Chemical Enhancers of Cytokine Signaling that Suppress Microfilament Turnover and Tumor Cell Growth

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Abstract

The transforming growth factor-β (TGF-β) family of cytokines regulates cell proliferation, morphogenesis, and specialized cell functions in metazoans. Herein, we screened a compound library for modifiers of TGF-β signaling in NMuMG epithelial cells using a cell-based assay to measure Smad2/3 nuclear translocation. We identified five enhancers of TGF-β signaling that share a core structure of diethyl 2-(anilinomethylene)-malonate (DAM), and D₅₀ values of 1 to 4 μmol/L. Taking advantage of the recently characterized Mga5 mutant phenotype of accelerated receptor loss to endocytosis, we determined that DAM-1976 restored the sensitivity of Mga5⁻/⁻ carcinoma cells to both TGF-β and epidermal growth factor (EGF). In Mga5 mutant and wild-type carcinoma cells, DAM-1976 enhanced and prolonged TGF-β- and EGF-dependent Smad2/3 and Erk activation, respectively. DAM-1976 reduced ligand-dependent EGF receptor endocytosis, actin microfilament turnover, and cell spreading, suggesting that the compound attenuates vesicular trafficking. Hyperactivation of intracellular signaling has the potential to suppress tumor cell growth and, in this regard, DAM-1976 represents a new pharmacophore that increases basal activation of Smad2/3 and Erk, inhibits microfilament remodeling, and suppresses carcinoma cell growth. (Cancer Res 2006; 66(7): 3558-66)

Introduction

The family of transforming growth factor-β (TGF-β) cytokines regulate cell cycle checkpoints, cell differentiation during embryogenesis, as well as matrix production and inflammation during postnatal life (1). Ligand activation of TGF-β receptor kinases (TβR) I and II heterodimers leads to phosphorylation of Smad2/3, recruitment of Smad4 (2), and translocation of these complexes into the nucleus where they bind various transcription factor complexes and regulate gene expression (3). TGF-β signaling stimulates expression of p21 and p27, which inhibit cyclin D/Cdk4 and the G₁-S cell cycle transition (4). Inactivating mutations in TβRⅡI and Smad4 genes promote expansion of premalignant cell populations. However, in late-stage cancers, TGF-β signaling promotes the invasive phenotype and cancer progression in cells that retain functional signaling machinery (5, 6). Epithelial-mesenchymal transition (EMT) in carcinoma cells is accompanied by loss of E-cadherin in adhesion junctions, membrane remodeling, and cell motility (7). EMT requires a balance between TGF-β/Smad2/3, and the antagonistic Ras/Erk and phosphatidylinositol 3-kinase (PI3K)/Akt oncogene pathways (8, 9). In this regard, TGF-β stimulates the expression of Pten phosphatase, a negative regulator of PI3K signaling (10). Cdk4 promotes G₁-S transition, in part by phosphorylating Smad2/3, which suppresses transcription of p21 (11). Smad2/3 binds to FOXO, also a negative regulator of cell cycle, whereas the PI3K/Akt pathway negatively regulates FOXO by blocking its transit into the nucleus (12). Therefore, chemical agents that control both Smad2/3 and Erk/PI3K pathways may have novel anticancer activities.

In postnatal life, TGF-β/Smad suppresses Erk/p38 kinase in activated leukocytes and suppresses inflammation (13). Interestingly, synthetic oleanane triterpenoids that act as suppressors of inflammation have shown to enhance TGF-β-dependent signaling (14). To identify additional enhancers of cytokine signaling, we developed a sensitive cell-based assay using quantitative immunofluorescence imaging to quantify Erk-p and Smad2/3 levels in the cytoplasm and the nucleus. We screened the Maybridge Diversity Set chemical library and identified five enhancers of TGF-β-dependent Smad2/3 nuclear translocation that share a core structure of diethyl 2-(anilinomethylene)malonate (DAM). In secondary assays to characterize the biological activity of DAM-1976, we took advantage of the recently characterized Mga5 mutation. Mga5 gene expression is up-regulated by Ras pathway activation (16, 17). We have shown that galectins bind to Mga5-modified N-glycans on glycoproteins, including the cytokine receptors (15). Mga5 gene expression is up-regulated by Ras pathway activation (16, 17). We have shown that galectins bind to Mga5-modified N-glycans on epidermal growth factor (EGF) receptor (EGFR) and TβR at the cell surface, which delays receptor loss to constitutive endocytosis and promotes sensitivity to cytokines (15). Tumor latency is longer and metastasis is reduced in polyomavirus middle T (PyMT) transgenic Mga5⁻/⁻ mice compared with PyMT transgenic Mga5⁻/⁺ mice (18). The PyMT Mga5⁻/⁻ mammary tumor cells display reduced sensitivity to multiple cytokines, including EGF, TGF-β, insulin-like growth factor, platelet-derived growth factor, and fibroblast growth factor (15). Here, we show that DAM-1976, a compound identified in our screen for modifiers of TGF-β signaling, rescues sensitivity to acute EGF and TGF-β in PyMT Mga5⁻/⁻ (229) cells, suggesting a mechanism of action that opposes membrane remodeling and endocytosis. In wild-type PyMT Mga5⁻/⁺ (2.6) tumors, DAM-1976 also enhances sensitivity to EGF and TGF-β, increases basal Erk and Smad2/3 activation, reduces microfilament remodeling, and selectively inhibits tumor cell proliferation.

Materials and Methods

Cell lines. Murine NMuMG epithelial cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in

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DMEM supplemented with 10% fetal bovine serum (FBS) and 10 μg/mL insulin (Life Technologies, Carlsbad, CA). Mammary tumor cell lines were established from spontaneous mammary carcinomas in mouse mammary tumor virus–PyMT transgenic mice on a 129sv/C2FVB background with either Mgat5+/+ or Mgat5−/− genotypes as previously described (18). The cell lines Mgat5+/+ (2.6) and Mgat5−/− (22.9) were generated from mammary tumors removed from littermate PyMT transgenic mice (15).

**Nuclear translocation assay.** NMuMG epithelial cells were plated in 96-well Corning tissue culture plates (Corning, Acton, MA) at a density of 5,000 per well in 100 μL DMEM, 10% FBS, and 10 μg/mL insulin (Life Technologies) for 24 hours. The medium was replaced with 100 μL TGF-β1 (R&D Systems, Minneapolis, MN) and Smad2/3 nuclear translocation was measured at the indicated times. Cells were either untreated or pretreated with 20 μM DAM-1976 for 2 hours, then stimulated with buffer or 50 μmol/L TGF-β for 45 minutes.

**High-throughput, cell-based screening assay.** For assay constancy, aliquots of NMuMG cells were stored in liquid nitrogen and expanded for 5 to 7 days immediately before their use in the screen. Cells were plated in 96-well plates manually, and cultured for 24 hours in DMEM plus 10% FBS. After a further 18 hours of serum starvation, the plates were placed on a fully automated system based on a ThermoCFS 205 robotic arm running on a 3-m rail. A Multimek96 outfitted with FPS100H slotted pins (V&P Scientific, Sunnyvale, CA) was used to transfer 200 nL of 5 mmol/L compound stocks dissolved in DMSO to the 50 μL cell culture. After 1-hour incubation with individual compounds at 10 μmol/L, TGF-β1-dependent Smad2/3 nuclear translocation was determined for 100 cells per well, generating a mean ± SE for each condition.

**Figure 1.** Identification of compounds that enhance TGF-β signaling. A, time course of Smad2/3 nuclear translocation in NMuMG mammary epithelial cells following the addition of TGF-β1. Cells were stained with anti-Smad2/3 antibodies, followed by a fluorescent-labeled secondary antibody as described in Materials and Methods. The nuclear and cytoplasmic staining intensity was determined individually for 100 cells per well, and nuclear minus cytoplasmic was used to represent the change in activation following addition of cytokine. Points, mean (n = 100); bars, SE (generally <4% for each assay point). B, sample of data from the primary compound screen for modifiers of Smad2/3 nuclear translocation in NMuMG cells. Points, mean nuclear translocation of Smad2/3 in well. C, dose responses for compound enhancement of TGF-β1-mediated Smad2/3 nuclear translocation in NMuMG cells. D50 values are listed in the box. D, time course of TGF-β1-dependent Smad2/3 nuclear translocation following compound sensitization of NMuMG cells. Cells were pretreated with DAM-1976 for 2 hours before addition of 50 μmol/L TGF-β1 and Smad2/3 nuclear translocation was measured at the indicated times. E, immunofluorescence images of NMuMG cells stained with anti-phospho-Smad2/3 antibodies. Cells were either untreated or pretreated with 20 μM DAM-1976 for 2 hours, then stimulated with buffer or 50 μmol/L TGF-β for 45 minutes.
final concentration) was added using a fixed tip multiprobe HT II. The 50 K compound library used in the screen was the Maybridge Diversity Set (Fisher, Hampton, NH). After incubating for 45 minutes with cytokine at 37°C, cells were fixed by the addition of 100 μL of 8% paraformaldehyde in PBS directly to the culture using a Multidrop 384. After 10 minutes, the cells were washed thrice with PBS (no Ca²⁺, no Mg²⁺) using a Biotek Elx405 Magna washer and immediately permeabilized for 2 minutes ± 5 seconds at 20°C by adding 100 μL of 100% methanol using the Multidrop 384, then washed thrice with PBS in the Biotek Elx405 Magna washer, and incubated with PBS plus 10% FBS for 1 hour at 20°C. Plates were then stored at 4°C until the start of the staining procedure 16 hours later, and this fully automated system was processed up to 28 plates per 8-hour shift. The automated staining of the cells was done as described for the manual method using the Biotek ELx405 washer and Multiprobe HTII. Following antibody staining, the plates were stored at 4°C in the dark for up to 48 hours until they could be read on the Cellomics Array Scan. The assay development procedure required careful attention to the stability of reagents, as well as each of the 15 operations on the robotic platform. The $Z'$ factor is defined as the ratio of separation band to dynamic range of the assay based on positive and negative control data in the assay. It takes the formula $Z' = 1 - \left[ \frac{3\sigma_{c+} + 3\sigma_{c-}}{\mu_{c+} - \mu_{c-}} \right]$ (19). The overall $Z'$ factor for the screen was 0.63.

**Immunoblot analysis.** Cells were lysed in TNTE [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, protease inhibitor cocktail (Sigma)] solution. Western blots were done with antibodies to phosphorylated Erk1/2 kinase (Thr202/Tyr204, Sigma) or to phosphotyrosine (4G10, Upstate, Chicago, IL).

**Immunofluorescent microscopy.** To monitor the adhesion junctions and actin microfilament, cells were grown in DMEM, 10% FBS on glass coverslips for 24 hours, and then treated with DAM-1976 for 24 to 48 hours. Cells were fixed with 3.7% formaldehyde for 15 minutes at 20°C and permeabilized with 0.2% Triton X-100 for 5 minutes at 20°C. Cells were blocked with PBS-10% FBS overnight at 4°C, incubated with 1:200 dilution of anti E-cadherin antibody (BD Biosciences, San Jose, CA) for 2 hours at 20°C. After three washes with PBS, cells were incubated with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin diluted 1:400 (Sigma) and a 1:2,000 dilution of Hoechst (Sigma).

### Table 1. Pharmacophore of DAM compounds identified in the screen for sensitizers of TGF-β1 signaling

<table>
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NOTE: The five compounds displayed activity as enhancers of TGF-β1-induced Smad2/3 nuclear translocation with $D_{50}$ of 1 to 4 μmol/L.
Cytotoxicity assay. Viable cell counts were measured using the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT)-based Cell Proliferation kit IIa (Roche, Mannheim, Germany), or with AlamarBlue (Sigma), following the suggested protocols. In brief, 1,000 cells per well were seeded in 96-well plates and cultured for 24 hours before addition of cytokine or no addition. For acute stimulation with TGF-β (50 pmol/L) or EGF (100 ng/mL), measurements of nuclear Smad2/3 at 45 minutes and nuclear Erk-p at 5 minutes, respectively, are shown. E, Mgtat5+/+(2.6) and Mgtat5−/−(22.9) cells treated with DAM-1976 for 48 hours in DMEM + 10% FCS, and stained for E-cadherin (green) and actin microfilaments with rhodamine-phalloidin (red).

Cell spreading. Cells were plated at 1,000 per well onto 96-well plates coated with 0.5 μg/well of fibronectin (Sigma). After 4-hour incubation at 37°C in serum-free DMEM, cells were fixed and stained with TRITC-phalloidin. Cell area was quantified by Array Scan and expressed as the mean ± SE of 100 cells per well. To measure microfilament turnover, cells growing in DMEM plus 10% FBS were treated with 100 μg/mL Latrunculin-A, an actin monomer-binding drug that renders the monomers incompetent for filament formation. At times after treatment, the cell area and microfilament density was measured following staining with TRITC-phalloidin.

Distribution of cytokine receptors. To measure surface EGFR, proteins were biotinylated by incubation with 0.5 mg/mL sulfo-NHS-LC-biotin (Pierce, Rockford, IL) in PBS (pH 8.0) for 1 hour at 4°C. Cells were lysed in TNTE [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, and Protease Inhibitor Cocktail (Sigma)]. Aliquots of cell lysate were immunoprecipitated with anti-EGFR antibody (Sigma), and biotinylated surface proteins were captured on streptavidin-agarose beads. Proteins were separated by SDS-PAGE and probed with anti-EGFR (Santa Cruz Biotechnology, Santa Cruz, CA) antibody.

Results

Identification of TGF-β signaling enhancers in a cell-based assay. To identify chemical modifiers of TGF-β signaling, we used fluorescence imaging combined with an automated system to quantify cytoplasmic and nuclear Smad2/3. The algorithm calculates the difference between mean nuclear and cytoplasmic
staining, and the method is more sensitive than conventional Western blotting (15). NMuMG mammary epithelial cells were grown in low serum conditions for 24 hours and stimulated with TGF-β1 to determine the optimal time and dose of cytokine for the screen. The cells displayed a 10-fold increase in nuclear Smad2/3 protein 20 minutes after addition of cytokine, which was sustained until 50 minutes, then declined thereafter with an apparent half-life of ~30 minutes (Fig. 1A). A dose of 50 pmol/L TGF-β produced ~80% saturation of Smad2/3 nuclear translocation. TGF-β did not stimulate Erk-p nuclear translocation and EGF did not stimulate Smad2/3 nuclear translocation, demonstrating specificity of the assays (data not shown). Our expectation was that chemical enhancers or inhibitors of Smad2/3 nuclear translocation would interact with a rate-limiting component of the signaling pathway.

We initially identified three compounds that enhanced TGF-β1-induced nuclear translocation of Smad2/3 in the first 10,000 of the 50,000 compounds in Maybridge Diversity set (Fig. 1B). Our primary hit rate for enhancers and inhibitors was 0.1%, and the confirmed hit rate upon retesting was 0.03%. Five enhancers displayed D50 values of 1 to 4 μmol/L (Fig. 1C), and shared a core chemical structure of diethyl DAM (Table 1). After an in silico structural screen of the rest of the library, we selected a subset of plates containing structurally related compounds and identified two additional hits (Fig. 1; Table 1). DAM-1976, with an EC50 of ~1 μmol/L, was selected for further characterization. DAM-1976 pretreatment of NMuMG cells enhanced maximum nuclear Smad2/3 levels after TGF-β stimulation and prolonged Smad2/3 nuclear residency (Fig. 1D). The effect of the DAM compounds on Smad2/3 phosphorylation following TGF-β stimulation was visualized in NMuMG cells by immunofluorescence microscopy (Fig. 1E).

**DAM-1976 sensitizes cells to EGF and autocrine TGF-β.** To determine whether DAM-1976 enhanced TGF-β1 signaling specifically or sensitized cells to multiple cytokines, we pretreated mammary carcinoma cells with the drug and compared sensitivity to EGF and TGF-β. DAM-1976 enhanced the sensitivity of cells to both cytokines with similar D50 values (Fig. 2A-D). Because EGF and TGF-β signaling pathways are distinct, it seems that DAM-1976 acts on a target that broadly regulates cytokine sensitivity, possibly at the level of receptor availability. Consistent with this suggestion, DAM-1976 pretreatment enhanced the sensitivity of Mgsat5+/+ (2.6) and Mgsat5−/− (22.9) tumor cells to EGF and TGF-β. The Mgsat5−/− (22.9) cells are deficient in retention of cytokine receptors at the cell surface, and consequently poorly responsive to TGF-β and EGF (15). Pretreatment of Mgsat5−/− (22.9) cells with DAM-1976 essentially rescues sensitivity to acute TGF-β and EGF (Fig. 2C and D). The initial characterization of the Mgsat5−/− defect showed that cytokine signaling could be rescued by blocking endocytosis with a 1-hour treatment of nystatin and K+ depletion (15). Nystatin disperses membrane cholesterol and disrupts caveolae-dependent endocytosis, whereas K+ depletion disrupts clathrin-assembly and coated-pits endocytosis. DAM-1976 may also disrupt receptor trafficking, causing a sustained increase in surface levels that delivers greater intracellular activation of transducers.

Mgsat5+/+ (22.9) tumor cells express the PI3K oncoprotein, which drives PI3K and Erk signaling, but the cells fail to undergo EMT as indicated by loss of E-cadherin in adhesion junctions.

![Figure 3. DAM-1976 delays EGFR internalization and prolongs Erk-p activation.](image-url)

- **A**, nuclear translocation of Erk1/2 in Mgsat5+/+ (2.6) cells pretreated with DAM-1976 for 2 hours and stimulated with EGF.
- **B**, phosphorylation of EGFR and Erk1/2 following stimulation with 100 ng/mL EGF for 10 minutes with and without DAM-1976 (5 μmol/L) pretreatment for 2 hours. Surface proteins were biotinylated with Sulfo-NHS-LC-biotin, captured on streptavidin-agarose beads, separated by SDS-PAGE, and probed with anti-EGFR antibodies. **C**, surface EGFR biotinylation at times after stimulation of Mgsat5+/+ (2.6) cells with EGF. Densitometry of Western blots following streptavidin-agarose pull-downs and probing with anti-EGFR. The data normalized to time 0 untreated.
Transfection of the mutant cells with an Mgat5 expression vector restored autocrine TGF-β signaling and EMT (15). DAM-1976 treatment alone increased basal Smad2/3 nuclear localization, indicating an enhancement of autocrine TGF-β signaling (Fig. 2A and C). Furthermore, DAM-1976 treatment of Mgat5−/− (22.9) cells induced loss of E-cadherin in adhesion junctions and redistribution of microfilaments from a cortical to a basolateral position (Fig. 2E). However, the microfilaments were disorganized and punctate, indicating shorter and branched forms of filamentous actin (F-actin). DAM-1976 treatment of NMuMG cells also disrupted adhesion junction and increased basolateral F-actin, a phenotype similar to TGF-β treatment of these cells (data not shown).

DAM-1976 slows endosomal trafficking and prolongs Erk activation. Next, we explored the possibility that DAM-1976 prolongs the activation state of signaling intermediates by slowing endocytosis and trafficking of signaling intermediates. Maximal Erk-p translocation to the nucleus occurs ~5 minutes after EGF addition and declines rapidly thereafter (Fig. 3A). DAM-1976 pretreatment of Mgat5−/− (2.6) cells enhanced peak levels of nuclear Erk-p and slowed the return to baseline (Fig. 3A). The levels of Erk-p were markedly higher in DAM-1976 pretreated cells 10 minutes after EGF stimulation compared with untreated cells, indicating a prolongation of receptor signaling (Fig. 3B). Activation of the EGFR stimulates its internalization via clathrin-coated pits and inactivation in the endosomes (20, 21). To measure the effect of DAM-1976 on EGFR endocytosis, Mgat5+/+ (2.6) cells were stimulated with EGF and, at times thereafter, surface labeled with sulfo-NHS-LC-biotin (Fig. 3C). Biotin-labeled EGFR was internalized at a 3-fold reduced rate in DAM-1976-pretreated cells compared with untreated cells (Fig. 3D). This suggests that DAM-1976 delays endocytosis of activated EGFR and thereby prolonged ligand-dependent receptor signaling. DAM-1976 induced a modest ligand-independent increase in nuclear Erk-p and Smad2/3 in Mgat5+/+ (2.6) cells, suggesting the compound sensitizes cells to autocrine stimulation.

DAM-1976 inhibits microfilament remodeling and slows tumor cell growth. The microfilament assembly in Mgat5+/+ (2.6) is largely cortical, whereas that of Mgat5−/− (2.6) cells span the substratum typical of the epithelial and mesenchymal...
phenotypes, respectively (Fig. 2E). In Mgat5−/− cells, DAM-1976 treatment increased the basolateral microfilament content and reduced cortical stress fibers, producing a phenotype similar to Mgat5+/+ (2.6) cells, either with or without drug (Fig. 2E). The basolateral microfilaments in Mgat5−/− (2.6) cells disassemble rapidly in the presence of latrunculin-A (LatA), an inhibitor of actin repolymerization, whereas LatA showed little effect in Mgat5+/+ tumor cells where basolateral filaments were constitutively very low (Fig. 4A). DAM-1976 inhibited LatA-dependent decay of basolateral microfilaments in Mgat5−/− (2.6) cells, indicating that DAM-1976 slows depolymerization of basal F-actin (Fig. 4B). Furthermore, DAM-1976 inhibited LatA-dependent retraction of Mgat5−/− (2.6) cells on fibronectin by 70%, but only by 10% for Mgat5−/− (22.9) cells and 20% for nonmalignant NMuMG cells (Fig. 4D).

DAM-1976 reduced Mgat5+/+ (2.6) and Mgat5−/− (22.9) tumor growth in tissue culture, but not that of nontransformed NMuMG epithelial cells under the same conditions (Fig. 4E). DAM-1976 treatment of Mgat5−/− (2.6) displayed a dose-dependent increase in G2-M and S phase cells, indicating that the compound delays cell cycle progression (Fig. 4F). To further explore the mechanism of DAM-1976-dependent inhibition of carcinoma cell growth, we cultured cells for 72 hours in the presence of increasing concentrations of either TGF-β or DAM-1976 and measured cell number and basal nuclear Erk-p and Smad2-p. Mgat5+/+ (2.6) tumor cells were compared with nontransformed MvLu epithelial cells and DR26 cells, an MvLu mutant cell line that lacks TβRII (22). TGF-β inhibited MvLu cell growth but not that of DR26 or Mgat5+/+ (2.6) cells (Fig. 4A). In contrast, DAM-1976 reduced Mgat5+/+ (2.6) cell growth, whereas MvLu and DR26 cells were less severely inhibited.

Figure 5. DAM-1976 inhibits tumor cell growth and stimulates basal Erk-p and Smad 2-p. Effects of (A) TGF-β and (B) DAM-1976 on cell number after 72 hours of growth in DMEM, 1% FBS measured by the AlamarBlue homogenous assay. Steady-state levels of (C and D) nuclear Smad 2-p (E and F) nuclear Erk-p in cells after 72 hours of growth in MEM, 1% FBS. DAM-1976 induced a greater loss of substratum attachment and death of Mgat5−/− (2.6) carcinoma cells compared with MvLu and DR26 cells, hence the absence of Erk-p and Smad 2-p data at >10 μmol/L. Representative of three experiments.
inhibited, suggesting a different profile of growth suppression than TGF-β (Fig. 5B). Basal levels of nuclear Smad2-p were higher in MvLu cells and increased slightly in the presence of TGF-β, whereas basal levels in Mgtat5+/− (2.6) cells and DR26 cells were lower and not altered by culturing with TGF-β (Fig. 5C). Nuclear Smad2-p increased in all of the cell lines cultured with DAM-1976, including DR26 cells (Fig. 5D). This suggests that DAM-1976 may enhance nuclear Smad2-p by a TGF-β-independent mechanism, as well as sensitizing the cells to acute TGF-β signaling (Fig. 2). TGF-β and DAM-1976 enhanced nuclear Erk-p in Mgtat5+/− (2.6) cells, whereas a decline or no change was observed for MvLu and DR26 cells (Fig. 5E and F). Therefore, increases in both Erk-p and Smad2/3 activation accompany selective growth suppression of tumor cells by DAM-1976. The invasive phenotype requires activation of Ras/Erk, PI3K/Akt growth pathways, and autocrine TGF-β/Smad2/3 signaling (8, 9). However, hyperactivated Ras/Erk signaling becomes toxic in tumor cells (23), and Smad2/3 signaling is well known to promote cell cycle arrest (10, 11, 24). Therefore, it seems likely that changes in these and other signaling pathways in DAM-1976-treated carcinoma cells may contribute to growth suppression.

Discussion

Five compounds sharing a core structure of DAM were isolated from the Maybridge Diversity Set chemical library as enhancers of TGF-β-dependent Smad2/3 nuclear translocation. These compounds access their targets in cells with D50 values of 1 to 4 μmol/L. Characterization of DAM-1976 activity suggests that these compounds inhibit microfilament remodeling and enhance sensitivity to cytokines by prolonging the trafficking and activation of signaling intermediates.

Oncogenic signaling downstream of receptor tyrosine kinases promotes vesicular trafficking and endocytosis (25). However, increases in membrane and microfilament remodeling in motile cells are balanced by mechanisms that protect cytokine receptors at the cell surface. In this regard, positive feedback from Ras/Erk signaling stimulates expression of the Mgtat5 gene (17), which strengthens galectin-glycoprotein cross-linking and retains surface cytokine receptors in invasive tumor cells (15). Mgtat5+/− (22.9) cells are deficient in cell surface receptors, and blocking endocytosis restores their sensitivity to EGF and TGF-β. DAM-1976 pretreatment of Mgtat5−/− (22.9) tumor cells showed a chemical rescue of this phenotype, and also enhanced responsiveness to EGF and TGF-β in Mgtat5−/− (2.6) cells. DAM-1976 enhanced autocrine TGF-β signaling as well as loss of E-cadherin in adherent junctions. However, DAM-1976 did not rescue cell spreading in Mgtat5−/− (22.9) cells, but rather inhibited spreading of the wild-type cells. This is consistent with the observations that although DAM-1976 enhances surface residency of receptors and sensitivity to cytokines, this may be secondary to the slowing of microfilament remodeling.

EGFR activation recruits Grb-2/She signaling complexes, but also Cbl, CIN85, and endophilins, which stimulate endocytosis and inactivation of EGFR in endosomes (20, 21). In contrast, TGF-β does not stimulate TβR endocytosis, but rather receptors are internalized at bulk endocytosis rates (26). Therefore the target(s) of DAM-1976 are likely to be mediators of microfilament turnover and/or vesicular trafficking, which affect receptor trafficking both before and after ligand binding. Receptor internalization and vesicular trafficking requires the Rab family of small GTPases and adaptor proteins (25). Activation of Arp2/3 complexes at the cell cortex stimulate actin polymerization required for endocytosis and cell shape changes (27). The cofilin/ADF protein accelerates actin filament turnover, and neural crest cells lacking n-cofilin display defects in F-actin bundling, cell polarization, and cell proliferation. The state of actin polymerization also regulates serum response factor (SRF) transcription factor activity and gene expression. Rho signaling induces F-actin polymerization and treadmilling, which reduces the pool of G-actin, releasing bound myocardin-related SRF coactivator (MAL) to translocate into the nucleus and activate SRF-dependent transcription (28). SRF forms regulatory complexes with Ets proteins and cooperates with Erk activation to stimulate early response genes. Agents that decrease actin treadmilling and MAL/SRF activity suppress early response gene expression. LatA blocks actin remodeling and increases G-actin levels, which retains MAL in the cytoplasm (29). DAM-1976 slows F-actin polymerization and actin treadmilling, which, in a similar manner, may disrupt cell proliferation through changes in MAL/SRF activity.

Screens for chemical inhibitors of cytokine signaling, such as EGFR/ErbB, have yielded new anticancer agents (30). Herein, we have taken a different approach; noting that hyperactivation of intracellular signaling can be selectively toxic to tumor cells, we identified chemical enhancers of cytokine signaling. Tumor cells treated with enhancer DAM-1976 show stabilized microfilaments, slower endocytosis of EGFR, and increased sensitivity to EGF and TGF-β cytokines. Tumor cells cultured with DAM-1976 display increases in basal nuclear Erk-p and Smad2/3, which, together with other changes in nuclear signal transducers, may contribute to growth suppression. In summary, the DAM compounds represent a new pharmacophore, with a novel activity profile, but further work is required to determine the molecular targets and clinical potential of the compounds.

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References

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