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Selective Inhibition of STAT3 Phosphorylation Using a Nuclear-Targeted Kinase Inhibitor

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Abstract

The discovery of compounds that selectively modulate signaling and effector proteins downstream of EGFR could have important implications for understanding specific roles for pathway activation. A complicating factor for receptor tyrosine kinases is their capacity to be translocated to the nucleus upon ligand engagement. Once localized in subcellular compartments like the nucleus, the roles for EGFR take on additional features, many of which are still being revealed. Additionally, nuclear localization of EGFR has been implicated in downstream events that have significance for therapy resistance and disease progression. The challenges to addressing the differential roles for EGFR in the nucleus motivated experimental approaches that can selectively modulate its subcellular function. By adding modifications to the established EGFR kinase inhibitor gefitinib, an approach to small molecule conjugates with a unique nuclear-targeting peptoid sequence was tested in both human and murine breast tumor cell models for their capacity to inhibit EGF-stimulated activation of ERK1/2 and STAT3. While gefitinib alone inhibits both of these downstream effectors, data acquired here indicate that compartmentalization of the gefitinib conjugates allows for pathway specific inhibition of STAT3 while not affecting ERK1/2 signaling. The inhibitor conjugates offered a more direct route to evaluate the role of EGF-stimulated epithelial-to-mesenchymal transition in these breast cancer cell models. These conjugates revealed that STAT3 activation is not involved in EGF-induced EMT, and instead utilization of the

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Supporting Information

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Supplemental figures; experimental Materials and Methods describing peptide, peptoid, and small molecule synthesis; and NMR and mass spectral data for synthesized molecules (PDF)

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Author Contributions

All work for this study, including from each of the authors, was conducted in the Department of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy at Purdue University, 575 Stadium Mall Drive, West Lafayette, Indiana, 47907, United States of America. M.D.B. designed and carried out the design, synthesis, purification, and characterization of piperazinyl gefitinib and each of the peptide or peptoid-based molecules. W.B. and R.A. conducted all blotting and imaging experiments. A.M.P. and Q.C. were involved in the design and experimental refinement of piperazinyl gefitinib synthesis. K.E.H. assisted with the synthesis of protected primary amines. M.D.B., A.M.P., V.J.D., and M.K.W. were involved in the overall design of the study and data analysis and coauthored the manuscript and Supporting Information.

Notes

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cytoplasmic MAP kinase signaling pathway is critical to this process. This is the first example of a conjugate kinase inhibitor capable of partitioning to the nucleus and offers a new approach to enhancing kinase inhibitor specificity.

Graphical abstract

The diversity of downstream signaling pathways mediated through epidermal growth factor receptor (EGFR) adds complexity to understanding the relative importance of this receptor in contexts of differential cell types and disease processes. As one of four members of the ErbB receptor tyrosine kinase family,¹ EGFR plays central roles in several cellular functions, including cellular proliferation, DNA synthesis, apoptosis, and initiation of epithelial-to-mesenchymal transition (EMT).^{2,3} Efforts to understand and target dysregulated EGFR signaling pathways have largely focused on the initiation and progression of human cancers including head and neck squamous cell carcinoma, lung, breast, colon, anal, pancreatic, ovarian, bladder, and esophageal.^{4–6}

While the cell surface receptor tyrosine kinase activities of EGFR have been intensely studied, evidence has continued to mount for significant roles for EGFR in subcellular organelles and compartments. The localization of additional receptor tyrosine kinases is also under investigation, with nuclear localization being a recurring theme.⁷ Some mechanisms have been proposed for transport of EGFR from the plasma membrane to the nucleus,^{8–12} mitochondria,¹³ and endoplasmic reticulum.¹⁴ Investigations of EGFR's role in these subcellular compartments continue to reveal importance in local signaling events.^{15–17} For example, EGFR entry into the nucleus is linked with functions including as a cotranscription factor,^{18–23} effector of DNA double stranded break repair,^{24,25} and PCNA stability on chromatin by phosphorylation at Y211 associated with reduced fidelity of mismatch repair.^{26,27} Despite these advances, many questions remain concerning the biological impact of intracellular EGFR and other growth factor receptors.

The utilities of protein tyrosine kinase inhibitors to address basic and disease-specific questions for signaling pathways is generally appreciated. Also evident are the significant efforts over the past two decades to target EGFR tyrosine kinase activity as a therapeutic strategy for cancers, with successes found in gefitinib (Iressa) and erlotinib (Tarceva) for EGFR mutant nonsmall lung cancers. However, a major challenge to understanding the

functional roles for compartmentalized EGFR stems from the lack of subcellular selective inhibitors. Indeed, as compared to the issue of target kinase binding, increasing the specificity of kinase inhibitors by modifying their subcellular localization is largely unexplored.

The selectivity of kinase inhibitors that target ATP sites is dependent upon relative concentrations of inhibitors and substrates. Therefore, localization within a compartment has the potential to shift or reveal underappreciated kinase substrates. The challenge of predicting these effects from basic *in vitro* biochemical profiling is difficult due to the unknown impact of compartmentalization. Molecular probes that can be rendered more compartment-specific could provide a suitable method for discerning functional roles in signaling pathways converging on or emanating from specific organelles or cellular locations.

Previous studies establish that EGFR functions can vary greatly in breast cancer, from induction of proliferation to EMT and apoptosis.^{28,29} In this work, we address the hypothesis that subcellular compartmentalization contributes to these differential functions of EGFR. To address this question, a strategy for subcellular compartmentalization of gefitinib has been used as an approach. Using a series of hybrid gefitinib conjugates that incorporate polycationic amide and NLS sequences, the selective targeting of nuclear EGFR kinase activity was evaluated in the human MDA-MB-468 and murine breast cancer models. A change in the overall substrate profile is observed that is consistent with compartment selectivity of EGFR action. This study reports for the first time the targeting of a receptor tyrosine kinase within the nucleus of cells as a useful strategy for subcellular selective pathway inhibition.

RESULTS AND DISCUSSION

BFA Does Not Affect EGFR-Mediated STAT3 Phosphorylation

The initial objective was to devise experimental approaches to address signaling bias downstream of EGFR. Previous reports suggest brefeldin-A (BFA) is capable of preventing ligand mediated translocation of EGFR to the nucleus.¹⁴ Furthermore, an EGFR complex with signal transducer and activator of transcription 3 (STAT3) has been previously isolated from nuclear lysates.¹⁹ Therefore, we hypothesized that BFA pretreatment would prevent EGF-mediated translocation of EGFR to the nucleus and inhibit EGF-induced phosphorylation of STAT3. However, as shown in Figure S1, BFA has no effect on EGF-stimulated phosphorylation of STAT3 in the MDA-MB-468 cell line. In contrast, BFA pretreatment did inhibit IL6-stimulated STAT3 phosphorylation, the mechanism of which is currently unknown. Since BFA was not able to affect EGF-stimulated phosphorylation of STAT3, this approach did not provide us with a means to investigate the role of nuclear EGFR in this process.

Design and Evaluation of Nuclear Targeting Agents

We next envisioned a more direct chemical probe as an alternative means to target nuclear kinase activity of EGFR. The chemical design strategy involves the modification of the

gefitinib to accommodate conjugation with polyamino- or polyguanidinium peptoids between five and nine residues to enhance cellular uptake.^{30–32} The use of peptide and peptoid-based carriers has been proposed to enhance the uptake and subcellular targeting of molecular agents.³³ Amino-peptoids are typically localized to the cytoplasm, whereas guanidinium-peptoids are trafficked to the nucleus.³⁰ Both types are characterized by multiple positively charged residues, but the differential sorting indicates that uptake kinetics and subcellular destination are dependent on the chemical side chains. In addition, the number of peptoid residues affects cellular uptake, with longer polycationic sequences reportedly taken into cells more rapidly.³¹ There is evidence that the efficiency of uptake of cell-penetrating peptides can differ between cell lines and warrants independent evaluations.³⁴

To assess the importance of these features in the cell models here, polyamino and polyguanidinium peptoid conjugates were prepared (Figure 1). Two polylysine peptoids (NLys) were synthesized that contained either seven or nine residues, each N-terminally modified with an aminohexanoic acid linker and 5-carboxyfluorescein (FAM). Previous studies have also shown that molecules can be targeted to the nucleus of cells through the use of nuclear localization sequences (NLSs) derived from viruses such as Human Immunodeficiency Virus 1 (TAT) or Simian vacuolating virus 40 (SV40).^{35,36} MBA-MB-231 triple negative breast cancer cells were exposed to the FAM tagged, 7-mer, 9-mer, TAT-peptide (sequence: GRKKRRQRRRPQ) or FAM alone for 3 h, fixed with paraformaldehyde and analyzed by confocal microscopy (Figure S2). No indication of cellular uptake by FAM alone was detected. Both the TAT-peptide and peptoid sequences enhanced the import of FAM into cells, and increasing the peptoid length from seven to nine residues appeared to increase the cellular uptake of fluorescent compounds. As noted in previous studies, NLys peptoid entry was characterized by a punctate intracellular distribution, being present inside vesicles. Interestingly, the TAT sequence did not display evidence of enhancing the nuclear localization of the dye during this time frame. Because of the apparent vesicle-restricted distribution of NLys-based compounds, the use of polyarginine peptoids (NArg) was pursued since they have previously been shown to increase nuclear distribution of a tagged molecule.³⁰ On the basis of the observations of peptoid length dependence on the efficiency of cellular entry, guanidinium-based compounds were also synthesized with a length of nine residues (Figure 1A).

To provide a comparative basis of testing for nuclear uptake, peptoid–SV40 peptide conjugates were prepared. The SV40 sequence used here (PKKKRKV) is naturally found on the surface of certain proteins and promotes nuclear transport through its recognition by importins, taking it through the nuclear pore complex.³⁷ Following the submonomer synthesis³⁸ of each respective cell-penetrating peptoid (CPPo) on Rink-amide resin, the NLS was assembled using basic Fmoc-based peptide synthesis conditions. The NLS and CPPos were separated by a six-carbon linker. An additional six-carbon spacer was placed on the N-terminus of the NLS, to which either a drug or FAM could be attached.

The cellular distribution of the FAM-conjugated NArg₉ containing molecules was evaluated in normal murine mammary gland cells transformed by overexpression of EGFR (NME)³⁹ and MDA-MB-468 human breast tumor derived cells. Both FAM-NArg and FAM-SV40-

NArg were efficiently taken into the cells. FAM-NArg was distributed inside the cytoplasm (Figure 2A, top), and the addition of the SV40 sequence enhanced the partition of the dye to the nuclear compartment (Figure 2A, bottom). MDA-MB-468 cells exposed to either FAM-NArg or FAM-SV40-NArg showed similar distributions with FAM-NArg primarily in the cytoplasm (Figure 2B, top), while nuclear compartmentalization was enhanced with FAM-SV40-NArg (Figure 2B, bottom).

Site of Inhibitor Conjugation

A rationale for sites of conjugation to receptor tyrosine kinase inhibitors was derived from inspection of the available structure–activity relationships for gefitinib.⁴⁰ In addition, the inspection of the available inhibitor-EGFR cocrystal structures for two drugs (PDB IDs: 2ITY (gefitinib); 1M17 (erlotinib)) indicated the potential for tolerated sites of conjugation with the CPPo (Figure S3). For gefitinib, an obvious substitution in the morpholino ring to affect a conversion to piperazine offered a logical strategy for conjugation. A synthesis of N-substituted-piperazinyl gefitinib (Pip-Gef) is provided in Scheme S1 that reveals a carboxylate site for direct coupling to peptoid sequences as shown in Scheme S2. The EGFR kinase inhibitory properties of the gefitinib core in a subset of compounds in Figure 1 were assessed using a commercial kinase assay and recombinant EGFR (Figure S4). The Gef-SV40-NArg and Gef-NArg retained useful inhibitory activities while Pip-Gef was observed to be a weaker inhibitor with respect to gefitinib. Noteworthy are a distinct set of piperazinyl-substituted gefitinib analogs recently reported with enhanced solubility's that retain cellular inhibitory activities.⁴¹

Drug-Peptoid Conjugates Alter STAT3 Phosphorylation

The cellular uptake and differential distribution of the peptoid-dye conjugates warranted further evaluation of the biochemical activity for the gefitinib-conjugates. ERK1/2 proteins located in the cytoplasm are activated by a variety of stimuli and translocate to the nucleus.⁴² The STAT3 transcription factor is also activated by various cytokines and growth factors and is able to translocate into and out of the nucleus independent of phosphorylation.^{43,44} The growth factor-dependent effects of the gefitinib-conjugates on EGFR, ERK1/2, and STAT3 were tested in the MDA-MB-468 cell model as well as two other model systems. The NME cells are a murine mammary tumor cell line whose transformation is driven by overexpression of EGFR, and the LM1 cells are derivatives isolated from lung metastases following orthotopic engraftment of the NME cells. Similar to the MDA-MB-468 cells, the NME cells express high levels of EGFR. In contrast, the metastatic derivatives of the NME cells⁴⁵ return to normal levels of EGFR, but the protein is disproportionally located in the nucleus, as opposed to the rest of the cell (Figure S5). For these studies, Gef-SV40, Gef-NArg, or Gef-SV40-NArg were selected for analysis since they were observed to be compartmentalized in the nucleus of cells (Figure 2). Erlotinib and gefitinib abrogated the phosphorylation of EGFR, STAT3, and ERK1/2 (Figure 3). Importantly, the NArg-based drug conjugates specifically blocked STAT3 phosphorylation while having no measurable effect on ERK1/2. Two control compounds used for these studies are Pip-Gef and the Gef-SV40 peptide conjugate (Schemes S1 and S2). These nonpeptoid-containing compounds had relatively little effect on the phosphorylation of EGFR and no effect on STAT3 or ERK1/2 phosphorylation. To assess the distribution of

phosphorylated STAT3 in the cell, 468 cells were stimulated with EGF and imaged using immunofluorescence (Figure 4). Unphosphorylated STAT3 showed distribution throughout the entire cell, both inside and outside the nucleus under nonstimulated conditions. Stimulation with EGF induced robust phosphorylation of STAT3 that was restricted to the nucleus, and pretreatment with Gef-SV40-NArg abrogated this response.

NArg-Based Compounds Disrupt STAT3 Phosphorylation in a Dose-Dependent Manner

To further investigate the bifurcation in EGF-mediated activation of ERK1/2 versus STAT3 signaling upon subcellular compartmentalization of gefitinib, MDA-MB-468 cells were exposed to Gef-NArg, Gef-SV40-NArg, or gefitinib in a two-fold series of concentrations up to 1 μ M for gefitinib or 5 μ M for each peptoid conjugate (Figure 5). Gefitinib was observed to nearly eliminate p-STAT3 and p-ERK1/2 within a 4-fold concentration range. Gef-NArg and Gef-SV40-NArg both disrupted p-STAT3 but had no effect on p-ERK1/2 even when administered at 10-fold higher concentrations. These data strongly implicate that compartmentalization of gefitinib allows for pathway specific inhibition of STAT3 versus ERK1/2 signaling.

Drug–Peptoid Conjugates Downregulate EGFR-Dependent but Not JAK-Dependent STAT3 Phosphorylation

In addition to EGF, STAT3 can be activated by other cytokines such as IL6, where cytoplasmic phosphorylation of STAT3 *via* Janus Kinase 1/2 (JAK1/2) leads to its nuclear translocation.^{19,46–48} To verify that our drug–peptoid conjugates were not affecting JAK-dependent STAT3 phosphorylation, the MDA-MB-468 cells were exposed to either EGF or IL-6 to activate EGFR- or JAK-dependent signaling cascades, respectively. Pretreatment with Gef-NArg, Gef-SV40-NArg, gefitinib, or the JAK1/2 inhibitor ruxolitinib was used to determine the effect on STAT3 and ERK1/2 phosphorylation (Figure 6). In the presence of EGF, Gef-NArg, Gef-SV40-NArg, and gefitinib disrupted the phosphorylation of STAT3, while only gefitinib affected the levels of p-ERK1/2. Conversely, in the presence of IL-6, none of the compounds had an effect on p-STAT3, with the exception of ruxolitinib. This selective effect was also observed for the Gef-NLys conjugate but not with the corresponding Gef-SV40-NLys (Figure S6). These results further demonstrate the potential specificity of kinase inhibitor conjugates for targeting EGF-mediated STAT3 activation. Furthermore, these data strongly suggest that EGF induces phosphorylation of a nuclear pool of STAT3 that is distinct from the pool that becomes phosphorylated *via* IL-6-JAK1/2 signaling. The directing of molecules to intracellular compartments has been well-studied in the past, but this work reports, to our knowledge, the first instance of incorporating a tyrosine kinase inhibitor into a NLS or CPPo and demonstrates a unique impact on downstream effectors.

The combination of both the peptide-based NLS and peptoid-based CPPo in these conjugates proved to enhance cellular uptake and activity. This approach for targeting molecules to the nucleus is to increase their lifetime and allow the cellular machinery to partition the drug to the nucleus using a nonlabile linker. The observation that the drug conjugates downregulate phosphorylation of STAT3, but do not affect ERK1/2, in contrast to gefitinib is a significant result. Both ERK1/2 and STAT3 can be found downstream of

EGFR, but their roles in cellular processes are quite different. ERK1/2 is found in the cytoplasm until activation, which forms dimers that translocate into the nucleus. The transcription factor STAT3, on the other hand, can be found in both the cytoplasm and nucleus, but phosphorylation state does appear to promote retention in the nucleus (Figure 4).^{43,44} The observations herein would be consistent with the Gef-NArg conjugates preventing EGFR-mediated STAT3 activation, while both molecules are in the nucleus, but a more complete mechanistic understanding would require further studies.

EGF-Induced Epithelial-Mesenchymal Transition (EMT) Utilizes the MAPK Pathway

As opposed to acting as a proliferative factor, previous studies demonstrate that EGF stimulation induces EMT in the MDA-MB-468 cells.³ Conflicting studies have mechanistically linked EGF-induced EMT to downstream activation of STAT3 and ERK1/2.^{49–51} The pathway specific modulation by our peptoid conjugates enabled further investigation of this process. As expected, addition of EGF to the MDA-MB-468 cells led to EMT visualized through loss of cell–cell junctions and gain in filamentous actin (Figure 7). The coaddition of our Gef-SV40-NArg conjugate actually enhanced this effect, leading to a more robust morphological change and the formation of actin filaments (Figure 7). These data strongly suggest that STAT3 signaling is not required for EGF-induced EMT. Consistent with the lack of STAT3 function in EGF-induced EMT, we also did not observe IL-6 to induce any morphological change in the MDA-MB-468 cells even though this growth factor robustly induces STAT3 phosphorylation in these cells (Figures 6 and 7). In stark contrast, EGF-induced EMT was completely blocked by addition of the MEK1/2 inhibitor Trametinib and the direct ERK2 inhibitor Vx11e (Figure 7). Taken together with our previous findings,³ these data indicate that EGF-induced EMT utilizes a cytoplasmic MAP kinase signaling pathway that does not require the activation of STAT3 in the MDA-MB-468 tumor cell line.

The discovery of ligands that selectively modulate signaling proteins downstream of EGFR could have important implications for understanding specific roles for pathway activation. STAT3 is an important signaling mediator in malignant disease, including breast cancer. Previous studies have demonstrated that suppressing signaling from EGFR-STAT3,⁵² as well as STAT3 signaling in general,^{53–57} can result in tumor cell apoptosis in breast, melanoma, leukemia, myeloma, and lung cancers. The feasibility of designing molecular tools that efficiently target specific segments of signaling pathways add value as chemical biology probes and potential future approaches to therapeutics.

METHODS

Synthesis of Conjugate Peptoid and Peptide Segments

A series of peptoid-based conjugates were synthesized using the methodology outlined in Scheme S2. These molecules consist of either amino- (NLys) or guanidinium- (NArg) based polycationic cell-penetrating peptoid (CPPo) sequences, with half of the total set also containing a peptide-based SV40 nuclear localization sequence. With the exception of negative controls that contained no drug, the conjugates were N-terminally tagged with either piperazinyl gefinitib (synthesis outlined in Scheme S1) or 5-FAM. The CPPo, NLS,

and drug/dye were each separated by a six-carbon spacer. A detailed procedure for the synthesis of the compounds can be found in the Supporting Information.

Cell Signaling and Immunoassays

Cells were seeded in a 24-well plate and grown overnight in complete media (DMEM + 10% FBS, penicillin/streptomycin, insulin). The next morning, the media were removed and replaced with serum-free media with inhibitors (1 μ M in DMSO). The cells were incubated for 6 h, and EGF (25 ng/mL) was added. The cells were incubated for 30 min. Then, the media were removed, and lysis buffer was added. The lysates were separated by SDS PAGE and transferred to a PVDF membrane. The membranes were probed for total and phospho-specific antibodies for ERK1/2, EGFR (phospho-Y845), STAT3 (phospho-Y705), and beta-tubulin. For immunofluorescence assays, cells were fixed in 4% formaldehyde for 20 min at RT and then the cells permeabilized in 0.1% Triton X-100 for 5 min at RT. The cells were washed with PBS, followed by PBS plus 2% BSA, and were incubated overnight at 4 °C with primary antibody (anti-STAT3 or phospho-STAT3-Y705) in PBS plus 2% BSA. After incubation, the cells were washed and again incubated for 1 h at RT with secondary antibody (donkey antimouse Alexa Fluor 488) in PBS plus 2% BSA. The cells were washed with PBS plus 2% BSA, PBS alone, followed by incubation with DAPI in PBS for 5 min at room temp.

Fluorescent Imaging of Peptoid Conjugates

NME or MDA-MB-468 cells were seeded on a 12-well plate and grown overnight in complete media (DMEM + 10% FBS, penicillin/streptomycin, insulin). FAM-NArg or FAM-SV40-NArg were added at a 10 μ M concentration in PBS, and the cells were incubated with the compounds for 1 h. Cells were then washed with PBS, fixed with 4% formaldehyde in PBS, washed again with PBS, and the nuclei stained with DAPI in PBS. Cells were imaged using the EVOS FL microscope.

Cell Culture

The MDA-MB-468 cells were obtained from the ATCC and cultured in DMEM containing 5% FBS and penicillin/streptomycin. NME cells were derived from normal murine mammary gland (NMuMG) cells and constructed to overexpress EGFR, as previously described.³⁹ NME tumor cells that underwent metastasis from the mammary fat pad were subcultured from the lungs of mice to constitute the LM1 cells line.⁴⁵ Both these cell lines were cultured in DMEM + 10% FBS, penicillin/streptomycin, and insulin (10 μ g/mL).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cell penetrating peptoid sequences and peptide-peptoid drug conjugates used in this study.

(a) Each peptoid-based compound consists of a nine-member amino- (NLys₉) or guanidinium- (NArg₉) based polycationic sequence. (b) For compounds containing a conjugated drug, piperazinyl gefitinib was coupled to either a peptide-peptoid hybrid consisting of SV40 (NLS) and NLys₉ or NArg₉ or simply an NLys₉ or NArg₉ peptoid sequence. Peptoid monomers, NLys and NArg, were prepared from 1,4-diaminobutane and 1,3-diaminopropane, respectively, as described in the Supporting Information.

Figure 2. NArg-peptoid conjugates enhancing nuclear uptake. EGFR overexpressing (a) NME or (b) MDA-MB-468 cells were incubated with either 10 μ M FAM-NArg or FAM-SV40-NArg for 1 h, fixed, and stained with DAPI. The cells were then imaged using an EVOS FL microscope. Scale bars are 100 μ m.

Figure 3.

Effect of nuclear-targeted NArg conjugates on the phosphorylation status of EGFR, ERK1/2, and STAT3 in murine and human tumor cells. (a) NME (top), LM1 (bottom), or (b) MDA-MB-468 cells were pretreated with erlotinib, gefitinib, Gef-SV40, or the NArg-based drug conjugates at concentrations of 1 μ M, and these cells were subsequently stimulated with EGF (25 ng/mL). “No Drug” (right panels) indicates the CPPo or NLS-CPPo sequences without gefitinib attached (i.e., containing an uncapped aminohexanoic acid at the N-terminus). NME and LM1 cells were lysed and probed for p-EGFR, p-STAT3, p-ERK1/2, total STAT3 (t-STAT3), and/or β -tubulin, and MDA-MB-468 cells were lysed and probed for p-STAT3, total STAT3 (t-STAT3), p-ERK1/2, and total ERK1/2 (t-ERK1/2).

Figure 4.

Gef-SV40-NArg disrupting nuclear accumulation of phosphorylated STAT3. MDA-MB-468 cells were cultured overnight in the presence of DMSO or Gef-SV40-NArg ($1 \mu\text{M}$) and subsequently stimulated with EGF (25 ng/mL) for 30 min. These cells were fixed and stained to visualize total (t-STAT3) and phosphorylated (p-STAT3) STAT3. Images were acquired using an EVOS FL microscope.

Figure 5. Dose response of NArg-based drug conjugates in MDA-MB-468 cells. A total of 468 cells were exposed to increasing concentrations of gefitinib, Gef-NArg, or Gef-SV40-NArg and subsequently stimulated with EGF (25 ng/mL). Cell lysates were probed for p-STAT3 (Y705), t-STAT3, p-ERK1/2, and β -tubulin.

Figure 6.

Drug-peptoid conjugates selectively downregulating EGF-dependent phosphorylation of STAT3. MDA-MB-468 cells were pretreated with 1 μ M Gef-NArg, Gef-SV40-NArg, gefitinib, or the JAK1 inhibitor ruxolitinib followed by stimulation with either EGF (25 ng/mL) or IL-6 (20 ng/mL). Cell lysates were probed for p-STAT3 (Y705), t-STAT3, p-ERK1/2, and β -tubulin.

Figure 7. EGF-induced EMT requiring cytoplasmic MAP kinase signaling. MDA-MB-468 cells were stimulated with EGF for 24 h in the presence of 1 μM of the indicated inhibitors (“NS” stands for “non-stimulated”). Cells were subsequently fixed and stained with FITC labeled phalloidin to visualize the actin cytoskeleton. The cells were then imaged using an EVOS FL microscope. Scale bars are 100 μm .