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Pharmacological Targeting of the Pseudokinase Her3

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Abstract

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Author contributions

N.S.G. oversaw all aspects of the experiments and manuscript preparation. T.X. and S.M.L. performed the chemical synthesis and structure-activity relationship analysis. T.X. performed hits/leads valuation by protein and cellular based assay with assistance from D.E., K.D.W., D.U. and M.E.D. K.D.W., D.G. and T.X. expressed and purified Her3 protein. T.X. and S.M.R optimized FRET based binding assay. T.S. performed molecular docking studies. S.B.F., J.A.M., K.D.W., D.G. and T.X. conducted mass spectrometry labeling experiment and analyses. T.X. and N.S.G. wrote the manuscript, and all coauthors participated in editing this manuscript.

Competing financial interests

C. Crews is founder and an equity shareholder in Arvinas, Inc., which is developing small molecule-induced protein degradation as a therapeutic methodology.

Supplementary information and chemical compound information are available online. Reprints and permissions information is available online too.

Her3 (ErbB3) belongs to the epidermal growth factor receptor tyrosine kinases and is well credentialed as an anti-cancer target but is thought to be “undruggable” using ATP-competitive small molecules because it lacks significant kinase activity. Here we report the first selective Her3 ligand, TX1-85-1, that forms a covalent bond with Cys721 located in the ATP-binding site of Her3. We demonstrate that covalent modification of Her3 inhibits Her3 signaling but not proliferation in some Her3 dependent cancer cell lines. Subsequent derivatization with a hydrophobic adamantane moiety demonstrates that the resultant bivalent ligand (TX2-121-1) enhances inhibition of Her3 dependent signaling. Treatment of cells with TX2-121-1 results in partial degradation of Her3 and serendipitously interferes with productive heterodimerization between Her3 with either Her2 or c-Met. These results suggest that small molecules will be capable of perturbing the biological function of Her3 and the approximately 60 other pseudokinases found in human cells.

Her3 (ErbB3) is a member of the epidermal growth factor receptor tyrosine kinases which also include Her1 (EGFR), Her2 and Her4. EGFR-dependent signaling frequently becomes deregulated in cancer due to receptor/ligand over-expression or oncogenic mutations that result in constitutive activation of the kinase domain. Activated mutants of Her1 and Her2 are successfully targeted by approved drugs for genotypically identifiable subsets of patients with non-small cell lung cancer (NSCLC) and breast cancer[1–4]. Her3 has not been the subject of small molecule drug discovery efforts because it has been historically considered a ‘pseudokinase’ due to the mutation of conserved residues Asp813 and Glu738 that are typically required for catalytic function[5–9]. A recent report suggests that Her3 may possess very weak kinase activity[7], but it is unknown whether this activity is required for Her3-dependent functions. Despite questions regarding its kinase activity, Her3 is well documented as an essential heterodimerization partner with epidermal growth factor receptor (EGFR) and Her2. Multiple studies have also shown that it interacts with c-Met, most notably in the context of drug resistance[10]. The Her3 kinase domain serves as an activator of EGFR kinase domain[5,6] andheterodimerization results in phosphorylation of specific tyrosine residues located near the C-terminus of the EGFR/Her3, Her2/Her3 or c-Met/Her3 dimers, providing a docking site for the phosphoinositide 3-kinase (PI3K) at the plasma membrane and eventual activation of the critically important downstream PI3K/Akt signaling network[11,12]. Her3 is over-expressed and deregulated in many cancers such as Her2 driven breast cancer, ovarian and non-small cell lung cancers (NSCLC)[13–16]. Furthermore, recent studies demonstrate that the up-regulation of Her3 phosphorylation can be the basis of resistance to approved EGFR and Her2 targeted drugs such as gefitinib and lapatinib[10,17–20]. This body of evidence suggests that antagonists of Her3 could be valuable anti-cancer therapeutics and has stimulated the development of numerous antibodies (*e.g.* AMG-888, MM-121, AV203, MEHD7945A) directed against the extracellular ligand-binding domain that are currently undergoing clinical evaluation and Pertuzumab, which blocks Her3/Her2 heterodimerization, has received regulatory approval for the treatment of breast cancer[20–24].

Although Her3 appears to play a critical role in a subset of breast, ovarian and NSCLCs there are currently no reported small molecules that can directly inhibit Her3 function. We sought to address the question of whether ATP-competitive ligands of Her3 would be

capable of antagonizing Her3 dependent signaling or growth. We hypothesized that ATP-competitive Her3 ligands may exhibit pharmacology for a number of potential reasons including: low level of Her3 kinase activity being essential, ATP-binding playing an important structural role, or because an ATP-competitive ligand could interfere with productive heterodimerization between Her2/Her3 or c-Met/Her3[10]. Here we report the use of screening and structure-based drug design to develop the first irreversible, ATP-competitive ligands of Her3 that form a covalent bond to Cys721 that is located on the 'roof' of ATP binding pocket. Chemical proteomics experiments demonstrate that our ligands can covalently modify Her3 in cells with excellent selectivity presumably because Cys721 appears to be uniquely present in Her3 based upon kinome-wide sequence alignments. Consistent with the notion that kinase activity is not important for Her3-dependent function, these compounds neither inhibit Her3-dependent signaling nor proliferation. Because siRNA-based approaches which deplete Her3 protein have proven effective in blocking Her3 functions in cell culture, we explored whether we could engineer covalent Her3 ligands to accomplish a similar outcome. Recently 'hydrophobic tagging' of proteins with an adamantane group using the HaloTag system has been reported to lead to the proteosomal-mediated degradation of the modified protein[25,26]. We therefore developed bi-functional molecules consisting of a covalent Her3 targeting small molecule linked to a hydrophobic adamantane moiety and demonstrated that these compounds can induce partial Her3 degradation by the proteasome while simultaneously inhibiting productive heterodimerization of Her3/Her2 and Her3/c-Met. It was also demonstrated that both an electrophilic group for the covalent bond formation and a hydrophobic tag are required for disruption of Her3 heterodimerization and inhibition of Her3-dependent proliferation. These bi-functional molecules are capable of blocking Her3-dependent signaling and growth at sub-micromolar concentrations in cells.

RESULTS

Acrylamide substituted compounds covalently modify Her3

In order to develop potent and selective covalent Her3 ligands, we first identified ATP-competitive ligands using high-throughput screening and then employed structure-guided drug design to introduce reactive moieties that could form a covalent bond with Cys721. Kinome-wide sequence alignments suggest that Cys721 is uniquely present in Her3 and therefore provides an excellent potential means to achieve selectivity[27]. A covalent inhibitor provides a number of potential advantages including higher potency, improved selectivity due to Cys721 being highly unusual across the kinome, and the ability to address pharmacological specificity by performing 'rescue' experiments with a Cys721 to serine mutation. We developed an ATP-competitive ligand binding assay using the fluorescence resonance energy transfer (FRET) based LanthaScreen™ Eu methodology[28] and screened a 1,500 member library consisting of both known and novel ATP-competitive kinase inhibitors. The most potent Her3 binders to emerge from the screen were five previously reported Src-family inhibitors: KIN001-111(A-770041)**(1)**[29], KIN001-051 **(2)**[30,31], (Fig. 1a) dasatinib[32], bosutinib[33] and KIN001-30 **(3)**, (Supplementary Results, Supplementary Fig. 1a) which possessed IC_{50}s below one hundred nanomolar (Fig. 1b). In order to develop covalent binders of Her3 that exploit the unique Cys721 residue, we docked these

compounds to the previously reported 2.80 Å Her3 crystal structures (3KEX[6] and 3LMG[7], Fig. 1c and Supplementary Fig. 1b). This modeling suggested that introducing a meta-acrylamide from the phenyl substituent of KIN001-111 might afford the correct trajectory for a conjugate addition with Cys721. After the iterative synthesis and assaying of approximately 100 pyrazolopyrimidine acrylamides, we developed the 'lead' compound TX1-85-1 **(4)** which possessed an IC_{50} of 23 nM in the binding assay (Fig. 1a, b). TX1-85-1 can be viewed as a molecular amalgam of the hinge binding moiety of KIN001-051 and the solubility enhancing tail of KIN001-111, combined with an acrylamide arm to reach Cys721 (Fig. 1c). As expected for a covalent inhibitor, the apparent IC_{50} decreases upon longer incubation with the protein and reaches a plateau at about 3 hours (Supplementary Fig. 2). Mass spectrometry was used to confirm the covalent addition of TX1-85-1 to recombinant Her3 kinase domain protein and subsequent proteolysis of Her3 with trypsin and MS^2 analysis revealed unique modification of Cys721 (Supplementary Fig. 3). We next investigated whether TX1-85-1 could form a covalent bond with Her3 in cells using a cellular competition binding assay. To enable these experiments we synthesized a biotinylated derivative (TX1-85-1-biotin) **(5)** that still preserves the ability to covalently bind to Her3 (Fig. 1a, Supplementary Fig. 4, IC_{50} = 50.7 nM). Cells were incubated with TX1-85-1 and then lysates were labeled with TX1-85-1-biotin in order to quantify the amount of Her3 that had escaped labeling by the initial incubation with TX1-85-1. We determined that incubation of PC9 GR4 cells (EGFR E746_A750/T790M) with 5 μM of TX1-85-1 for 8 hours resulted in complete protection of Her3 from subsequent labeling with TX1-85-1-biotin (Fig. 1d). This result suggests that TX1-85-1 (MW = 580Da) can pass through the cell membrane and is capable of complete 'target engagement' with Her3 intracellularly. To examine the specificity with which TX1-85-1 modifies Her3 we performed a live cell chemical proteomics experiment using the KiNativ® approach[34–36] which demonstrated potent binding to Her3 as well as to Lyn, Her2 and several other Src family kinases (Supplementary Table. 1). Binding to Lyn, Her2 and several other Src family kinases was expected as these kinases were equipotently bound by the non-acrylamide screening hit compound KIN001-111[29], suggesting that interaction with these targets is non-covalent. Collectively these results suggest that TX1-85-1 is capable of covalently modifying Cys721 of Her3 *in vitro* and in cells.

TX1-85-1does not stop Her3 dependent signaling or growth

We next evaluated the ability of TX1-85-1 to inhibit Her3-dependent signaling and growth. We utilized two established lung cancer cell lines, PC9 GR4 (EGFR E746_A750/T790M), HCC827 GR6 (EGFR E746_A750/MET amplification)[18,37] and an ovarian cancer cell line, Ovcar8 that we reconfirmed to be 'addicted' to Her3 using siRNA mediated depletion of Her3 (Supplementary Fig. 5). TX1-85-1 possessed an anti-proliferation EC_{50} of greater than or equal to approximately 10 μM for all three cell lines (Fig. 2a). At a concentration of TX1-85-1 sufficient to fully label Her3 in cells (5 μM) there was no growth inhibition of PC9 GR4 cells and no inhibition of the phosphorylation of Akt, an important downstream effector of Her3 (Fig. 2b). These results suggest that despite successful target engagement of Her3 in cells by TX1-85-1, the compound is not capable of inhibiting Her3-dependent function under the conditions investigated.

TX1-85-1-Adamantane conjugates induce Her3 degradation

Recent reports have suggested that covalent modification of proteins with hydrophobic small molecules can result in their proteasome mediated degradation[25,26,38,39]. While a detailed mechanistic understanding for this phenomenon is unknown, there is considerable evidence that cells have evolved sophisticated protein homeostasis machinery that can eliminate unfolded and otherwise damaged proteins which are presumably engaged by this hydrophobic tagging strategy. Among the reported hydrophobic tags, we selected the adamantane group due to its ability to densely introduce hydrophobicity with a minimal increase in molecular weight. A series of compounds possessing various linkers connecting TX1-85-1 with an adamantane moiety were synthesized and confirmed to bind potently with Her3, identifying a representative molecule (TX2-121-1) **(6)** demonstrating an IC_{50} of 49.2 nM in the protein based binding assay. To confirm that the selectivity of TX2-121-1 remained similar to that exhibited by TX1-85-1 we performed KiNativ® profiling. The selectivity of TX2-121-1 was very similar to TX1-85-1 and displayed most potent binding to Her3 (90.3%). TX2-121-1 off-target labeling list included Her2, EGFR, several Src family kinases such as Src, Yes and Lyn which, as expected, is the same as the off-target list of TX1-85-1. (Supplementary Table 1). To investigate whether TX1-85-1 and TX2-121-1 could directly inhibit EGFR or Her2 we performed enzymatic assays (Z′-LYTE, Invitrogen, Supplementary Table 2) and cell proliferation assays using the Ba/F3 EGFR VIII cell line (Supplementary Fig. 6). TX2-121-1 does not directly inhibit the enzymatic activity of EGFR or Her2 nor inhibit EGFR-dependent proliferation at concentrations below 10 μM.

We next examined whether adamantane-derivatized Her3-binders could induce degradation of Her3 and thereby inhibit downstream signaling. As part of this analysis we also evaluated the importance of the electrophilic warhead by preparing TX2-135-2 **(7)**, where the reactive acrylamide moiety is replaced with the non-reactive propyl amide (Fig. 3a). Treatment of starved PC9 GR4 cells with TX2-121-1 at concentrations of 0.5 and 2 μM for 12 hours resulted in partial degradation of Her3 and inhibition of phosphorylation of downstream Her3 effectors: Erk and Akt, after stimulation with Neuregulin (NRG) (Fig. 3b). The non-covalent analog TX2-135-2 and compounds lacking the adamantane group (*e.g.* TX2-120-1 **(8)** and TX1-85-1) were less effective at inducing Her3 degradation or blocking signaling. Growth assays also demonstrated that the acrylamide group substituted compounds possess cell growth EC_{50} about 7-fold lower than the propionamide containing compounds which are incapable of covalent bond formation (Fig. 3c). Analysis of the structure-activity relationships for this compound series demonstrates that degradation is dependent on the type and the length of linker.

To confirm that the observed pharmacology is Her3-dependent we engineered PC9 GR4 cells to possess either native Her3 containing the reactive Cys721 or Her3 containing a non-reactive serine mutation, C721S. The mutation was confirmed by Sanger DNA sequencing of ErbB3 (Supplementary Fig. 7). We verified target engagement using a TX1-85-1-biotin conjugate compound to perform pull-down assays. Indeed, TX1-85-1-biotin effectively pulled down Her3 WT, but not Her3 C721S suggesting that the acrylamide motif of TX1-85-1-biotin forms a specific covalent interaction with C721 but does not form a covalent bond with C721S Her3 (Supplementary Fig. 8).

We performed cell proliferation assays against PC9 GR4 cells expressing either wild-type or C721S mutant Her3 with TX1-85-1 and TX2-121-1 and their corresponding propylamide-controls (TX1-85-3 **(9)** and TX2-135-2) in order to investigate the importance of the electrophilic acrylamide and the adamantane moiety. These experiments demonstrate a 6-fold rightward shift in EC_{50} for C721S Her3 (EC_{50}= 5.5 μM for PC9 GR4 Her3 C721S) versus wild-type Her 3 expressing cells (EC_{50} =0.9 μM for PC9 GR4 WT Her3) only for TX2-121-1 which possesses both an electrophilic warhead and an adamantane moiety (Fig. 4a, Supplementary Fig. 9) and the downstream signaling western blot is consistent with the antiproliferation result (Fig. 4b). All other compounds: TX1-85-1, TX1-85-3 and TX2-135-2 have similar EC_{50} on both PC9 GR4 Her3 WT and PC9 GR4 Her3 C721S. These results demonstrate that in this cellular system, effective inhibition of Her3-dependent growth requires both covalent modification and the presence of the adamantane moiety. Incidentally, we also observe of the two reversible controls that TX2-135-2 bearing the adamantane group is more antiproliferative than TX1-85-3 (Supplementary Fig. 9).

To investigate the generality of these findings, we further tested the antiproliferative activity of TX2-121-1 against a panel of cell lines that are Her3-dependent (HCC827 GR6, PC9 GR4 and Ovcar8) and Her3-independent (Ovcar5, A549) (Fig. 4a, **bottom**). Interestingly, TX1-85-1, which does not possess the adamantane tag, was capable of partially depleting Her3 protein and inhibiting signaling in Ovcar8 cells and HCC2935 cells despite possessing an EC_{50} of greater than 10 μM in these lines (Fig. 4c and Supplementary Fig. 10). While the EC_{50}s are in the single digit micromolar range, there is a trend towards enhanced potency in the Her3-dependent cell lines. We analyzed Her3 expression in all these cells by western blot which demonstrated that Her3-dependent cells generally express higher level of Her-3 relative to the Her3-independent cell lines (Supplementary Fig. 11).

Hsp70, Hsp90 and the proteasome aid Her3 degradation

Proteasome-mediated degradation is dependent on the ATP-dependent molecular chaperones proteins Hsp70 and Hsp90 for sensing correct protein folding [25]. To investigate the involvement of these proteins in the mechanisms underlying our adamantane-derivatives we examined the ability of the reportedHsp70 inhibitor 4-[1,1′-biphenyl]-4-yl-3,4-dihydro-6-methyl-2-oxo-5-[(phenylmethoxy)carbonyl]-1(2H)-pyrimidinehexanoic acid (116-9e) and the Hsp90 inhibitor 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG) to modulate the ability of TX2-121-1 to induce Her3 degradation. Treatment of cells with a combination of 116-9e (1.0 μM) or 17-AAG (0.1 μM) and TX2-121-1 (0.5 μM) resulted in enhanced degradation of Her3 relative to treatment with either agent alone (Fig. 5a). We also confirmed that the proteasome inhibitor MG132 diminished the ability of TX2-121-1 to induce Her3 degradation (Fig. 5a). A cartoon depiction of a potential degradation mechanism is shown (Fig. 5b).

TX2-121-1 prevents Her3 heterodimerization

We noted that the effect of TX2-121-1 on Her3-dependent signaling appeared to be more dramatic than might be anticipated based upon the moderate amount of Her3 degradation (Fig. 3b). We hypothesized that TX2-121-1 might also antagonize Her3-dependent signaling by interfering with productive heterodimerization between Her3 and Her2 or c-Met. To

investigate this possibility, we treated PC9 GR4 cells with compounds at 1 μM for 6 hours followed by wash-out to remove non-covalently bound drug. The amount of Her2 and c-Met associated with Her3 was determined by western blot of SDS-PAGE resolved immunoprecipitates of Her3. These experiments demonstrated that only the adamantane modified TX2-121-1 could effectively antagonize the ability of Her3 to associate with Her2 and c-Met, in contrast to the non-adamantane TX1-85-1 and the non-covalent TX2-135-2. Moreover, we also see that TX2-121-1 interferes with Her2/Her3 heterodimerization more than c-Met/Her3 heterodimerization (Fig. 5c).

DISCUSSION

Here we report the development of the first small molecule Her3 ATP-competitive binder TX1-85-1, which can covalently modify Her3 via conjugate addition to Cys721. Treatment of cells with TX1-85-1 at single digit micromolar concentrations results in covalent modification of Her3 with kinome selectivity consistent with the chemotype from which the compound was derived. Despite successful target engagement by TX1-85-1, proliferation and Her3-dependent functions including phosphorylation of downstream effector Akt are not affected in PC9 lung carcinoma cell lines. Nevertheless, despite being a very poor inhibitor of the growth of Her3-dependent cells lines such as Ovcar8 and HCC2935 (EC_{50}>10 μM), TX1-85-1 is capable of inducing partial degradation of Her3 protein and attenuating Her3-dependent signaling. These results are consistent with studies that have shown that some non-covalent kinase inhibitors of Her2 (lapatinib) and b-Raf (vemurafenib) can also lead to partial degradation of their kinases targets[40].

Overall these results are consistent with the designation of Her3 as a ‘pseudokinase’ possessing very low kinase activity. However, it remains unclear whether Her3 kinase activity plays any significant biological role and we cannot exclude the possibility that some residual Her3 activity remains following inhibitor treatment. In order to circumvent this limitation we developed bivalent hydrophobically tagged adamantane derivatives, such as TX2-121-1 that are capable of antagonizing Her3-dependent signaling at single digit micromolar concentrations. Treatment of cells with TX2-121-1 at concentrations producing covalent modification of Her3 results in partial degradation of the target (Her3) via the proteasome and disrupts the ability of Her3 to heterodimerize with obligate partners such as Her2 and c-Met. We demonstrated that both the electrophilic acrylamide and the adamantyl moiety are required for this effect. We further show that this observed pharmacology is ‘on-target’ to Her3 based on the ability of a Cys721Ser Her3 mutant to rescue the degradation, signaling and proliferation effects to the level observed with the non-covalent ligand. Our most optimized compound, TX2-121-1, can induce preferential death of Her3-dependent cell lines with an EC_{50} in the range of 0.8–1.4 μM.

Despite the progress made with this approach a number of key limitations and questions remain. Most importantly from a translational perspective, TX2-121-1 is only capable of inhibiting Her3-dependent signaling at single digit micromolar concentrations in contrast to clinically useful inhibitors of catalytically active oncogenic kinases which are typically active at single or double digit nanomolar concentrations. Further potency improvements will likely be required to have a realistic chance of achieving sufficient target engagement in

in vivo models and eventually in the clinic. How to improve ATP-competitive Her3 inhibitors to achieve the desired therapeutic effects remains unclear and will depend on which mechanisms prove dominant upon further study. For example, it has been hypothesized that Her3 may function primarily as a structural scaffold to support signaling pathways. In this scenario TX2-121-1 may exert Her3-dependent pharmacology through an allosteric mechanismthereby disrupting interactions between Her3 and other proteins such as Her3 or cMet consistent with results presented here and distinct from conventional kinase inhibitors, which block catalytic function[41–43]. Structural studies of Her3 in complex with both covalent inhibitors such as TX2-121-1 and interacting proteins such as Her2 will likely shed light on this question. Additionally, the kinetics of covalent chemistry will require additional consideration and optimization. From our labeling studies with recombinant Her3 protein, we can only achieve partial labeling of Her3 after a three hour incubation which is the maximum time Her3 maintains stability in our binding assay. This suggests that compared with other reported covalent kinase inhibitors, our Her3 ligands exhibit relatively slow labeling kinetics. This is also consistent with the fact that we do not observe a significant improvement in IC_{50} under the conditions of our binding assay between our most potent non-covalent and covalent Her3 ligands. These results suggest that further optimization of the covalent Her3 binding moiety may lead to more effective compounds.

An additional challenge when optimizing these compounds is our lack of a detailed mechanistic insight into how TX2-121-1 induces degradation and disruption of Her3/Her2 or Her3/c-Met heterodimers. While we have pharmacological evidence for the potential involvement of Hsp70, Hsp90 and the proteasome, we do not know to what extent ubiquitination of Her3 is involved in the degradation and whether the reported E2 and E3 ligases for Her3 such as Nrdp1[46–48] and Chip[49] are involved in the mechanism. Understanding the biochemical basis for how the compound's hydrophobic moiety recruits the protein homeostasis network will allow for development of more precise assays that can guide the optimization of the linker and hydrophobic moiety. Finding the minimal functionality required for this phenomenon will allow us to reduce the molecular weight of the target compounds which would contribute greatly to improving the 'drug-like' properties and pharmacological parameters. The ability of TX2-121-1 to disrupt protein-protein interactions between Her3 and Her2 is impressive but elucidating the mechanistic basis for this phenomenon will require a study of Her3 protein structure and dynamics. Furthermore, improving the properties of Her3-targeted inhibitors may require exploration of the less potent reversible scaffolds that were identified in the initial screen that may provide better platforms for introducing electrophilic groups that are more ideally situated to react with Cys721.

Our work has implications for the design of other bifunctional small molecule kinase inhibitors and for the prospects of designing small molecules that can perturb the biological function of other pseudokinases. Bivalent compounds possessing a protein targeting moiety and a hydrophobic tag could be developed for a number of targets including either currently druggable or 'undruggable' proteins. For example, targets like kinases have targetable enzymatic activity but perhaps distinct and more profound pharmacology might be expected from a compound that induces protein degradation or antagonizes a requisite protein-protein

interaction. Many kinases require hetero or homo-dimerization to enable productive signaling. For example, vemurafenib which targets mutant b-raf and leads to transient responses in metastatic melanoma also induces undesired transactivation of c-Raf as a result of b-Raf/c-Raf heterodimerization. Bivalent b-Raf compounds that could induce b-Raf degradation or that could antagonize b-raf containing homo and heterodimers would not be expected to have this effect. Further research is justified to investigate the scope of this strategy for kinases and other targets of therapeutic interest.

Online Methods

Chemistry

We synthesized all compounds using established procedures. See the Supplementary methods for synthetic schemes and procedures.

Her3 kinase domain purification

Constructs containing residues 665-1001 of human Her3 kinase were expressed using a baculovirus/insect cell system with a N-terminal hexahistidine and GST fusion protein in a manner similar to that developed for expression of the EGFR kinase domain[50]. All purification steps were performed at 4 °C. Cells were lysed by sonication in Buffer A (50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, 5% glycerol, 5 mM TCEP) containing 10 mM imidazole and protease inhibitor mixture (Roche). After centrifugation for 1 hour at 40000 g, clarified lysates were applied to NTA-Agarose (Qiagen), washed with Buffer A containing 20 mM imidazole and retained proteins were eluted with Buffer A containing 250 mM imidazole. Buffer exchanged to 50 mM sodium phosphate, pH 7.5, 150 mM sodium chloride, 5% glycerol, 10 mM imidazole, 5 mM TCEP using an econo desalting column (Biorad) and subjected to cleavage with recombinant hexahistidine-tagged TEV overnight. Applied to NTA-Agarose and the flow-through containing purified Her3 was captured. This was concentrated using a centrifugal concentrating device (Milipore), further purified using gel filtration on a Superdex 75 column (GE Healthcare) with buffer containing 50 mM MOPS pH 7, 50 mM Ammonium sulfate, 1 mM TCEP and concentrated to 6 mg/ml for storage.

Her3 labeled with TX1-85-1

Labeling with TX1-85-1 was accomplished by incubating purified Her3 protein at a concentration of 15 μM (0.5 mg/ml) with 150 μM TX1-85-1 in buffer containing 50 mM MOPS pH 7, 50 mM ammonium sulfate, 1 mM TCEP and 5% glycerol at room temperature for 2 hours.

LanthaScreen® technology (Life Technologies) kinase binding assay

Binding potency (IC_{50}) was measured by the Life Technologies LanthaScreen® Eu kinase binding assay, which is based on the binding and displacement of an Alexa Fluor 647 labeled ATP-competitive kinase inhibitor scaffold (Kinase Tracer 178). Binding of the tracer to the kinase is detected using a europium-labeled anti-tag antibody (anti-GST). Purified ErbB3 (665-1001) protein was obtained from Life Technologies at a concentration of 1.19 mg/ml in 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 0.02% Triton® X-100, 2

mM DTT, 50% Glycerol. The typical experimental procedure was to dilute ErbB3 protein to 30 nM with Kinase Buffer A (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij-35) followed by mixing the ErbB3 protein 1:1 with 12 nM LanthaScreen® Eu-anti-GST antibody solution which was also diluted in Kinase Buffer A. Prior to use, the antibody tube was thawed and centrifuged at approximately 10,000 g for 5 minutes and the amount used for the assay was aspirated from the top of the solution. This centrifugation step will eliminate spurious data points that can arise on occasion due to any particulates in the product. Next, 5 µl of the mixture of ErbB3 protein and Eu-anti-GST was added to 5 µl of test compound solution per well in Corning 384 plates. Then, 5 µl of Kinase Tracer 178 was added at a concentration of 39 nM per well. The plates were then incubated for 3 hours at 4 °C and read with a Perkin Elmer EnVision®.

Cell culture and materials

HCC827 Gefitinib Resistant 6 (GR6) cells, PC9 Gefitinib Resistant 4 (GR4) cells, Ovcar5, and Ovcar8 cells were cultured in 60mm plate with 10% fetal bovine serum (FBS), in Roswell Park Memorial Institute medium (RPMI) medium. HCC2935 cells were grown in RPMI 1640 media with 5% Fetal bovine serum, and 1x Pen/Strep/Amphotericin B.

Immunoblotting

All cell lines were cultured as described in the manuscript. When the confluence reached 80%, cells were treated with compounds at the indicated concentration. After 4 hours, cells were washed with medium for three times. Lysis buffer included 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, Roche PhosSTOP phosphatase inhibitor cocktail tablets and Roche Complete Protease inhibitor cocktail tablets. Lysis was accomplished by addition of lysis buffer and rotating end-to-end for 0.5. Lysates were centrifuged in a microfuge at 14,000 rpm for 20 min at 4 °C and the supernatant collected. Protein concentrations were normalized using a Pierce ® BCA protein assay kit. The assay was calibrated using a standard bovine serum albumin (BSA). Samples were run on a 4%-12% SDS-PAGE gel at 110 V. After transferred, the PVDF membrane was probed with antibodies: Phospho-Her3 antibody, Cell Signaling 4791; Her3 antibody, Santa Cruz sc-285; Phospho-Akt (Ser473) antibody, Cell Signaling 4060; Akt antibody, Cell Signaling 9272; Phospho-p44/42 MAPK (Erk1/2) (Thr202/Thr204), Cell Signaling 9101; p44/42 MAPK (Erk1/2), Cell Signaling 9102.

Her3 shRNA construct and lentiviral infection

A Her3 shRNA construct cloned in pLKO.1 puro vector was previously discribed[10]. Vector containing a non-targeting (NT) shRNA was used as a negative control. Briefly, 3000 cells per well were infected with 10% viral supernatant. After 24 hours of incubation, media was replaced with and without selection antibiotic Puromycin (2 µg/ml). Growth and inhibition of growth was assayed on day six. Her3 WT and C721S constructs were cloned into JP1520 retroviral vector (C-Flag). Briefly, 500,000 cells per 10 cm dish were infected with 100% viral supernatant. After 48 hours of incubation, media was replaced with selection antibiotic puromycin (2 µg/ml). Transduced cells PC9 GR4 Her3 (Her3 WT) and PC9 GR4 C721S (Her3 C721S) were maintained in puromycin.

Anti-proliferation assay

The anti-proliferation assay was carried out using 96 well white bottom plates. 2000–4000 cells were seeded per well with a final volume of 100 μl and incubated for 3 days after adding and titrating indicated concentration of compounds. The cell viability was measured via CellTiter-Glo® Luminescent Assay. In a typical experiment, 10 μl CellTiter-Glo® reagent was added per well. The plate was mixed and shaked for 2 minutes to induce cell lysis at room temperature and allowed to equilibrate at room temperature for approximately 10 minutes to stabilize luminescent signal. The plate was then measured with Perkin Elmer EnVision®. The cell numbers were normalized by the DMSO control. And the EC_{50}s were calculated using GraphPad® Prism.

Mass spectrometry analysis of intact Her3

TX1-85-1 treated Her3 (~1 μg) was loaded onto self-packed reverse-phase column (1/32 in. outer diameter x 500 μm inner diameter, with 5 cm of POROS 10R2 resin). After desalting, protein was eluted with an HPLC gradient (0%–100% B in 4 min, A = 0.2 M acetic acid in water, B = 0.2 M acetic acid in acetonitrile, flow rate = 10 μl/min) into a QSTAR Elite mass spectrometer (AB SCIEX, Toronto) scanning m/z 330–1500. Mass spectra were deconvoluted using MagTran1.03b2 software[51].

Protease digestion and nano LC/MS analysis of peptide fragments

TX1-85-1 treated Her3 (~1 μg) was diluted with ammonium bicarbonate buffer (pH 8.0), reduced with 10 mM DTT for 30 min at 56 °C, alkylated with 22.5 mM iodoacetamide for 30 min (room temperature in the dark), and digested overnight with trypsin at 37 °C. Digested peptides (~2 pmol) were injected onto a self-packed pre-column (4 cm POROS10R2) and eluted into the mass spectrometer (LTQ OrbitrapXL, ThermoFisher Scientific) using an HPLC gradient (0%~35% B in 20 min, A = 0.2 M acetic acid in water, B = 0.2 M acetic acid in acetonitrile, flow rate ~30 nl/min)[52]. The top 10 ions in each MS scan (image current detection; resolution=30K) were subjected to CAD (electron multiplier detection, relative collision energy 35%, q = 0.25). Dynamic exclusion was enabled with a repeat count of 1 and exclusion duration of 15 seconds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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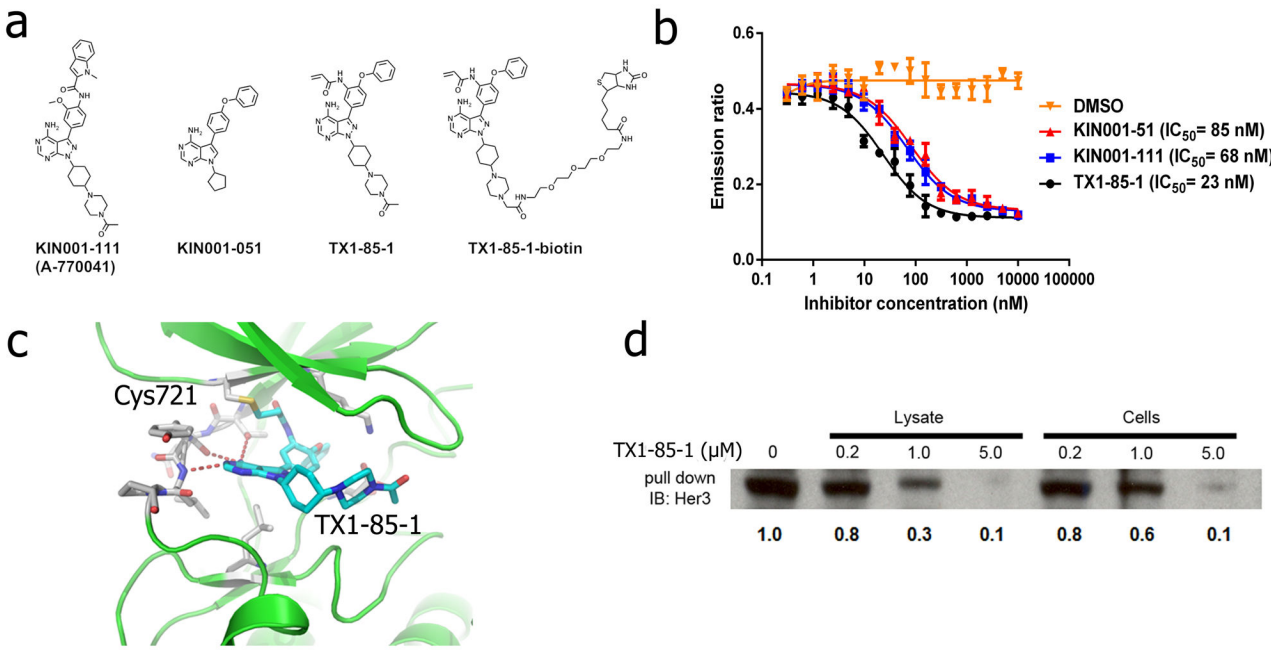

Figure 1. Hit identification and development of the Her3 irreversible inhibitor TX1-85-1

(a) Chemical structures of the representative lead compounds identified by FRET binding assay, TX1-85-1 and biotin conjugated TX1-85-1. (b) *In vitro* kinase FRET binding assay results for top screening hits and TX1-85-1. Purified recombinant Her3 kinase domain (665-1001) was used in conjunction with Lanthascreen® technology (Invitrogen). TX1-85-1 shows the highest potency with an IC_{50} of 23 nM. Each condition was tested in triplicate. Data represent mean values ± s.d. (c) Docking study of TX1-85-1 with Her3 x-ray crystallography models (PDB ID 3KEX[6], 3LMG[7]). An acrylamide substitution is predicted to form a covalent bond with Cys721 in Her3 via Michael Addition. (d) Her3 pull down experiment with TX1-85-1-biotin. PC9 GR4 cells were pre-treated with TX1-85-1 for 8 hours before lysis or TX1-85-1 was directly added to cell lysate at the indicated concentrations. Streptavidin beads were used to recover compound labeled Her3 which was detected with an anti-Her3 antibody.

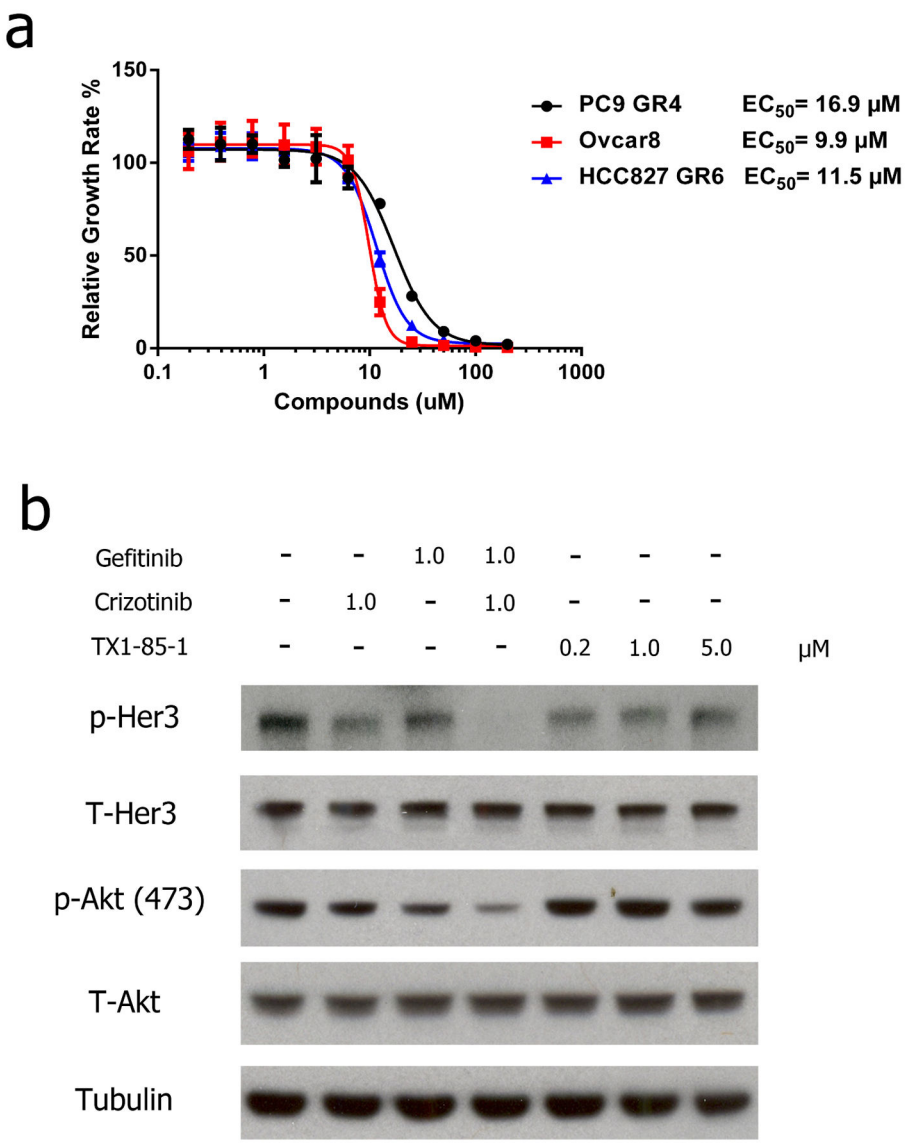

Figure 2. TX1-85-1 is insufficient to inactivate the Her3/PI3K/AKT pathway explaining lack of growth inhibition for Her3 dependent cells

(a) EC_{50}s of TX1-85-1 for several Her3 dependent cell lines. Anti-proliferative activity was achieved by TX1-85-1 at micromolar concentrations. Each condition was tested in triplicate. Data represent mean values ± s.d. (b) Her3/PI3K/AKT signaling analysis on the PC9 GR4 cell line. Western blotting for proteins in the Her3 signaling axis showed lack of specific inhibition in the low micromolar range.

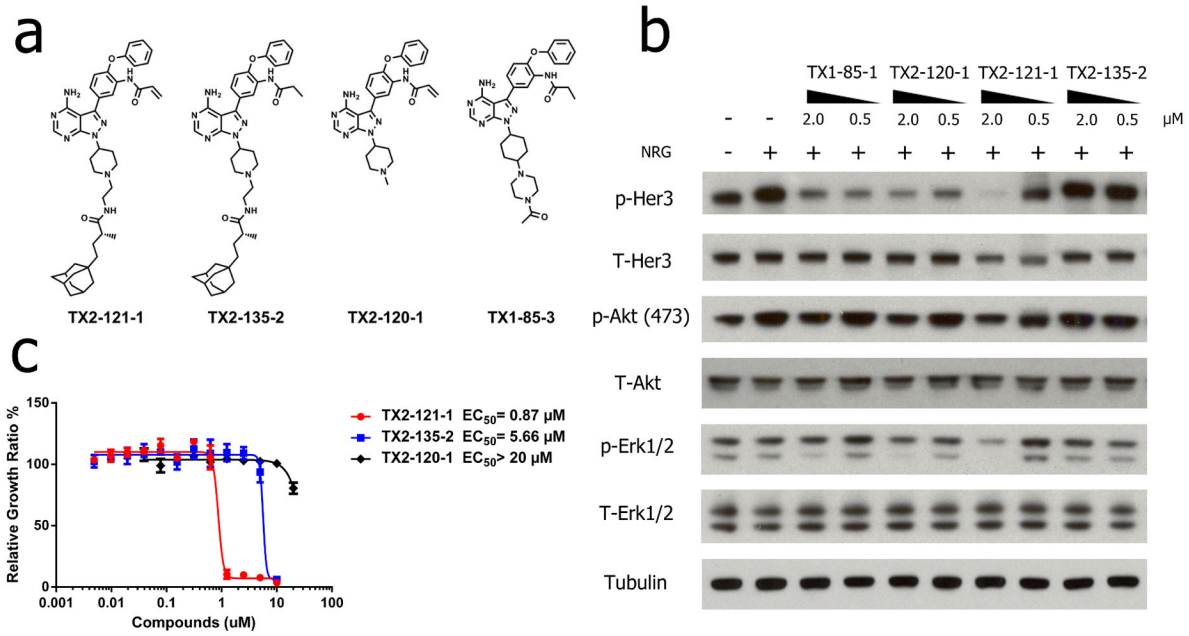

Figure 3. Adamantane conjugated compounds induce Her3 degradation

(a) Chemical structure of representative adamantane tagged compound TX2-121-1, negative control without adamantane and negative control lacking a nucleophilic warhead. (b) Her3/PI3K/AKT signaling analysis. Western blots were performed on lysates from serum starved PC9 GR4 cells treated with adamantane tagged compounds followed by 30 min treatment of 100 ng/ml NRG. Her3 was induced by NRG (lane 2) but degraded by 2 μM TX2-121-1 (lane 7). This was accompanied by decreases in p-Akt and p-Erk. (c) Anti-proliferative activity of adamantane conjugated compounds. TX2-121-1 which includes the reactive acrylamide group is about 7 fold more potent against PC9 GR4 cell line than negative controls which contain an unreactive propionamide group. The EC_{50} was not achieved for a compound lacking the adamantane group. Each condition was tested in triplicate. Data represent mean values ± s.d. In summary these results suggest that both adamantane and the electrophilic warhead contribute to potency of the compound.

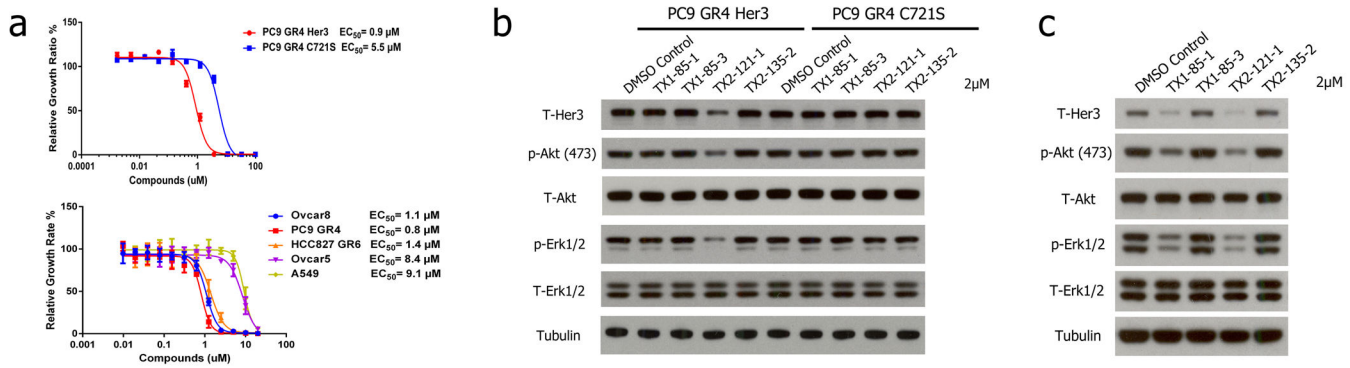

Figure 4. C721S Rescue from compound-induced degradation

(a, top) Effect of Her3 C721S on PC9 GR4 cell viability. Treatment with TX2-121-1 gives EC_{50}s which are significantly lower for PC9 GR4 C721S cells compared to PC9 GR4 Her3 cells suggesting a rescue of cell viability by the Cys721Ser mutation which prevents covalent modification of Her3 by TX2-121-1. (a, bottom) Anti-proliferative effects of TX2-121-1 on Her3 dependent and Her3 independent lines. EC_{50}s are lower for Her3 dependent lines as compared to Her3 independent lines suggesting that antiproliferative effects of TX2-121-1 are related to specific interactions with Her3 as compared to other off-target effects. Each condition was tested in triplicate. Data represent mean values ± s.d. (b) Native PC9 GR4 and PC9 GR4 C721S cells were treated with adamantane tagged compounds at 2 μM and then probed by western blot for changes in Her3 pathway signaling. Treatment with TX2-121-1, which includes both adamantane and electrophilic components, results in lower levels of detectable Her3, p-Akt and p-ERK (lane 4) while control compounds which lack the adamantane and/or electrophile do not. This effect of TX2-121-1 treatment is reversed by the Cys721Ser Her3 mutation. (c) Western blot analysis of Ovcar8 cells treated with the indicated compounds at 2 μM.

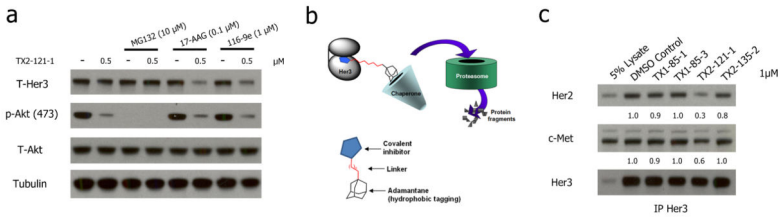

Figure 5. Mechanism of TX2-121-1 compound-induced degradation and interferences with Her2/Her3 and c-Met/Her3 interactions

(a) Proteasome degradation pathway inhibitors block TX2-121-1 mediated effects while Hsp70 and Hsp90 inhibitors potentiate them. The Hsp90 inhibitor 17-AAG and the Hsp70 inhibitor 116-9e decrease detectable levels of Her3 protein in PC9 GR4 cells while the proteasome inhibitor MG132 protects Her3 from TX2-121-1 induced degradation, suggesting that the proteasome plays a central role in the degradation mechanism. (b) Cartoon for adamantane mediated degradation of Her3. Bulky hydrophobic chemical moieties are directed in cells to the proteasome degradation pathway. By tethering these substituents to Her3 using covalent chemistry, Her3 is also directed to the proteasome.(c) Her3 pull down. Treatment of PC9 GR4 cells for 6 hours followed by pull down using Her3 results in detection of Her2 and c-Met. Treatment with TX2-121-1 results in decreased levels of Her2 and c-Met suggesting that TX2-121-1 disrupts the interaction between Her3 and those proteins.