Selective Inhibition of ADAM Metalloproteases as a Novel Approach for Modulating ErbB Pathways in Cancer

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Abstract Purpose: ErbB receptor signaling pathways are important regulators of cell fate, and their dysregulation, through (epi)genetic alterations, plays an etiologic role in multiple cancers. ErbB ligands are synthesized as membrane-bound precursors that are cleaved by members of the ADAM family of zinc-dependent metalloproteases. This processing, termed ectodomain shedding, is essential for the functional activation of ErbB ligands. Recent studies suggest that elevated levels of ErbB ligands may circumvent the effectiveness of ErbB-targeted therapeutics. Here, we describe the discovery and preclinical development of potent, selective inhibitors of ErbB ligand shedding.

Experimental Design: A series of biochemical and cell-based assays were established to identify selective inhibitors of ErbB ligand shedding. The therapeutic potential of these compounds was assessed in multiple in vivo models of cancer and matrix metalloprotease–related toxicity.

Results: INCB3619 was identified as a representative selective, potent, orally bioavailable small-molecule inhibitor of a subset of ADAM proteases that block shedding of ErbB ligands. Administration of INCB3619 to tumor-bearing mice reduced ErbB ligand shedding in vivo and inhibited ErbB pathway signaling (e.g., phosphorylation of Akt), tumor cell proliferation, and survival. Further, INCB3619 synergized with clinically relevant cancer therapeutics and showed no overt or compounding toxicities, including fibroplasia, the dose-limiting toxicity associated with broad-spectrum matrix metalloprotease inhibitors.

Conclusions: Inhibition of ErbB ligand shedding offers a potentially novel and well-tolerated therapeutic strategy for the treatment of human cancers and is currently being evaluated in the clinic.
instance, increased levels of the EGFR ligand transforming growth factor-α (TGF-α) have been observed in trastuzumab-treated breast cancer patients with progressive disease (6). Similar results were obtained in a preclinical model of trastuzumab resistance that showed a concordant increased sensitivity to an EGFR-selective TKI (7). Moreover, a nonbiased analysis of patients receiving gefitinib described significantly elevated expression of the ErbB ligands amphiregulin and TGF-α in nonresponders, agreeing with in vitro data suggesting that high ErbB ligand concentrations can circumvent the effectiveness of an EGFR-targeted agent (8, 9) or the less selective ErbB kinase inhibitor GW572016 (10). These data are consistent with the preclinical observation that targeting the EGFR pathway at multiple points (ligand binding and ATP pocket) may be more efficacious than either method in isolation (11, 12)—a hypothesis currently being evaluated clinically. Finally, even in patients who do respond to EGFR-selective TKIs, the emergence of secondary mutations in the EGFR kinase domain affords resistance to the ATP-competitive TKIs (13–16). Although this is an evolving field, these and other data support the following concepts: (a) although ErbB-targeted agents have shown clinical promise, there is significant room for improvement; (b) a multifaceted approach to inhibiting multiple ErbB pathways would be expected to improve therapeutic response; and (c) inhibiting the activation of multiple ErbB ligands represents a logical and perhaps clinically relevant point of intervention.

Although certain broad spectrum matrix metalloprotease (MMP) inhibitors (e.g., marimastat) can inhibit EGFR ligand shedding in vitro (among other activities), this has not been investigated in vivo nor has the therapeutic hypothesis been tested clinically due to the lesser potency of these drugs against key ADAM protease—relative to MMPs (see below)—and their inability to be tolerated at higher doses (17). Here, we describe the discovery and preclinical development of selective inhibitors of ErbB ligand shedding and show that these compounds inhibit ErbB ligand shedding and tumor growth in vivo without inducing joint fibroplasias, the dose-limiting toxicity of many MMP inhibitors. These compounds possess the pharmacologic properties making them ideal candidates to test the therapeutic potential of sheddase inhibition for the treatment of cancer in a clinical setting.

Materials and Methods

In vitro metalloprotease and pharmacology assays. Sheddase inhibitors were synthesized at Incyte Corp as described (18). Human MMPs, ADAM9, and ADAM10 were purchased from R&D Systems (Minneapolis, MN) and assayed as recommended by the manufacturer. The peptide substrate (7-methoxycoumarin-4-yl) acetyl-Pro-Leu-Gly-Leu-[3,2,4-dinitrophenyl]-l-2, 3-diaminopropionyl)-Ala-Arg-NH2 was used for the MMP assays as recommended by the manufacturer (R&D Systems). Partially purified porcine ADAM17 was obtained from spleen as described (19). The fluorogenic peptide substrate, (7-methoxycoumarin-4-yl)-acetyl-Pro-Leu-Ala-Gln-Ala-Val-[3,2,4-dinitrophenyl]-l-2, 3-diaminopropionyl)-Arg-Ser-Ser-Ser-Arg-NH2, was used for the ADAM9, ADAM10, and ADAM17 assays following the manufacturer's recommendations (R&D Systems). INCB3619 was also screened against a panel of G-protein coupled receptors and ion channels for inhibitory activity (ExpressProfile, Cerprep, Inc., Paris, France).

Cell culture and generation of stable cell lines. Cell lines were obtained from American Type Culture Collection (Manassas, VA) and were cultured as recommended by the supplier. Retroviral transduction was used to generate derivatives of the NMuMG and Rat-1 cells as described previously (20). The coding region of the human cDNA for TGF-α was cloned into pMSCVpuro (BD Biosciences, Mountain View, CA) following amplification from a pool of human cDNA (BD QUICK Clone). Human umbilical vascular endothelial cells were obtained from Cambrex Corp (East Rutherford, NJ) and treated following the manufacturer's protocols.

Analysis of ErbB ligand shedding and its effect. The TGF-α cleavage assay used SCC9 cells stimulated with 10 μmol/L phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO) for 30 min in serum-free medium following a 30-min pretreatment with DMSO or metalloprotease inhibitor. Supernatants were collected and assayed for soluble TGF-α by a commercially available ELISA kit (R&D Systems). Alternatively, NMuMG or Rat-1 cells were grown for 24 h in the presence of DMSO or sheddase inhibitor, and supernatants were analyzed in a similar manner. Amphiregulin release was measured in supernatants of HCT116 stimulated with phorbol 12-myristate 13-acetate (1 μmol/L, 30 min) using an ELISA kit (R&D Systems). The heparin-binding EGF-like growth factor (HB-EGF) cleavage assay used Chinese hamster ovary cells stably transfected with human placental alkaline phosphatase–tagged HB-EGF (In Vitro Biology Department of Incyte Corporation, Wilmington, DE). The transfected Chinese hamster ovary cells were grown in 96-well plates and treated with DMSO or compound for 10 min followed by phorbol 12-myristate 13-acetate stimulation (2 μmol/L, 45 min). Alkaline phosphatase activity was assessed using a luminescent substrate (Roche Molecular Biochemicals, Indianapolis, IN). Herregulin cleavage was assessed using Chinese hamster ovary-K1 cells transiently transfected with the human hergulin-β1 cDNA in pCDNA3.2-DESTTM (In Vitro Biology Department of Incyte Corporation) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Following transfection, the cells were grown in serum-free medium for 24 h. Cells were pretreated with DMSO or compound for 15 min and stimulated with phorbol 12-myristate 13-acetate (1 μmol/L, 60 min). Herregulin-β1 was quantified in the supernatants using a commercially available human NRG1-β1/HRG1-β1 ELISA kit (R&D Systems) according to the manufacturer's instructions.

Conditioned medium experiments used NMuMG-T cells grown in the presence of DMSO or INCB3619 for 24 h. Medium was collected and concentrated (Amicon Centriprep, Millipore Corp., Billerica, MA) and applied to serum-deprived (6 h) A431 cells for 30 min. Protein extracts were then subjected to immunoblotting using antibodies against total EGFR and phosphorylated EGFR (Tyr1173) antibodies (Cell Signaling, Beverly, MA).

Primary head and neck cancer cells were obtained from a consenting patient with squamous cell carcinoma of the buccal mucosa (approved by the University of Pittsburgh Institutional Review Board). The patient was not previously treated with standard therapies. Cells were maintained in DMEM, 10% fetal bovine serum, and 2 mmol/L glutamine, and passed 1:10 weekly. Epithelial cells were separated from fibroblasts using selective trypsinization. For proliferation assays, cells were seeded at 104 per well in the in a 96-well plate and allowed to attach overnight. The medium was then replaced with 10 μL of low serum medium (2% fetal bovine serum) containing compound or DMSO and maintained for 5 days. Viability was assessed following manufacturer's protocols (Promega Cell-Titer Glo, Madison, WI) and percentage inhibition of growth was determined relative to the DMSO control. Amphiregulin shedding was determined from 4 × 105 cells per well in 12-well plates, which were allowed to attach overnight. The medium was replaced with low serum medium (2% fetal bovine serum) containing compound or DMSO and incubated overnight. Centrifuged medium was collected and analyzed following

3 C. Arteaga, personal communication.
to identify potent, selective, and well-tolerated inhibitors of a subset of the ADAM family of metalloproteases, herein called sheddases. Sheddases are responsible for the cleavage and activation of all three types of ErbB ligands: (a) those that bind solely to EGFR (e.g., TGF-α), (b) those that bind both EGFR and ErbB4 (e.g., HB-EGF), and (c) those that bind ErbB3 and/or ErbB4 (e.g., heregulin; reviewed in refs. 5, 22). Importantly, the ultimate goal was to discover molecules with drug-like properties, including oral bioavailability and good pharmacokinetics. To this end, we synthesized a number of novel hydroxamic acid-derived metalloprotease inhibitors (18), 3 culminating in the identification of a series of molecules exemplified by INCB3619 (Fig. 1A).

The sheddases—ADAMs responsible for processing the membrane-bound ErbB ligands—have been reported to include ADAM17 (also known as tumor necrosis factor-α converting enzyme) and ADAM10 (22–29). We thus established biochemical assays using recombinant proteins and fluorogenic peptide substrates and screened proprietary hydroxamic-based compounds (19) for inhibition of ADAM10/ADAM17 enzyme activity. Hydroxamic acids have previously been shown to affect HB-EGF shedding through inhibition of an unknown enzyme (30). Compounds from our collection that showed >50% inhibition of ADAM10 and/or ADAM17 at a concentration of 200 nmol/L were further characterized against a larger panel of metalloproteases and ADAM proteins to assess selectivity. A structure-activity relationship was established and provided direction for the synthesis or more potent and/or selective compounds. Using this strategy, INCB3619 was identified as a potent inhibitor of ADAM17 and ADAM10 activity with IC50 values of 14 and 22 nmol/L, respectively. Moreover, INCB3619 shows excellent selectivity both within the ADAM family (e.g., IC50 for ADAM9 is >5 μmol/L) and against a panel of MMPs (Table 1). Functional cell-based assays confirmed the ability of INCB3619 to inhibit the shedding of TGF-α, HB-EGF, amphiregulin, and heregulin with nanomolar potencies (Fig. 1B). Compounds that possess MMP activity but lack ADAM10/17 activity did not block ErbB ligand shedding in these assays (see below).

In addition to metalloprotease selectivity, multiple structural analogues were screened against a panel of >50 G-protein coupled receptors and ion channels (Cerep, Inc., ExpressProFile) and no significant binding or inhibition was observed at 1 μmol/L (data not shown). Pharmacokinetic analysis of INCB3619 indicated that it was well-absorbed orally (10 mg/kg but had a half-life of <2 h in the mouse). 5 As such, for in vivo administration, we took advantage of the excellent solubility of this compound and used implantable osmotic pumps. In larger species, such as dog, the half-life of INCB3619 was significantly longer (6.1 h). Improved pharmacokinetics in canine and nonhuman primates is a common feature of hydroxamic acid–based inhibitors. Therefore, continuous infusion in rodents is a better reflection of the predicted human pharmacokinetics and also serves to specifically test the therapeutic hypothesis by avoiding maximal drug exposures sufficient to potentially inhibit an array of MMPs nonspecifically.

In vitro and in vivo sequelae of selective ADAM inhibition. To begin to assess the biological effect of inhibiting ErbB ligand shedding, we first engineered a cellular model reliant on

5 J.S. Fridman and M. Pan, unpublished data.
TGF-α expression for its transformed phenotype (Fig. 2A). NMuMG cells (immortalized mammary epithelial cells) were transduced with either an empty expression vector (NMuMG-V) or a vector encoding TGF-α (NMuMG-T). After confirming the ability of transfected cells to produce TGF-α, we tested the ability of INCB3619 to inhibit the shedding of TGF-α from the cell surface by analyzing tissue culture supernatants from cells grown in the presence or absence of compound (Fig. 2B). In contrast to INCB3619, which inhibits ADAM10 and ADAM17, neither INCB8765 (ref. 9; an ADAM10 selective inhibitor; Fig. 2B, △) nor an inhibitor of MMPs that is both ADAM10 and ADAM17 sparing (Fig. 2B, ■; ref. 9) inhibited TGF-α shedding. This agrees with the knockout data implicating ADAM17 as the enzyme responsible for TGF-α shedding (24, 31). The ability to potently inhibit ErbB ligand cleavage was not specific to INCB3619 as additional compounds having similar enzymatic activities as INCB3619 were also able to do so (data not shown). Moreover, this pharmacology was not limited to cells of epithelial origin as nearly identical results were obtained using immortalized fibroblasts (Fig. 2C). To extend these findings beyond contrived cell lines, we investigated the effect of sheddase inhibition on EGFR ligand release and cell proliferation in primary tumor cells surgically isolated from a consenting patient with squamous cell carcinoma of the buccal mucosa. This tumor type was chosen for investigation based on the clinical efficacy observed with antibodies that inhibit EGFR ligand binding. Using these cells, we show that INCB3619 can inhibit the release of amphiregulin (Fig. 2D) in primary tumor cells in addition to ErbB ligands in established cell lines. Treatment with INCB3619 resulted in a significant decrease in proliferation of these primary tumor cells (Fig. 2E). The EC_{50} for proliferation was estimated to be near the EC_{90} for amphiregulin shedding, suggesting that the ADAM sheddases may require marked inhibition for efficacy in this model system. Although the results in these primary cells are consistent with our cell line data, we cannot conclude that similar effects will be observed in all cancer cells. In contrast, in normal cells (human umbilical vascular endothelial cells or primary fibroblasts), there was no effect on proliferation at concentrations >10 times those effective on the tumor cells (data not shown).

Although these experiments show that selective sheddase inhibition can diminish the release of EGFR ligands from the cell surface, they do not show that this can affect EGFR signaling. To illustrate that inhibition of the release of TGF-α can reduce EGFR pathway activation, we treated the EGF-responsive A431

Fig. 1. INCB3619 exemplifies a series of potent and selective sheddase inhibitors that prevent the proteolytic processing of multiple ErbB proligands. A, the chemical structure of INCB3619. B, INCB3619 inhibits the shedding of TGF-α, amphiregulin (AR), HB-EGF, and heregulin (HRG) in cell-based assays.
cells with conditioned medium harvested from INCB3619-treated NMuMG-T cells. Treatment of NMuMG-T cells with INCB3619 markedly inhibited activation of EGFR in the A431 cells, confirming that NMuMG-T cells release a soluble factor capable of activating EGFR and that this is prevented with a selective sheddase inhibitor (Fig. 2F). Similar experiments in which A431 cells were first incubated with an EGFR-neutralizing antibody showed no EGFR activation after addition of conditioned medium from NMuMG-T cells grown in the presence or absence of INCB3619 (data not shown), consistent with the conclusion that selective sheddase inhibition prevents activation of an autocrine TGF-α loop. Likewise, we have previously shown in multiple autonomous cell-based systems that pharmacologic or genetic inhibition of ADAM10/17 can inhibit ErbB receptor phosphorylation and downstream signaling (9). However, as none of the cell lines studied here overexpress HER2, we did not detect significant changes to the amount of HER2 receptor phosphorylation (data not shown).

The ability of sheddase inhibition to reduce TGF-α shedding in vivo (Fig. 3A) as well as EGFR-driven tumor growth was investigated in both tumors of fibroblast (Fig. 3B; 42% TGI) and epithelial origin (Fig. 3C; 38% TGI). In agreement with data that has been generated using less selective compounds, the direct effect of sheddase inhibition addition on ex vivo cellular proliferation of established cell lines was not dramatic (refs. 32–35; data not shown)—similar to what was observed by others using the C225 anti-EGFR antibody. This was not unexpected as any of a number of signaling pathways may be sufficient to drive proliferation in in vitro established cell culture systems and/or to activate the ErbB receptors via crosstalk among signaling pathways. However, three-dimensional tumor growth in vivo is different than growth in culture and ErbB signaling may play a more crucial role. Supporting this hypothesis, it has been shown that xenografts arising from cultured cells significantly up-regulate key players in ErbB signaling (36). Therefore, evaluating the effect of sheddase inhibition in the more clinically relevant in vivo setting was imperative.

To determine if sheddase inhibition could be as efficacious as direct kinase inhibition using a low molecular weight TKI, such as gefitinib (Iressa), we treated mice bearing well-established MCF-7 human breast cancer xenografts with either INCB3619 (60 mg/kg/d) or a maximally tolerated regimen of gefitinib (100 mg/kg/d) and concluded that both treatments resulted in equivalent tumor inhibition (50% and 48% TGI, respectively, and 19-day TGD for both; Fig. 4A). Further, in support of the targeted nature of INCB3619 and gefitinib, neither agent showed efficacy against MDA-MB-231 xenografts, in which proliferation is driven, not by EGFR, but by mutated k-Ras (ref. 37; Fig. 4B and data not shown). In contrast, the nonselective MMP inhibitor marimastat, which inhibits both MMPs and sheddases (Fig. 1B), was efficacious (69% TGI; Fig. 4B), implying that the antitumor effect of marimastat is due to MMP inhibition and that INCB3619 is a selective compound and that its antitumor activity is not a result of its somewhat weak potency against MMP2. Indeed, a selective ADAM 10/17 inhibitor with 10-fold greater selectivity against MMP2 (INCB9725) retained its ability to inhibit tumor growth similarly to INCB3619 in the MDA-MB-435 tumor model (INCB3619: TGI, 35%; TGD, 10 days; INCB9725: TGI, 44%; TGD, 10 days; not shown).

As we and others have hypothesized that the antitumor effects obtained by inhibiting a single node of an ErbB pathway in isolation using one treatment modality may not be optimal, we investigated the potential of improving responses to an EGFR TKI by concomitantly treating with INCB3619. Mice with s.c. human non–small cell lung carcinoma xenografts were treated with gefitinib, INCB3619, or the combination and tumor growth was followed over a 2-week period (Fig. 4C). As single agents, neither compound was efficacious; however, in combination, INCB3619 and gefitinib markedly inhibited tumor growth. Therapeutic benefit was obtained without notation of compounding toxicity. Similar results have been obtained using erlotinib in place of gefitinib (data not shown). These data are consistent with that recently published exemplifying the ability of INCB3619 to potentiate the effects of gefitinib in vitro (9). Collectively, these data support that a sheddase inhibitor may be an efficacious and well-tolerated treatment modality for affecting ErbB pathways in cancer as a single agent or in combination with other targeted agents.

Because particular cancer types have been linked to elevated ErbB ligand expression, and hence increased EGFR activity (reviewed in ref. 38), we evaluated the activity of INCB3619 in a xenograft model of one such tumor type, head and neck squamous cell carcinoma. Subcutaneous tumors were established using the SCC4 cell line. As platinum-based therapeutic regimens are the standard of care for inoperable tumors of this type, we compared the efficacy of INCB3619 to the cytotoxic agent cisplatin, alone or in combination with INCB3619. INCB3619, as a single agent, nearly completely inhibited tumor growth during the treatment period (30% TGI) and subsequently resulted in a 13-day TGD (Fig. 5A). In contrast, cisplatin was mostly ineffective (0% TGI and a 2-day TGD) although it was administered near its maximally tolerated dose (Fig. 5A and data not shown). Although cisplatin was lackluster as a single agent, the addition of INCB3619 was synergistic (66% TGI and a 24-day TGD), producing sporadic tumor regressions (in three of seven mice). Importantly, INCB3619 did not exacerbate the toxic effects of cisplatin, as assessed by animal weights and clinical observations (data not shown). To understand the

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**Table 1.** INCB3619, as opposed to the broad-spectrum MMP inhibitor marimastat, potently and selectively inhibits ADAM10 and ADAM17, compared with a panel of other zinc-dependent metalloproteases

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<th>Enzyme assays</th>
<th>IC50 (nmol/L)</th>
<th>Marimastat IC50 (nmol/L)</th>
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<tr>
<td>ADAM8</td>
<td>1,000</td>
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<td>ADAM9</td>
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<tr>
<td>MMP14</td>
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Abbreviation: ND, not determined.
mechanism behind the observed synergy, we analyzed similarly treated tumor samples *ex vivo*. Immunohistochemical analyses indicated that INCB3619 alone was sufficient to modestly decrease tumor cell proliferation nearly by nearly 10% (Fig. 5B, top) and activation of the Akt/PKB survival signaling pathway (Fig. 5B, bottom). This latter effect likely accounts for the increased responsiveness to the cytotoxic insult induced by treatment with cisplatin (Fig. 5A and C). However, even as a single agent, INCB3619 was sufficient to induce tumor cell apoptosis (Fig. 5C, top right). Similar synergism was observed in

![Fig. 2. Selective sheddase inhibition prevents proteolytic processing of ErbB ligands and activation of an EGFR receptor ligand autocrine/paracrine loop. A, transduction of the immortalized murine mammary epithelial cell line NMuMG with TGF-α results in an aggressively tumorigenic cells. NMuMG cells were transduced with an empty retroviral expression vector (●) or one expressing TGF-α (▲). Pooled populations of transduced cells were injected s.c. into immunocompromised mice, and tumor growth was followed over time. B, ErbB ligand shedding was evaluated in NMuMG cells expressing TGF-α in the presence of increasing concentrations of INCB3619 (▲), the ADAM10 selective compound INCB8765 (△), or the ADAM10/17-sparing MMP inhibitor INCB8278 (○). C, TGF-α shedding was also inhibited in rat fibroblasts (Rat-1-T) grown for 24 h in the presence of DMSO (control) or INCB3619, thus indicating that this effect is not cell type specific. D, INCB3619 also potently inhibited amphiregulin shedding from primary tumor cell cultures of a treatment naive head and neck cancer patient. E, INCB3619 inhibits the proliferation of primary head and neck carcinoma cells. Cells were grown in low serum (2%) for 5 d in the presence of increasing concentrations of INCB3619 or DMSO, and proliferation was assessed using a luciferase-based assay (see Materials and Methods for details). No effect on proliferation was observed in normal cells (human umbilical vascular endothelial cells or BJ fibroblasts) at similar concentrations of INCB3619 (not shown). F, inhibition of ligand shedding resulted in inhibition of EGFR activation in serum-deprived (6 h) A431 cells treated with conditioned medium from TGF-α-transduced cells grown in the presence or absence of INCB3619 (2 μmol/L) for 30 min as determined by immunoblot analysis. An EGFR blocking antibody similarly inhibited EGFR activation (not shown).
a breast cancer xenograft model (MDA-MB-435) when INCB3619 was used in combination with paclitaxel, thus indicating that these effects were not particular to a specific tumor type or chemotherapeutic combination (paclitaxel, 10-day TGD; INCB3619, 20-day TGD; combination, 50-day TGD; Fig. 5D and E). INCB3619 also improved the therapeutic responses to other cytotoxic agents in models of pancreatic, non–small cell lung, and colon cancer (data not shown).

Although INCB3619 and related molecules are potent and highly selective, they are not completely devoid of MMP activity. For example, selectivity against MMP2 has proven difficult due to the similarity of its active site to those of ADAM10 and ADAM17.6 As discussed above, although the efficacy of selective sheddase inhibitors do not require MMP2 activity, it is possible that inhibition of MMP2 has potential

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6W. Yao, unpublished observations.

Fig. 3. Sheddase inhibition reduces the levels of circulating TGF-α and retards tumor growth in vivo. A, TGF-α levels were determined in plasma from mice bearing Rat-1-T tumors before treatment and 2 d after treatment with INCB3619 (120 mg/kg/d; n = 8). B, Rat-1-T tumors were treated with vehicle (gray) or INCB3619 (black; 60 mg/kg/d) for 7 d, and tumor size was determined (n = 7). C, carcinoma growth was also reduced by sheddase inhibition as exemplified by the epithelial NMuMG-T tumors treated with INCB3619 (black columns; 60 mg/kg/d) or vehicle (gray columns) for 7 d (n = 13).

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Fig. 4. Selective sheddase inhibition is equally as efficacious as an EGFR TKI, and their combination results in improved tumor growth inhibition. A, s.c. human breast cancer xenografts (MCF-7) were treated with INCB3619 (60 mg/kg/d) or gefitinib (100 mg/kg/d) for 2 wk and their tumor volumes were measured (n = 8). B, MDA-MB-231 xenografts, which have a mutated K-ras and which are gefitinib insensitive, were treated with INCB3619 (●) or marimastat (△) (60 mg/kg/d) for 14 d, and tumor size was followed over time (n = 9). C, sheddase inhibition improves the therapeutic response to EGFR kinase inhibition. Well-established A549 non–small cell lung carcinoma tumor xenografts were treated for 14 d with vehicle (■), INCB3619 (△; 60 mg/kg/d), gefitinib (●, 100 mg/kg, bid), or a combination of the two agents (○). Tumors were measured twice weekly using calipers. Points, mean tumor volumes (n = 7/group); bars, SE.
INCB3619 inhibits tumor cell proliferation and survival signaling resulting in synergism with cytotoxic chemotherapy. A, SCC4 xenografts were treated with vehicle (■), INCB3619 (●; 60 mg/kg/d × 14 d, horizontal arrow), cisplatin (▲; 10 mg/kg, 2×/wk × 2, vertical arrows), or a combination of the two agents (○), and tumor growth was followed over time (n = 7). B, tumors were harvested similarly treated mice 8 h after the first dose of cisplatin and inhibition of cellular proliferation (Ki67, top) and survival signaling (p-Akt, bottom) was examined by immunohistochemistry (n = 4). C, apoptosis was also assessed using TUNEL on these same samples (n = 4). Arrows, examples of apoptotic cells. D, INCB3619 also synergizes with taxanes in a human breast cancer xenograft model. S.c. well-established MDA-MB-435 tumors were treated with vehicle (■), INCB3619 (●; 60 mg/kg/d × 14 d, horizontal arrow), paclitaxel (▲; 10 mg/kg, 2×/wk × 2, vertical arrows), or a combination of the two agents (○), and tumor growth was followed over time (n = 6/group). Points, mean tumor volumes; bars, SE. E, apoptosis was also assessed using TUNEL on tumor samples collected 24 h after the first dose of taxane (n = 3). Arrows, examples of apoptotic cells.
Similar steady-state drug exposures were achieved for both compounds (INCB3619, 691 ± 228 nmol/L; marimastat, 448 ± 74 nmol/L). Thus, sheddase inhibition can be shown to be both efficacious and devoid of the toxicity that prevented the exploration of this mechanism in earlier clinical trials with drugs such as marimastat. In addition, INCB3619 is somewhat active against MMP12, and studies in knockout mice have suggested a potential role for MMP12 in the vascularization of murine lung tumor metastasis via the reduced formation of angiostatin—a reported endogenous angiogenesis inhibitor and substrate of MMP12 (39). The role of such molecules in the human condition, however, is a point of contention. Indeed, recent clinical data supports a beneficial role of MMP12 inhibition in non–small cell lung carcinoma, in which MMP12 expression correlates with metastasis while inversely correlating with relapse-free survival (40). Taken together, these data suggest that the selective sheddase inhibitors, such as INCB3619, are capable of preventing the functional activation of ErbB ligands and have therapeutic potential both as single agents and when used in combination with additional therapeutics. Furthermore, these compounds are extremely well tolerated in xenograft experiments and do not exacerbate the toxicities of cytotoxic therapies. In addition, we evaluated toxicologic variables indicative of hematologic, renal, and hepatic dysfunction and found no significant effect in rodents after continuous administration of INCB3619 at a dose significantly greater than that required for efficacy (Table 2). Moreover, untransformed cells (e.g., human umbilical vascular endothelial cells or primary fibroblasts) were resistant to the growth-inhibitory effects of INCB3619 at concentrations 10 times higher than those effective in transformed cells. These findings suggest selective ADAM10/17 inhibitors, such as INCB3619, have the potential to effect tumor growth and survival while being better tolerated than the broad-spectrum MMP inhibitors.

**Discussion**

Targeted therapies for the treatment of cancer have advanced significantly in recent years and continue to show great promise. The challenges that the medical and scientific communities now face involve the identification of populations that will derive clinical benefit from these agents and the delineation of an optimal strategy for combining these agents with current cytotoxic regimens to improve response rates while decreasing exposure to unnecessary side effects. Nonetheless, significant advances are rapidly being made. For example, the identification of the mechanism(s) of resistance to some of these new therapies, such as imatinib (41–43) and the EGFR-selective TKIs (14, 15), has occurred within a short time after these agents were introduced as approved therapies. As such, complementary or alternative therapies are actively being pursued. Our identification of potent, selective inhibitors of the ErbB ligand sheddases that are efficacious, well tolerated, and have suitable drug-like properties represents a new opportunity to affect clearly validated oncology targets. These molecules prevent the release of ligands for multiple ErbB family members representing a new class of pan-ErbB inhibitors.

There is strong evidence that ligand shedding is critical for the transforming potential of EGFR (reviewed in ref. 44) and increased ErbB ligand expression is a common event in human cancers (38). This latter observation may be explained, in part, by observations demonstrating metalloprotease-dependent ErbB ligand shedding is increased upon activation of a number of diverse signaling pathways linked to human cancers (e.g., WNT, prostaglandin E2, and LPA; refs. 3, 5, 22, 23). Further, correlative clinical and preclinical data suggest that ErbB pathways may compensate for one another, thus...
Their mechanism of tumor growth inhibition from that of mutated K-ras do not show efficacy in a xenograft model, which has a spectrum MMP inhibitors, the sheddase-selective compounds dent metalloprotease inhibitors, they are highly selective against a broad panel of MMPs and thus do not share their musculoskeletal side effects. In addition, unlike broad-spectrum MMP inhibitors, the sheddase-selective compounds do not show efficacy in a xenograft model, which has a mutated K-ras, and do not show efficacy in in vitro angiogenesis systems (i.e., inhibition of human umbilical vascular endothelial cell proliferation, migration, invasion, and tube formation; data not shown), further distinguishing their mechanism of tumor growth inhibition from that of MMP inhibitors. It remains unclear why this latter class of agents failed in the clinic but it is intriguing to find that some MMP activities have been linked to the generation of antiangiogenic molecules (e.g., endostatin and tumstatin; ref. 50). It is therefore tempting to speculate that the effect of these antiangiogenic products of MMP activity may be greater in human cancer than in the mouse or that in the preclinical mouse models, the concentration of MMP inhibitors was high enough to affect the sheddases, thus partly explaining their efficacy against some tumor xenografts.

In this report, we have shown that selective sheddase inhibitors can prevent the release of ErbB ligands from cell lines and primary patient tumor cells and that this can result in inhibition of ErbB signaling pathways in vitro and in vivo. Although we cannot conclude that inhibition of sheddases in addition to ADAM10 and ADAM17 can be important for this activity (e.g., ADAM12 or ADAM15), we have clearly shown that MMP sparing protease inhibition is sufficient for efficacy. Moreover, our previous in vitro studies have shown that selective genetic (RNA interference) inhibition of ADAM17 and not ADAM9 or ADAM15 is sufficient to affect ErbB signaling pathways (9). These molecules thus have antitumor activity in tumor models reported to be sensitive to perturbations in ErbB pathways, but not elsewhere (e.g., MDA-MB-231 tumors). Although sheddase inhibitors do possess significant single-agent antitumor activity, their ability to inhibit survival pathways such as Akt and our preclinical findings demonstrating additive or synergistic effects support the use of combination therapy in the clinic. This is further substantiated by their excellent tolerability and their lack of exacerbating the toxicity associated with standard chemotherapeutics or targeted agents. Further, combining sheddase inhibitors with other ErbB inhibitors may thwart multiple mechanisms of resistance, such as kinase (gatekeeper) mutations or increased ligand expression. As such, selective sheddase inhibitors may complement the existing arsenal of anticancer agents; hence, clinical investigation of sheddase inhibition is undoubtedly warranted.

Table 2. Hematologic and clinical chemistry variables are not altered upon treatment with INCB3619

<table>
<thead>
<tr>
<th></th>
<th>RBC ((\times 10^9/\text{mL}))</th>
<th>RET ((\times 10^9/\text{mL}))</th>
<th>WBC ((\times 10^9/\text{mL}))</th>
<th>ALB (g/dL)</th>
<th>TP (g/dL)</th>
<th>GLOB (%)</th>
<th>ALT (units/L)</th>
<th>BILI (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>10.23 ± 0.43</td>
<td>425.4 ± 60.47</td>
<td>8.23 ± 1.86</td>
<td>3.22 ± 0.08</td>
<td>5.62 ± 0.11</td>
<td>2.40 ± 0.04</td>
<td>47.00 ± 4.03</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>INCB3619</td>
<td>10.88 ± 0.14</td>
<td>388.5 ± 14.53</td>
<td>7.05 ± 0.83</td>
<td>2.88 ± 0.16</td>
<td>5.12 ± 0.23</td>
<td>2.23 ± 0.08</td>
<td>58.83 ± 11.57</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>Statistically significant</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

NOTE: Immunocompetent mice were treated with vehicle or INCB3619 (45 mg/kg/d) by continuous infusion for 7 d (\(n = 6/\text{group}\)). Whole blood was evaluated by looking at RBC counts, reticulocytes, and WBC. Plasma was used to quantify the levels of albumin, total protein, globulin, alanine aminotransferase, and bilirubin, changes in which may indicate altered hepatic and/or renal function.

Abbreviations: RET, reticulocytes; ALB, albumin; TP, total protein; GLOB, globulin; ALT, alanine aminotransferase; BILI, bilirubin.

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