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14. ABSTRACT ErbB-2 is overexpressed in 25-30% of breast cancers and is a promising therapeutic target with clinically proven outcome for the treatment of breast cancer. In breast cancers with erbB-2 overexpression, abnormal cell proliferation is caused by the extremely high tyrosine kinase activity of erbB-2. Hence, inhibition of the erbB-2 kinase activity is a promising strategy for the treatment of breast cancer. Through a powerful computerized structure-based 3D-database searching, we have discovered several potent and selective small molecule kinase inhibitors of erbB-2. One such promising lead compound potentially inhibits the kinase activity of erbB-2 both <i>in vitro</i> and <i>in vivo</i> , while it has little effect on the kinase activity of EGFR, displaying an excellent selectivity between Her-2 and EGFR. Furthermore, this lead compound was shown to effectively inhibit tumor growth in multiple human breast cancer xenograft models with high Her-2 overexpression and have little effect on tumor growth in xenograft models with high level of EGFR but low level of Her-2. Our studies demonstrate that potent and selective erbB-2 kinase inhibitors may have a great therapeutic potential as a novel and molecularly targeted therapy, either alone or in combination with other therapies, for the treatment of breast cancer.					
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INTRODUCTION:

Her-2/neu/erbB-2 gene encodes a 185 kD transmembrane glycoprotein that has partial homology with other members of the epidermal growth factor receptor (EGFR) family. ErbB-2 is overexpressed in 25-30% of breast cancers and it has been associated with a high risk of relapse and death. Therefore, therapies targeted at erbB-2 have a great therapeutic potential for the treatment of breast cancers. Indeed, the recently completed phase III clinical trial of anti-Her2 antibody Herceptin has provided evidence that systemic administration of Herceptin alone and in combination with cytotoxic chemotherapy in patients with erbB-2 overexpressing primary tumors, can increase the time to recurrence and overall response rates in metastatic breast cancer. Hence, erbB-2 is a therapeutic target with clinically proven outcome for the treatment of breast cancer.

In breast cancers with erbB-2 overexpression, abnormal cell proliferation is caused by the extremely high tyrosine kinase activity and resulting high level of signal transduction. Theoretically, erbB-2 kinase inhibitors that are capable of entering cell, blocking this extremely high tyrosine kinase activity and shutting-down the signal transduction pathway mediated by erbB-2 may be used as potential therapeutic agents for the treatment of breast cancer. A number of criteria should be considered in the development of small molecule kinase inhibitors, including good potency, selectivity, cell permeability, bioavailability, appropriate pharmacokinetics and non-toxicity. Although a potent, erbB-2 specific small-molecule kinase inhibitor capable of entering the cell is likely to have a great therapeutic potential for the treatment of breast cancers, to date, very few potent and erbB-2 specific small-molecule kinase inhibitors are reported.

In this DOD IDEA grant, we propose to discover potent and erbB-2 specific kinase inhibitors through structure-based drug design approach. To investigate the possibility of designing selective erbB-2 inhibitors, we have modeled the erbB-2 and EGFR kinase domains. Our molecular modeling studies showed that erbB-2 has a highly conserved ATP binding site in its kinase domain as compared to other receptor kinases, but it has two unique residues (Cys805 and Ser783) located at the ends of ATP binding region and different from those in most other receptor kinases. It is of note that the Cys is common for members of EGFR family (EGFR, erbB-2 and erbB-4) but the Ser residue is unique for erbB-2. We note that using the unique Cys

residue common for the EGFR family, a class of highly potent and selective irreversible kinase inhibitors has been successfully designed with the aid of molecular modeling and has good *in vivo* efficacy. Thus our molecular modeling studies and the recent success of designing potent and selective kinase inhibitors for the EGFR family lead us to hypothesize that the kinase domain of erbB-2 may have sufficient structural distinctions from other receptor kinases for the design of selective erbB-2 kinase inhibitors. In order to carry out successfully the design of erbB-2 selective kinase inhibitors, we propose to employ a powerful, computerized structure-based 3D-database searching strategy to search over more than 500,000 small organic compounds and natural products to identify promising potential erbB-2 kinase inhibitors that can effectively interact with the erbB-2 ATP binding site. The discovery of novel, potent and selective erbB-2 kinase inhibitors represents the first but important step to develop such inhibitors as a novel therapy, alone or in combination with other therapies, for the treatment of breast cancer in near future.

The following three specific tasks were proposed in our original proposal.

Task #1. Structure-based discovery of potential erbB-2 selective kinase inhibitors

Task #2. Investigation of the potency and selectivity of potential erbB-2 kinase inhibitors

Task #3. Evaluation of *in vivo* anti-tumor activity and toxicity of most promising erbB-2 kinase inhibitors

In the following sections, we described our accomplishments for each specific task.

Body of the final report

Task 1. Molecular modeling, Structure-based database searching, and computational docking

- **1.a. Further refinement of the erbB-2 and EGFR models.**

Experimental 3D structure (including the kinase domain) of either erbB-2 or EGFR has not been determined. Fortunately, the structures of the kinase domain of a number of receptor tyrosine kinases have been determined through X-ray crystallography with high resolutions. Protein kinases, including erbB-2 and EGFR, have an active and inactive conformation. Analysis of the X-ray structures of several kinases, including insulin receptor kinase and FGFR kinase showed that when an ATP analog or a small molecule kinase inhibitor bound to the ATP binding site of the kinase domain, the kinase always assumes an active conformation. Accordingly, it is hypothesized that erbB-2 and EGFR also assume an active conformation when bound to an inhibitor.

We have used three template protein structures all with high resolution to model the active conformations of erbB-2 and EGFR. The first is the structure of the kinase domain of the insulin receptor kinase bound to an ATP analog and a peptide substrate, which was determined with X-ray crystallography to an accuracy of 1.9 Å (PDB code: 1IR3). The second is the structures of the kinase domain of the human FGFR1, either alone or in the complex with a small molecule kinase inhibitor SU4984, or SU5402, or PD173074, whose structures were determined with X-ray crystallography to an accuracy of from 2.0 to 2.5 Å (PDB codes: 1FGK, 1AGW, 1FGI and 2FGI). The third is the structure of the human tyrosine protein C (SRC), whose structure was determined with X-ray crystallography to an accuracy of 1.5 Å (PDB code: 1FMK). These proteins share the highest degree of homology with erbB-2 and EGFR in their kinase domains among all the structures in the Protein Databank (PDB). The rationale of using structures of three different kinase domains for homology modeling is to investigate if significant differences were found with modeled structures when different template structures were used. The sequence alignment between the kinase domains of erbB-2 and EGFR and these template proteins is shown in Figure 1.

Based upon the sequence alignment, erbB-2 and the insulin receptor kinase domains have an amino acid identity of 35% and a similarity of 52%; erbB-2 and the FGFR1 and kinase domains have an amino acid identity of 37% and a similarity of 55%; erbB-2 and the SRC kinase domains have an amino acid identity of 41% and a similarity of 55%. There is 10% of amino acid gap (insertion and deletion) between erbB-2 and the insulin receptor kinase domains, 8% gap between erbB-2 and the FGFR1 and kinase domains and 5% gap between erbB-2 and the SRC kinase domains. Accurate modeling of these gap regions is a major challenge and problem in homology modeling at the present time. Fortunately, the major gap is located in a loop region connecting two helices in all the three template proteins, which is approximately 20 Å away from the ATP/inhibitor binding site. Therefore, the potential problem with accurate modeling the gap region is of minimal significance in our structure-based design erbB-2 kinase inhibitors. It is of note that even in the X-ray structures of insulin receptor, SRC and FGFR1, the coordinates of the loop region residues were missing, due to the high flexibility of the loop region.

Figure 1. Sequence alignment between erbB-2, EGFR, Insulin receptor kinase, FGFR1, and SRC tyrosine kinase and this alignment was used for homology modeling. The residues that form the ATP binding pocket are highlighted.

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VKVLGSGAFGTVYKG-IWIPDGENVKIPVAIKVIRENTSPKANKEILDEAYVMAGVGSP-YVSRLLGI (erbB2)
IKVLGSGAFGTVYKG-LWIPEGEKVIPVAIKELREATSPKANKEILDEAYVMASVDNP-HVCRLGI (EGFR)
GKPLGEGAFGQVLA-EAIGLPNRVT-KVAVKMLKSDATEKDLSLISEMEMMKMIGKHKNIIINLLGA (1IR3)
LRELQGSGFSGMVYEGNARDIIKGEAETRVAVKTVNESASLRERIEFLNEASVMKGFTCH-HVVRLLGV (1FGK)
EVKLQGCFCGEVWMTW-----NGTTRVAIKTLKPGTMSPE--AFLQEAQVMKKL-RHEKLVQLYAV (1FMK)

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(Helix region) (loop region)

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_____
CL-TSTVQLVTQLMPYGCuLLDHVRENrGRrLGrSD-----LLNWCM-QIAKGMSYLEDVRLVHRDLA
CL-TSTVQLITQLMPFGcLLDYVREhKNdIGsQY-----LLNWCV-QIAKGMYLEDRLVHRDLA
CTQDGPLYVIVEYASKGNLREYLQARR-QLSSK-----LVS-CAYQVARGMEYLASKKCIHRDLA
VSKGQPTLVVMElMAHGDlKSlYLRSLRPEAENNPGRPPPTLQEMIQMAAEIADGMAYLNaKKFVHRDLA
-VSEEPiIYIVTEYMSKGSLLDFLkGETGK-----YLRLPQLVDMAAQIASGMAYVERMNYVHRDLR

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ARNVLVKSPNHVKITDFGLARLLDIDETEHADGGKVPIKWMALeSILRRRFTHQSDVWSYGVTVWELM...
ARNVLVKTPQHVKITDFGLAKLLGAEeKEYHAEGGKVPIKWMALeSILHRIYTHQSDVWSYGVTVWELM...
ARNVLVTEDNVMKIADFGLA-----DYYKKG-RLPVKWMAPEALFDRIYTHQSDVWSFGVLLWEIF...
ARNCMVAHDFTVKIGDFGMTRDIETDRKG---GKGLLPVRWMAPESLKDGvFTTSSDMWSFGVLLWEIT...
AANILVGENLVCKVADFGLAR-----FPIKWTAPeEAALYGRFTIKSDVWSFGILLTELT...

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Many homology modeling studies, including blind structure predictions from the Critical Assessment of Structure Prediction (CASP) experiments, have shown that when the homology

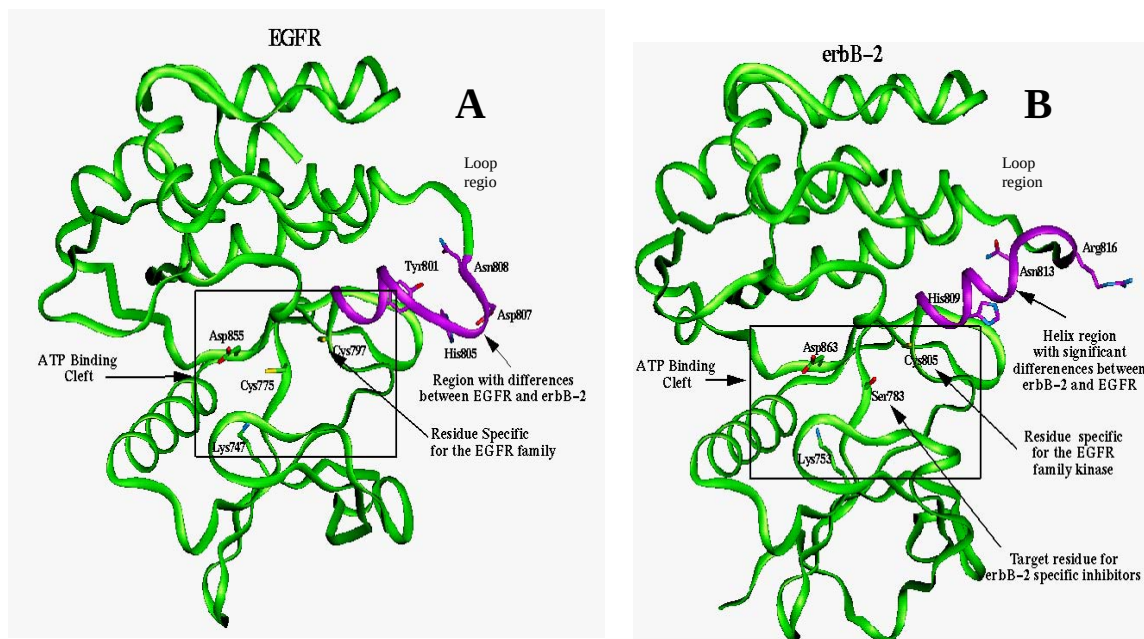


Figure 2. 3D structure representation of erbB-2 and EGFR derived from homology modeling using 11R3 as the template structure. The crucial differences in residues between erbB-2 EGFR and other kinases in their ATP binding sites are highlighted, including a unique cysteine residue (Cys805 in erbB2 and Cys775 in EGFR) and a number of residues in the helix region.

between the target and template proteins are more than 30%, an accurate structure of the target protein can be reliably obtained. Therefore, the high degree of homology (35-41% of amino acid identity and 52-55% of amino acid similarity) between erbB-2, or EGFR, and these three different template proteins should allow us to achieve an accurate modeling of the 3D structure of the erbB-2 and EGFR kinase domains.

The sequence alignment between EGFR kinase domain and these three different kinase domains is also provided in Figure 1. The amino acid identity and similarity between EGFR and these three kinase domains are comparable to that between erbB-2 and these three kinase domains. The amino acid identity is 35-38%, the amino acid similarity is 54-55% and the gap is 4-5%. Similar to erbB-2, the gap is located in the region connecting the two helices and 20 Å away from the ATP binding site. Therefore, it is expected that an accurate 3D structure of the EGFR kinase domain can be obtained based upon the X-ray structures of these template proteins using homology modeling approach.

A homology modeling program, MODELLER, developed by Dr. Sali at Rockefeller University has been shown to be able to provide a fairly accurate 3D structure for a target protein when there is at least 30% of sequence homology between the target and the template protein. The predictive ability of MODELLER program has been validated through a number of unbiased and blind structural prediction tests. Accordingly, MODELLER was chosen to model the 3D structures of the erbB-2 and EGFR kinase domains based upon the sequence alignment shown in Figure 1 and three X-ray structures of insulin receptor kinase (pdb code: 1IR3), FGFR1 kinase domain (pdb code: 1FGI), and SRC tyrosine kinase (pdb code: 1FMK).

With such high degree homology between target proteins and template proteins, it is expected that homology modeling should provide accurate structural information for the main chain of the proteins. However, the side chain conformations may need to be further refined, although MODELLER does use a side chain library to model the side chain conformations. For this purpose, we have carried out extensive molecular dynamics simulations using the CHARMM program. The force field used in the simulations to represent the proteins is the latest CHARMM force field (version 24). To accurately model the solvent environment, TIP3P water model was used to represent the water molecules. It is of note that the CHARMM force field was developed specifically to be compatible with the TIP3P water model for modeling proteins and DNAs. Constant temperature MD simulations were carried out at 300 K for 1000 ps or longer. Since it is believed that the main chain conformations were modeled fairly accurately, constraints were applied to main chain atoms. The refined structures of erbB-2 and EGFR kinase domains using IR3 as the template structure are shown in Figure 2.

It is important and instructive to compare the structures of erbB-2 and EGFR kinase domains with the experimental structures of other kinase domains to gain a fundamental understanding on the structural similarity and difference between these kinases. Such analysis should provide the structural basis for the structure-based design of selective and potent kinase inhibitors.

Based upon our modeled structures, we found that erbB-2 and EGFR have a very similar ATP binding site in terms of general shape as compared to other receptor kinases such as insulin receptor tyrosine kinase. However, among the residues that form the ATP binding site, there exist significant differences between the EGFR family (erbB2 and EGFR) and other kinases. Therefore, it is expected that potent and highly selective inhibitors can be designed against the

EGFR family kinase. Indeed, a number of highly potent and selective reversible EGFR family kinase inhibitors have been reported.

It is of note that a cysteine residue (Cys805 in erbB-2 or Cys797 in EGFR), located on one end of a helix immediately outside the ATP binding pocket (Figure 2), is quite unique for EGFR and erbB-2 and is not shared by other receptor kinases. Using this single unique cysteine for the EGFR family, potent and highly selective, irreversible kinase inhibitors for the EGFR family have been successfully designed[6; 12; 16].

Comparison between the modeled structures of erbB-2 and EGFR showed that inside the ATP binding site, the apparent residue difference is Ser783 (Figure 2) in erbB-2, whose corresponding residue is Cys775 in EGFR. It is of note that a number of residues located on the helix immediately outside the ATP binding pocket differ between erbB-2 and EGFR. For example, His809 in erbB-2 aligns with Tyr801 in EGFR, Asn813 in erbB-2 aligns with His805 in EGFR and Arg116 in erbB-2 aligns with Asn808 in EGFR. The residue differences between erbB-2 and EGFR will be used as our specific targeted residues for the design of erbB-2 selective inhibitors. The apparent residue differences with this helix between EGFR and erbB-2 may also translate into a conformational difference with this helix in terms of both orientation and conformational flexibility. It is of note that the Cys805 in erbB-2 or Cys797 in EGFR is located at the end of this helix, whose conformation will be affected by a conformational change in the helix. A key element in our proposal is to explore these structural differences between erbB-2, EGFR and other kinases for inhibitor design to achieve high specificity.

- **1.b. Performance of structure-based database searching on both the ACD and NCI 3D-database to identify potential kinase inhibitors.**

In the last few years, we have built four large 3D-databases of small molecules, which consist of more than 650,000 organic synthetic compounds and natural products. These large 3D-databases are valuable resources for drug design and have been used successfully in several projects for novel lead discovery. We have performed structure-based searching on these databases consisting of 650,000 compounds, including the National Cancer Institute's 3D-database of 225,00 structurally diverse synthetic compounds and natural products, to identify

potential erbB-2 kinase inhibitors using the modeled erbB-2 structure. First we used the DOCK program developed by Dr. Kuntz at the UCSF to determine whether compounds can fit into the ATP binding site of erbB-2 in terms of their shape. For the top 10,000 compounds that satisfy the shape criterion, we used the program MCDOCK developed in our laboratory to further evaluate which potential inhibitors most effectively interact with erbB-2 kinase domain binding site using van der Waals and electrostatic interaction energy and taken account the conformational flexibility of these potential inhibitors. To avoid selecting highly charged molecules, the electrostatic interaction energy was scaled by a factor of 10. The top 2000 compounds with most favorable interaction energy identified through the MCDOCK calculations were further examined to identify compounds with low molecular weight (<800), simple chemical structures. About 500 compounds met such criteria. Of which, we selected 200 compounds with sufficiently structural diversity as potential erbB-2 kinase inhibitors for biological screening, as detailed below.

Task 2. Biological Confirmation of Potential Kinase Inhibitors

2.a. Initial cell based screening for activity and specificity.

First, we used the human breast cancer cell line MDA-453 that overexpresses erbB-2 resulted from gene amplification to screen these potential erbB-2 kinase inhibitors for their ability to inhibit erbB-2 auto phosphorylation. Eleven out of 200 candidate compounds inhibited more than 90% of erbB-2 phosphorylation at 100 μ M. We then carried out a dose-dependent phosphorylation assay in MDA-453 cell line that overexpresses erbB-2 and MDA-468 cell line that overexpresses the EGFR to test both the potency and selectivity. Of which, 7 compounds were found to have relative selectivity in inhibiting erbB-2 phosphorylation in MDA-453 cells *versus* in inhibiting EGFR auto-phosphorylation the MDA-468 cells. The chemical structures of these lead compounds are shown in **Chart I** and their activity is summarized in **Table 1**.

Char I. Chemical Structures of novel erbB-2 small molecule inhibitors discovered from structure-based 3D-database searching.

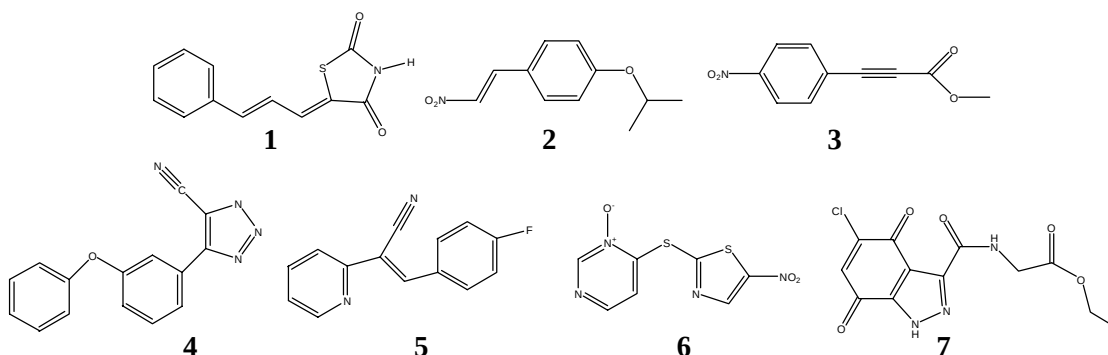


Table 1. Summary of the cell-based inhibitory activity of seven novel lead compounds.

	1	2	3 (B17)	4	5	6	7
MDA-453, IC ₅₀ , μ M	50	5	2.5	25	25	25	15
MDA-468, IC ₅₀ , μ M	>100	>400	>400	400	>400	>400	>400
Selectivity EGFR v.s. erbB-2	>2	>80	>160	8	>8	>8	>27
Inhibitor Type	R*	I**	I	R	R	R	I

*R: reversible inhibitor; **I: irreversible inhibitor

As can be seen from Table 1, these compounds have an IC₅₀ from 2.5 to 50 μ M in cell-based inhibition erbB-2 phosphorylation. The most potent compound **3 (B17)** has an IC₅₀ value of 2.5 μ M, and importantly, **B17** showed a selectivity more than 160-fold between its potencies in inhibition of erbB-2 and EGFR auto-phosphorylation (**Fig. 3**). In addition to **B17**, a number of these compounds may be considered as novel leads. Compound **2** has an IC₅₀ of 5 μ M and a selectivity between erbB-2 and EGFR over 80-fold. Compounds **4** and **5** both have an IC₅₀ value of 25 μ M in inhibition of erbB-2 auto-phosphorylation and **5** also showed more than 8-fold selectivity between erbB-2 and EGFR. Molecular modeling studies showed that specific

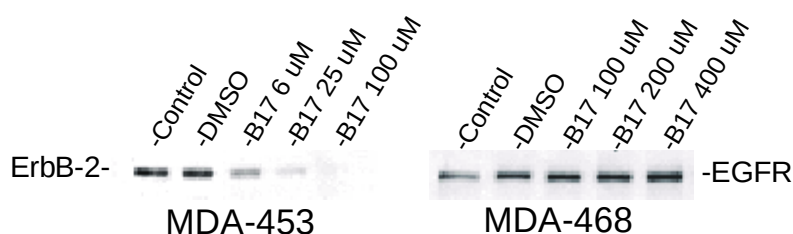


Figure 3. MDA-453 and MDA-468 cells were grown in 12-well plates to 90% confluence and then changed into serum-free medium overnight. Cells were treated with B17 for 30 min and extracts were made for western blotting with anti-phosphotyrosine mAb.

modifications with these compounds can be made to dramatically improve their potency. These compounds represent new classes kinase inhibitors with novel chemical scaffolds. Furthermore, **2** and **3 (B17)** display approximately 100-fold selectivity between erbB-2 and EGFR. Therefore, a number of these compounds may be considered as promising initial leads for further optimization to improve their potency and selectivity. Since **3 (B17)** is the most potent lead among these compounds, we have carried further characterization on **3 (B17)**, as detailed below.

2.b. Inhibition of ligand-induced phosphorylation and further testing of specificity using model cell lines.

To further assess that the potency and selectivity of **B17**, we utilized several model cell lines. The NIH-3T3 cells were transfected with either EGFR, erbB-2 or the chimeric EGFR (extracellular) and erbB-2 (intracellular) receptor. The chimeric EGFR (extracellular) and erbB-2 (intracellular) receptor has an EGF ligand binding site in its extracellular domain but has an

intracellular erbB-2 kinase domain. Thus, although both EGFR and chimeric EGFR/erbB-2 depend on addition of EGF to induce phosphorylation, they have different kinase domains. Overexpression of erbB-2 receptor resulted in a high level of auto-phosphorylation in these cells. A more erbB-2 specific kinase inhibitor, such as **B17** should inhibit the EGF induced phosphorylation in the NIH 3T3 cells transfected with the chimeric EGFR/erbB-2 receptor but should be much less active in inhibiting the phosphorylation induced by EGF in the NIH 3T3 cell line transfected with EGFR. Moreover, it was also predicted that **B17** should also inhibit the auto-phosphorylation activity in the NIH 3T3 cell line overexpressed erbB-2. As shown in **Fig. 4**, **B17** selectively inhibits the EGF-induced erbB-2 kinase activity in the NIH 3T3 cells

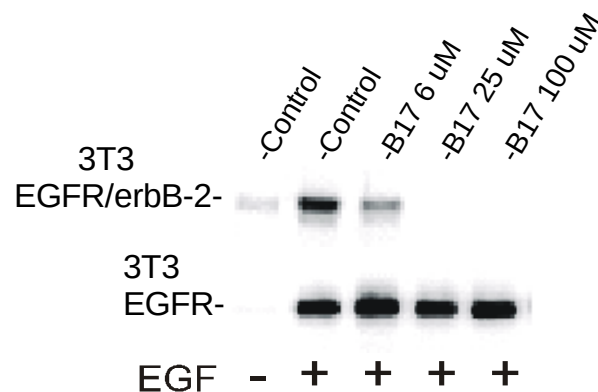


Figure 4. Inhibition of the ligand-induced phosphorylation by B17 in NIH3T3 cells transfected with either wild-type EGFR or chimeric EGFR/erbB-2 receptor kinase. Cells were treated same as in Fig. 3, but with addition of EGF at 10nM for 5 min.

transfected with the chimeric EGFR/erbB-2 receptor with an IC_{50} value of 1.3 μ M, but did not show appreciable inhibition of the EGFR kinase activity in 3T3-EGFR cells with concentrations up to 100 μ M. **B17** also inhibits the auto-phosphorylation of erbB-2 in the NIH 3T3/erbB-2 cells with same potency. Thus, we demonstrated that **B17** is a potent and selective erbB-2 specific kinase inhibitor, with a selectivity approximately 100-fold between erbB-2 and its closely related family member EGFR using isogenic model cell lines with same genetic backgrounds.

To further assess its selectivity, we have tested **B17** against insulin receptor or insulin-like growth factor receptor I kinase (NWT21) in 3T3 transfected cells, and PDGF (BB) receptor

in wild-type 3T3 cells treated with PDGF (BB). We also used endothelial cell line HUVEC to test the effect of **B17** on VEGF receptor kinase and FGF receptor after treated with VEGF or bFGF, respectively. In all these tests, no appreciable inhibition was observed with **B17** against these kinases (data not shown).

2.c. Inhibition of erbB-2 and EGFR mediated MAP kinase activity.

ErbB-2 and EGFR mediate the down-stream MAP kinase activity. It was thus predicted that a selective kinase inhibitor against erbB-2 such as **B17** should significantly inhibit the erbB-2 mediated MAP kinase activity in MDA-453 but not the EGFR mediated MAP kinase activity in MDA-468. Indeed, our results showed that the MAP kinase activity in MDA-453 was inhibited by **B17** with a potency similar to that in inhibition of erbB-2 auto-phosphorylation, while the MAP kinase activity in MDA-468 was not inhibited with concentration up to 400 μ M. These results indicated that **B17** specifically inhibits the erbB-2 mediated but not EGFR mediated MAP kinase signaling pathway in cells.

2.d. Inhibition of cell proliferation and selectivity.

We further tested the ability of **B17** in inhibition of cell growth using a number of cell lines overexpressed either erbB-2 or EGFR, or neither. Among a number of cell lines we used, MDA-453 overexpresses erbB2, while MDA-468 overexpresses EGFR and MDA-231 overexpresses neither. **B17** had an IC_{50} value of 0.3 μ M in MDA-453. In MDA-468, **B17** had 25% of inhibition at 5 μ M ($IC_{50} > 5 \mu$ M). Thus, **B17** displays a relative selectivity of more than 15-fold between cell lines overexpressed erbB-2 and EGFR. In control cell line MDA-231, **B17** also has an estimated IC_{50} value more than 5 μ M, 15-times less potent than its activity in MDA-453 overexpressed erbB-2.

Task 3. Evaluation of *in vivo* anti-tumor activity and toxicity of most promising erbB-2 kinase inhibitors

3.a. *in vivo* antitumor activity in human breast cancer xenograft models

Our *in vitro* studies showed that B17 is a potent and selective inhibitor against erbB-2. To further test its therapeutic potential, we have synthesized a large quantity of B17 and evaluated B-17 for its activity in inhibition of tumor growth in human breast cancer xenografts. First, we have tested **B17** for its antitumor activity in BT-474/M1 xenograft model of human breast cancer in nude mice. The results are shown in **Figure 5**.

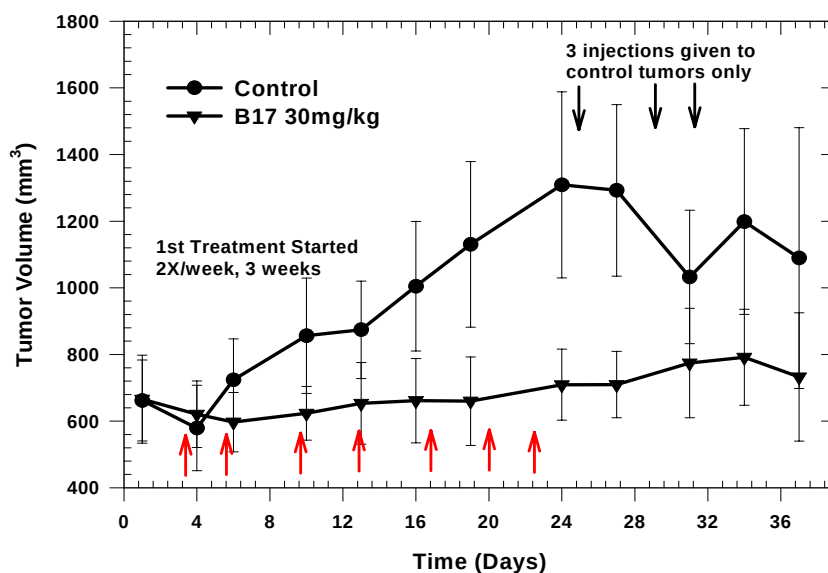


Figure 5. Antitumor activity of **B-17** in BT-474/M1 xenograft model of human breast cancer in nude mice.

As can be seen, **B17** is effective in inhibition of tumor growth. During the treatment period, the tumors did not grow significantly, while control tumors treated with vehicle had double in volume. Significantly, three injections of **B17** into animals bearing very large tumors (>1000 mm³) reduced the tumor size. Our data thus suggested that B17 is effective in inhibition of tumor growth in BT-474/M1 human breast cancer xenografts with overexpression of erbB-2.

We have further evaluated the anti-tumor activity of B-17 *in vivo* using an additional human breast cancer cell line (MDA-316/DYT2) with erbB-2 overexpression in the xenograft model (**Figure 6**). In the MDA-316/DYT2 (human breast carcinoma cells, ER positive and subclone DYT2), we administered the B-17 at the second day after cell inoculation. With **B-17** injected at 30mg/kg twice a week for three weeks, 80% tumor growth inhibition (8-10 tumors in each group) was achieved in the treated group of mice as compared to the control group with no treatment (Figure 7). It is noted that none of the mice showed weight loss or any other signs of toxicity at the dose regime used.

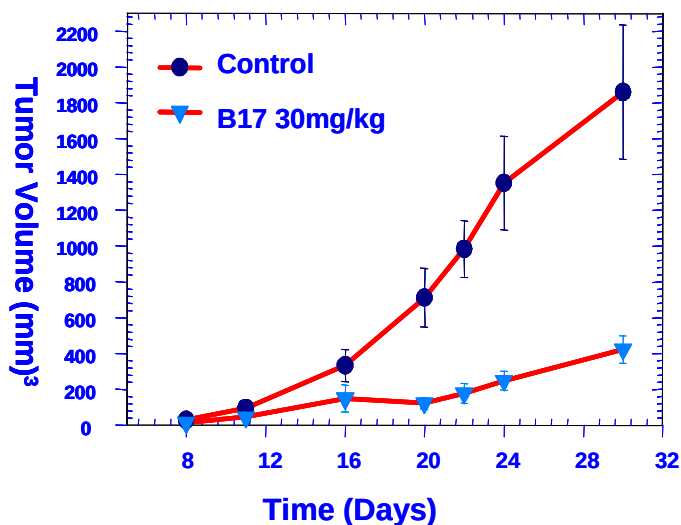


Figure 6. *In vivo* inhibition of tumor growth by **B17** in MDA-MB-361/DYT2 human xenograft model in nude mice.

Taken together, our *in vivo* data with two xenograft models of human breast cancer (BT-474/M1 and MDA-361/DYT2) demonstrated that B-17 has a potent anti-tumor activity *in vivo* and inhibition of the erbB-2 kinase activity using a fairly potent and erbB-2 specific small molecule kinase inhibitor has the therapeutic potential for the treatment of cancers with erbB-2 overexpression.

3.b. *In vivo* inhibition of erbB-2 phosphorylation and MAP kinase activity by B17.

We next investigated if **B17** can achieve *in vivo* modulation of erbB-2 or EGFR phosphorylation under systemic administration in animals.

For this purpose, we have utilized BT-474/M1 or A431 xenografts model (BT-474/M1 was kindly provided by Dr. C. Benz, UCSF) which formed progressive tumors in nude mice. We first treated mice bearing BT-474/M1 or A431 tumor intraperitoneally (i.p) with **B17** at 100 mg/kg. We excised tumors at 24 hours after treatment, then prepared homogenates of the tumors and determined levels of phosphorylated erbB-2 or EGFR by western blotting analysis. Treatment with **B17** almost completely suppressed erbB-2 phosphorylation and MAP kinase activation (**Figure 7**), while having no appreciable inhibition of on EGFR phosphorylation in A431 cells (data not shown). It is of note that **B17** had no effects on total erbB-2 protein, suggesting that its inhibition in erbB-2 kinase activity is not due to inhibition of erbB-2 expression or enhancing protein degradation.

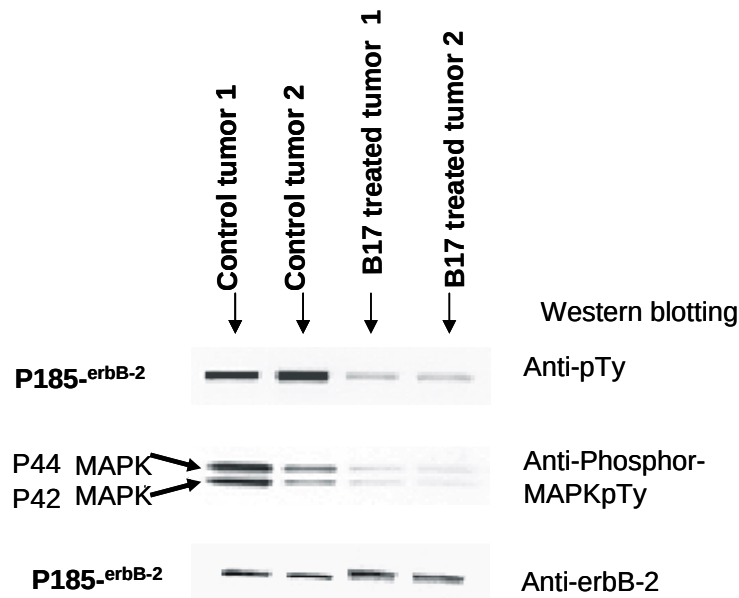


Figure 7. *In Vivo* Effects of **B17** on Phosphorylation of erbB-2, MAP kinase and total erbB-2 in tumor tissues bearing BT-474/M1 xenograft (24 Hours after Intraperitoneal Treatment)

Key Research Accomplishments

- a. Our computational structure-based database searching has led to the discovery of several classes of structurally novel, potent and selective non-peptide small-molecule inhibitors of erbB-2. Of which, **B17** has achieved sub-micromolar potency in inhibition of erbB-2 phosphorylation and cancer cell growth in human breast cancer cell lines with overexpression of erbB-2.
- b. Our *in vitro* studies using isogenic model cell lines and diverse human breast cancer cell lines have clearly demonstrated the activity and selectivity of **B17** and provided in-depth insights into its molecular mechanism of action.
- c. Our *in vivo* studies have shown that **B17** is highly effective in inhibition of tumor growth in animal models of human breast cancer and provided insights into its mode of action.

Our data thus strongly suggested that B17 represents a promising lead compound for further optimization and development as a new class of anticancer therapy for the treatment of human breast cancer with overexpression of erbB-2.

Reportable Outcomes

1. Several classes of novel potent and selective small-molecule inhibitors of erbB-2 have been discovered. These inhibitors represent promising initial lead compounds for further development.
2. **B17** represents the most promising lead compound with potent *in vitro* and *in vivo* activities. We plan to pursue extensive optimization of this lead compound to further improve its potency, selectivity and pharmacokinetic properties. This may ultimately lead to the development of a new class anticancer therapy for the treatment of human breast cancer with erbB-2 overexpression.
3. A manuscript on the discovery and detailed *in vitro* and *in vivo* study has been written and will be submitted for publication once the revisions are completed.

Conclusions:

Her-2/erbB-2 is a clinically proven and highly promising molecular target for human breast cancer. Our studies have lead to the discovery of several classes of potent, selective and novel small-molecule inhibitors against erbB-2. Of which, B17 is a highly promising lead compound for further optimization and development as a new class of anticancer therapy for the treatment of human breast cancer with overexpression of erbB-2. In summary, our studies have accomplished all the tasks and goals we have outlined in our initial proposal.