and glucocheirolin acted on residues at the same position as these well-known inhibitors. This may imply that these small molecules also have the potential to inhibit the target protein. Previous studies reported that hamamelitannin and glucocheirolin also showed anti-proliferative effects on colon cancer (58, 59). Taken together, these data may suggest that hamamelitannin and glucocheirolin are able to desensitize cells to E_2 or EGF stimulation cell growth through their ability to inhibit $ER\alpha$ and PI3K.

In the second screening, we found eight compounds which were able to counteract the stimulation of EGF treatment (Figures 6 and 7). One of them was aminopterin, which we found inhibited PI3K downstream signaling of phospho-AKT in the MCF-7 and T47D cell lines. The results presented in Figures 8, 9 and 11 suggest that aminopterin has the potential to act as a dual inhibitor of PI3K and KRAS. A phase II study showed that aminopterin led to the remission of acute lymphoblastic leukemia in children (60), while in another study it was effectively used against immune-related diseases such as rheumatoid arthritis (61). Our study is the very first of its kind, and revealed that aminopterin has the

Figure 11. Dual inhibitor ability assay. Breast cancer cells were starved overnight (indicated as S) and then treated with pemetrexed and aminopterin at different concentrations for 2 h and stimulated by 1 nM 17 beta-estradiol and 1 ng/ml epidermal growth factor for 30 min. After incubation, cells were harvested and analyzed for the expression pattern of phosphorylated and non-phosphorylated target proteins in serine/threonine kinase 1 (AKT) and mitogen-activated protein kinase kinase (MEK) involved in downstream processing of the signal.

ability to act as a dual inhibitor of PI3K and KRAS. This suggests, in turn, that aminopterin may be of benefit in patients with ER-positive breast cancer who be disease has become resistant to endocrine therapies.

Furthermore, we found that aminopterin exhibits a molecular structure (Figure 10) highly similar to that of folic acid. Aminopterin is an antifolate drug like the currently used drugs methotrexate and pemetrexed, both of which can inhibit one-carbon transfer reactions in nucleotide synthesis. In our study, we further found that aminopterin reduced the phosphorylation of p-AKT, even in the MCF-7 R cell line, and reduced the phosphorylation of p-MEK in breast cancer cell lines under E₂ and EGF stimulation. However, methotrexate, whose structure is most similar to that of aminopterin, did not inhibit phosphorylation in the downstream signaling of these target proteins. Comparing the structures of the four compounds, we assume that aminopterin may have better inhibitory ability due to the R form structure on the right-side carbon chain, while pemetrexed provides partial inhibition of phosphorylation due to the nitrogen-containing heterocyclic on the left side (Figure 10). Of particular note is the fact that pemetrexed has reported benefit on recurrent, metastatic or advanced breast cancer in some clinical reports (62-64). Thus, it can be speculated that a structural modification of aminopterin might be instrumental in improving the current pemetrexed regimen.

Collectively, we identified four potential candidate compounds for targeting ER α , PI3K, KRAS, and SRC using structure-based virtual screening. These findings may provide important insight into the development of new therapeutic approaches that target multiple RTKs-mediated activated pathways in breast cancer.

Conflicts of Interest

The Authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as no conflict of interest.

Authors' Contributions

YHD and GYC acquired the data. WYC and JCY contributed to the concept and design of the project. YHD and JCY analyzed and interpreted the data. YHD and JCY performed the literature review and wrote the article. GYC, WCH, CHT and YCW revised the article.

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