RG7212 Anti-TWEAK mAb Inhibits Tumor Growth through Inhibition of Tumor Cell Proliferation and Survival Signaling and by Enhancing the Host Antitumor Immune Response

Xuefeng Yin1, Leopoldo Luistro1, Hua Zhong1, Melissa Smith1, Tom Nevins1, Kathleen Schostack1, Holly Hilton1, Tai-An Lin1, Theresa Truitt1, Denise Biondi1, Xiaqian Wang1, Kathryn Packman1, Jim Rosinski1, Windy Berkofsky-Fessler1, Jian-Ping Tang1, Saumya Pant1, David Geho1, Suzana Vega-Harring2, Mark DeMario1, Hy Levitsky3, and Mary Simcox1

Abstract

Purpose: To explore the role of TWEAK in tumor growth and antitumor immune response and the activity and mechanism of RG7212, an antagonistic anti-TWEAK antibody, in tumor models.

Experimental Design: TWEAK-induced signaling and gene expression were explored in tumor cell lines and inhibition of these effects and antitumor efficacy with RG7212 treatment was assessed in human tumor xenograft-, patient-derived xenograft, and syngeneic tumor models and phase I patients. Genetic features correlated with antitumor activity were characterized.

Results: In tumor cell lines, TWEAK induces proliferation, survival, and NF-κB signaling and gene expression that promote tumor growth and suppress antitumor immune responses. TWEAK-inducible CD274, CCL2, CXCL-10 and -11 modulate T-cell and monocyte recruitment, T-cell activation, and macrophage differentiation. These factors and TWEAK-induced signaling were decreased, and tumor, blood, and spleen immune cell composition was altered with RG7212 treatment in mice. RG7212 inhibits tumor growth in vivo in models with TWEAK receptor, Fn14, expression, and markers of pathway activation. In phase I testing, signs of tumor shrinkage and stable disease were observed without dose-limiting toxicity. In a patient with advanced, Fn14-positive, malignant melanoma with evidence of tumor regression, proliferation markers were dramatically reduced, tumor T-cell infiltration increased, and tumor macrophage content decreased. Antitumor activity, a lack of toxicity in humans and animals and no evidence of antagonism with standard of care or targeted agents in mice, suggests that RG7212 is a promising agent for use in combination therapies in patients with Fn14-positive tumors. Clin Cancer Res; 19(20); 1–13. ©2013 AACR.

Introduction

TWEAK and the TWEAK receptor, Fn14, are upregulated in cancer, and TWEAK-induced signaling promotes multiple processes known to contribute to tumor growth. This TNF superfamily ligand has been described as mediating diverse effects ranging from proliferation to apoptosis. Pathway upregulation in cancer suggests a tumor-promoting function and a rationale for inhibition as a therapeutic strategy. Reports of TWEAK-induced apoptosis in tumor cell lines argue against this. We therefore explored the biology of TWEAK by evaluating signaling, gene expression, and phenotypic changes in TWEAK-stimulated cells and the effect of a TWEAK blockade on tumor growth in mice. TWEAK mediates signaling through Fn14 to promote inflammation (1), cell proliferation (2–5), cell survival (6), and angiogenesis (7). TWEAK mRNA is detected in a wide range of cell types (8), and TWEAK protein has been shown to be expressed by inflammatory and tumor cells (9–11). Fn14 is expressed in multiple cell types, but expression levels are low in normal cells and have been shown to increase with tissue injury, in inflammatory diseases, and in cancer (12). Pathway expression has been associated with poor outcome in patients with cancer. TWEAK and Fn14 expression are negatively correlated with patient overall and disease-free survival in renal cell carcinoma (RCC; ref. 13), and Fn14 is a negative prognostic factor in breast cancer (14), gastric cancer (15), and glioma (16). TWEAK and Fn14 are expressed in neuroblastoma cell lines and primary tumors, and increased levels of both are observed in high-stage
Translational Relevance

A blockade of TWEAK signaling with anti-TWEAK mAb, RG7212, inhibits tumor growth in models with Fn14 expression and pathway activation. RG7212 decreased proliferation and serum immunomodulatory cytokines and chemokines, increased tumor apoptosis, and altered immune cell distribution in mice. Similar pharmacodynamic changes, including decreases in tumor pERK and altered tumor immune cell content, were observed in patients with evidence of tumor shrinkage in phase I clinical testing.

tumors (17). In vitro, TWEAK stimulation of neuroblastoma cell lines increased survival, partially via activation of NF-κB signaling, and induced the release of matrix metalloprotease-9 (MMP-9), suggesting that TWEAK may play a role in invasiveness in neuroblastoma. Silencing of TWEAK and Fn14 using siRNA significantly reduced survival. Fn14 upregulation can occur as a consequence of overexpression (14) or activating mutations in oncogenic receptor tyrosine kinases (18).

TWEAK induces proliferation and survival signaling in tumor and endothelial cells via mitogen-activated kinases and canonical and noncanonical NF-κB pathways associated with tumor growth and survival. Here, we show these effects in tumor cell lines and that a TWEAK blockade with an anti-TWEAK mAb, RG7212, results in significant tumor growth inhibition (TGI) in multiple tumor models expressing Fn14. These in vitro and in vivo data are consistent with tumor-promoting effects shown with other TNF family receptors lacking death domains (19, 20) and with TWEAK data in disease models in animals and pathologic conditions in humans (21, 22).

In contrast to data showing TWEAK proinflammatory, proangiogenic, and proliferation-inducing effects in disease models in animals (5, 23–25) and association of pathway activation with human disease (21, 22, 26–28), some published in vitro studies have shown that TWEAK induces apoptosis via upregulation of TNFα (29) or in combination with IFNγ in a small number of tumor cell lines (8). In the absence of IFNγ, apoptosis induction was modest (30) or required relatively high TWEAK concentrations (29). In vivo, surprisingly both RG7212 and agonistic anti-Fn14 antibodies result in tumor growth inhibition in xenograft tumor models. RG7212, although sharing complex pathway biology with these agents, is herein characterized as a pathway antagonist. RG7212 antitumor activity is accompanied by downmodulation of TWEAK-induced proliferation and survival signaling, decreased transcription of proinflammatory, mitogenic, survival and immunomodulatory genes and by altered tumor immune cell composition. Although TWEAK induced changes in multiple pathways, the dominant effect is on NF-κB signaling, inducing changes in genes encoding secreted mitogens and immunomodulatory cytokines and receptors. These dominant effects point to a mechanism of action of RG7212 that involves reconfiguration of the tumor microenvironment to promote tumor growth and escape from immune destruction. These pharmacodynamic effects in mice have also been observed in patients in a phase I study.

Antitumor efficacy has been shown in mice with agonistic anti-Fn14 antibodies, BIIB036 and PDL192 (enavatuzumab, ABT-361; refs. 12, 29, 30). These antibodies have distinct properties, but each shows a complex mechanism of action including engagement of Fc receptors resulting in receptor clustering effects and eliciting antibody-dependent cell-mediated cytotoxicity (ADCC) and activation of NF-κB signaling (12, 16, 29, 31). In contrast, although pathway biology is complex due to TWEAK activation of multiple downstream signaling pathways and pathway expression in multiple cell types, RG7212 acts by simply antagonizing TWEAK/Fn14 signaling. Here, we show that neither TWEAK nor RG7212 treatment impacted viability in 292 tumor cell lines.

In malignancies, constitutive activation of NF-κB with induction of survival and inflammation-promoting genes is a major event leading to the initiation and progression of cancer (32).

Anti-Fn14 antibodies result in elevation of undesirable proinflammatory cytokines that are associated with human pathology, including the development of pancreatitis (32–34). In the phase I study, pancreatitis was observed in patients treated with PDL192 at a dose that achieved the drug exposure target for antitumor efficacy in mice. No complete or partial responses and 2 patients with stable disease were reported.

We show that these cytokines are induced by TWEAK in vitro and that RG7212, in contrast to PDL192, decreases these cytokines in the serum and tumors of treated mice. Importantly, RG7212-induced cytokine changes are observed in tumor models where complete TGI is observed. BIIB036 has also shown antitumor efficacy in mice that is dependent on full Fc effector function and is observed regardless of tumor cell line sensitivity to the antibody in vitro (29) with minimal cytotoxicity only in combination with IFNγ. In summary, both Fn14 antibodies have shown that in vivo efficacy is at least partially mediated by ADCC and neither has shown significant cytotoxicity in vitro in models where antitumor activity was observed when grown as xenografts in mice, arguing direct cell killing is not the dominant mechanism of action. Expression of the pathway in nontumor cells, including immune cells (9), and modulation of immunomodulatory cytokines and chemokines with tumor pathway modulation by either a TWEAK or Fn14 antibody likely contribute to the mechanism of action of these agents.

TWEAK signaling promotes cell survival through induction of AKT phosphorylation (31) and activation of NF-κB leading to upregulation of anti-apoptotic genes including BCL2L1 (BCLXL) and BCL2L2 (BCLW) (6). TWEAK induces mitogen-activated protein kinase (MAPK) signaling in tumor and endothelial cells (32). TWEAK has been shown to induce endothelial cell proliferation and tube
formation in vitro (2) and to promote angiogenesis (33) and tumor growth (34) in vivo. TWEAK plays a role in innate and adaptive immune antitumor response. Its absence in TWEAK-knockout mice was shown to enhance both innate and adaptive immune responses to tumor challenge (35).

As the biology of TWEAK in tumor cells is controversial and has not been broadly profiled, we examined signaling and gene expression changes in a panel of tumor cell lines, investigating the impact of TWEAK on viability and gene expression changes in a panel of tumor cell lines, investigating the impact of TWEAK on viability in vitro. The reversal of these TWEAK-induced effects and tumor growth inhibition with an antibody blockade was then explored in vivo in xenograft models in immunodeficient and in transplanted tumors in immunocompetent mice. Because of the potential complexity of host and tumor cell interactions in mouse tumor models, we immunized hamsters to generate a fully cross-reactive antibody directed against soluble TWEAK that was humanized to produce RG7212. The impact of immune function on antitumor efficacy was explored by using a range of mouse genetic backgrounds and by testing the effects of specific immune cell depletion on antibody activity. RG7212 acts as a TWEAK antagonist, blocking TWEAK-induced signaling and cytokine production, promoting apoptosis, altering immune cell distribution, and inhibiting tumor growth without toxicity in mice. In phase I testing, evidence of tumor regression, with pharmacodynamic changes consistent with those observed in mice and without toxicity, was observed in patients (36).

Materials and Methods
Detailed experimental procedures are available in Supplementary Experimental Procedures.

Cell lines
Human U2OS, HCT116, ACHN, AsPC1, Calu-3, Caki-1, AsPC-1, Panc-1, SJA, SK-MES-1, MDA-MB-231, KPL4, U118MG, MDA-MB-436, HEC1A, 22Rv1 and murine Lewis Lung carcinoma (LLC1) were purchased from the American Type Culture Collection and identities were confirmed via supplementary Experimental Procedures. Human U2OS, HCT116, ACHN, AsPC1, Calu-3, Caki-1, AsPC-1, Panc-1, SJA, SK-MES-1, MDA-MB-231, KPL4, U118MG, MDA-MB-436, HEC1A, 22Rv1 and murine Lewis Lung carcinoma (LLC1) were purchased from the American Type Culture Collection and identities were confirmed via supplementary Experimental Procedures.

Mice
Athymic nu/nu (nude) mice were purchased from Charles River Laboratories, and SCID-beige mice were from Taconic Farms. C57BL/6J and Balb/c mice were obtained from Jackson Laboratory. Mice used in studies were approximately 6 to 8 weeks old. All procedures conducted according to protocols approved by the Roche Animal Care and Use Committee.

Generation of anti-TWEAK mAbs and ELISAs
RG7212 was generated using conventional immunization and hybridoma screening techniques. ELISA details (TWEAK binding, TWEAK:Fn14 blocking, TWEAK quantitation, cytokine, and cleaved cytokeratin) are described in Supplementary Information.

Gene expression profiling
Affymetrix gene array and PCR array studies described in Supplementary Experimental Procedures.

Profiling of immune cells in mouse spleen, whole blood, and Pan02 tumors
Single-cell suspensions were prepared from spleen and mouse tumors using gentleMACS dissociator and corresponding kits following manufacturer’s recommendations (Miltenyi Biotec). Dissociated cells and leukocytes were incubated with fluorescence-labeled antibodies followed by washes using PBS containing 0.5% bovine serum albumin (BSA). Labeled cells were fixed in 1% formaldehyde solution, and the differential cell populations were analyzed using the imaging flow cytometer ImageStreamX (Amnis Corporation). Antibodies are listed in Supplementary Information.

Tumor and tumor cell lysates
Protein lysate preparation for immunoblotting and ELISA and antibodies used are in Supplementary Information.

Statistical analysis
In all studies, values are expressed as mean ± SEM or box plot summaries, as indicated. Statistical analyses were conducted by unpaired Student t test, or Mann–Whitney U test, or linear regressions, as indicated. Differences were considered statistically significant at P < 0.05. Additional details can be found in the Supplementary Experimental Procedures.

Results
Anti-TWEAK mAb properties
RG7212 was generated using conventional immunization and hybridoma screening techniques (Supplementary Methods). As cross-reactive antibodies were of interest for modeling host and tumor-derived TWEAK effects in mice, Armenian hamsters were immunized with human soluble TWEAK, followed by a boost with soluble murine TWEAK. A hamster antibody with high affinity and strong neutralizing activity was selected for generation of a chimeric antibody. RG7212, a humanized IgG1 mAb, was generated from the chimeric antibody using standard humanization methodologies. mRG7212 is a murine IgG2a chimeric version of RG7212. Four IgG1 monoclonal anti-TWEAK mAbs were used in these studies. Identical binding and neutralization properties are shown in Supplementary Table S1. For simplicity, mAbs in this report are designated RG7212 and mRG7212.

RG7212 is a fully cross-reactive, binding human, rat, and murine TWEAK with high affinity (Fig. 1A and B) and blocking TWEAK binding to Fn14 and TWEAK-induced proliferation and cytokine secretion (Fig. 1B). The amino acid sequence of Cynomolgus monkey TWEAK is identical
to human TWEAK. Human TWEAK binds mouse Fn14 (34) and mouse TWEAK binds to human Fn14 (Fig. 1C). Cross-functionality and the cross-reactivity of RG7212 support modeling of the host- and tumor-derived TWEAK biology in animal studies. A RG7212 pharmacokinetic study in ACHN-tumor bearing mice was conducted for use in combination with efficacy data to estimate the target drug exposure resulting in an antitumor effect (Fig. 1D). In safety studies, animals were dosed to achieve exposures exceeding the drug levels required for antitumor efficacy, based on single and multiple dose-level pharmacokinetic (PK) studies, monkeys and rats were selected for toxicology studies based on RG7212 cross-reactivity and health authority guidelines for rodent and non-rodent species selection. In repeat dose good laboratory practice (GLP) studies, intravenous administration of RG7212 at doses up to 100 mg/kg/wk [rats; area under curve (AUC) 204 mg.h/mL, Cmax 2,670 µg/mL] and 50 mg/kg/wk (monkeys; AUC 211 mg.h/mL, Cmax 2,110 µg/mL) for 13 weeks was well-tolerated, with no test article-related findings. These results suggested a favorable safety profile for pursuing RG7212 in clinical studies.

**RG7212 inhibits growth in human tumor xenograft, transplanted syngeneic mouse tumor, and patient-derived tumor graft models**

RG7212 antitumor efficacy was shown in multiple tumor models, including cell line- and patient-derived human xenografts and transplanted mouse tumor xenograft models (Supplementary Table S2). Complete tumor growth inhibition to regression was observed in ACHN (RCC), MDA-MB-231 (breast), Caki-1 (RCC), and Calu-3 (non–small cell lung cancer, NSCLC) models with the highest level of Fn14 expression and detectable TWEAK expression (Fig. 2A–D and Supplementary Table S2). The antitumor effect of RG7212 is dose-dependent after twice weekly administration. RG7212 treatment resulted in statistically significant TGI with biweekly dosing (Fig. 2A). Together with the pharmacokinetic data from the same study (Fig. 1D), the estimated average steady state concentration (Cavg) and estimated trough concentration to achieve a 90% antitumor activity are 261 ± 88.2 µg/mL and 167 ± 56.8 µg/mL, respectively.

No antitumor activity was observed with RG7212 treatment in either 22Rv1 prostate or KPL4 breast cancer models.
lacking Fn14 expression (Fig. 2D and Supplementary Table S2), nor was RG7212 active in HEC1A human endometrial carcinoma xenografts (Supplementary Fig. S1A), a cell line reported to undergo TWEAK-induced apoptosis in vitro (29). RG7212 did not inhibit and, more importantly, did not accelerate tumor growth as might be expected with a TWEAK blockade if TWEAK signaling was a negative regulator of tumor growth.

RG7212 antitumor efficacy was assessed in additional xenograft models (Supplementary Table S2) with a range of Fn14 expression (Supplementary Fig. S1B), including evaluation of the activity of murine RG7212 in 5 transplanted, syngeneic tumor models (Supplementary Fig. S1C). Significant antitumor activity was observed in syngeneic Pan02 and RENCA, models with the highest level of Fn14 expression (Supplementary Fig. S1D). Activity in the aggressive Pan02 model was modest but comparable to the best response observed with standard-of-care cytotoxic agent gemcitabine (37) or immunomodulating agents (38). Tumor growth and tumor-induced immunosuppression is multifactorial and single-agent therapies are unlikely to be curative, but RG7212 monotherapy activity and lack of toxicity suggest that combination therapies should be explored.

Combination studies were conducted in Fn14-expressing syngeneic, cell line- and patient-derived tumor models (Supplementary Table S2). In Pan02 tumors, mRG7212 increased the level of antitumor efficacy of gemcitabine from 51% to 67% TGI \((P < 0.05; \text{Supplementary Fig. S1E})\). RG7212 increased the activity of cisplatin from 33% TGI \((P < 0.05)\) to 61% \((P < 0.05)\) in patient-derived xenograft (PDX; Champions Oncology, Inc.) PULM009 (Supplementary Fig. S1F) and increased the activity of cisplatin from 34% to 50% TGI \((P > 0.05)\) in the paclitaxel-resistant PDX ST013 ovarian model.

In cell line–derived xenograft models, addition of RG7212 did not antagonize standard of care agents or targeted therapies (Supplementary Table S2). In Calu-3, combination of RG7212 with cisplatin, B20 4.1 (cross-reactive anti-VEGF mAb), or both increased antitumor efficacy compared to the monotherapy treatments. Docetaxel efficacy was increased in both the MDA-MB-436 breast and Calu-3 models when combined with RG7212.
Monotherapy and combination therapies, where combination agents were dosed at the respective maximum tolerated dose, were well tolerated with no significant body weight loss (≥20%, P < 0.05) or evidence of gross toxicity. Exome sequencing data showed that RG7212 was active in models from cells expressing either mutant or wild-type BRAF, CDKN2A, KRAS, TP53, and TRAF2 (Supplementary Fig. S1G). Mutations were not identified in TWEAK or Fn14 genes in responders or nonresponders. All responsive models express wild-type MAP3K14 (NIK), and a mutation (T763A) was found in nonresponder AsPC1. All models assessed in vivo expressed wild-type PIK3CA. Lack of activity in models was not correlated with mutations in CD40, LTBR, TRAF3, or TAC1, associated with constitutively activated NF-xB (39). A TRAF2 mutation of uncertain functional significance was identified in responder Caki-1. Expression analysis suggests that downstream pathway expression and activation markers may be useful in addition to Fn14 in predicting antitumor efficacy.

Quantitative PCR was used to characterize cell lines that respond, or are refractory, to RG7212 treatment when grown as xenografts in mice. mRNA expression with and without TWEAK stimulation was compared using a custom PCR array (Supplementary Fig. S2A) that included NF-xB genes and TWEAK- modulated genes. Additional candidate response prediction markers significantly elevated at baseline in responders were: IKBKG (NEMO), MAP3K14 (NIK), STAT3, STAT5B, and TRAF1 (Supplementary Fig. S2B). NIK and TRAF1 are TWEAK-inducible, suggesting that the pathway is activated at baseline in responders possibly reflecting pathway dependence. NEMO is required for canonical and NIK for noncanonical NF-xB activation (40), suggesting that both pathways are critical and activated in responsive, TWEAK-dependent tumors.

**TWEAK induces proliferation and survival signaling in tumor cells**

As there are conflicting reports in the literature regarding the functional consequences of TWEAK stimulation in cells, proliferation, survival, and gene expression profiles were assessed in multiple Fn14-expressing cells. These studies were also useful for generating pharmacodynamic biomarker candidates for evaluation in efficacy studies and clinical testing. TWEAK stimulation induces proliferation of U2OS osteosarcoma cells and HUVECs up to 2-fold but in tumor cell lines that are most responsive to RG7212, when grown at tumor xenografts in mice and in a large panel of tumor cell lines, no significant increase or decrease in cell number occurred with ligand treatment (Supplementary Fig. S3A–S3D). Neither TWEAK nor RG7212 treatment affected the viability of 299 tumor cell lines assessed in CellTiterGlo (Supplementary Fig. S3D) or in efficacy lines in MTT assays (Supplementary Fig. S3C). TWEAK did not induce apoptosis in tumor cells, as measured by caspase-3 activation, cytookeratin-18 or caspase-3 cleavage, or detection of cytoplasmic nucleosomes (Supplementary Fig. S3E–S3H).

Signaling changes in TWEAK-stimulated tumor cells are consistent with the magnitude of these observed effects on proliferation and survival. TWEAK stimulation leads to modest increases in ERK and AKT phosphorylation but dramatic increases in NF-xB signaling with induction of IKBx and NF-xB p65 phosphorylation (Fig. 3A and Supplementary Fig. S3L and S3P). TWEAK signaling is blocked by RG7212 in tumor cells that respond to the antibody when grown as xenografts in vivo (Fig. 3A) and is not induced in tumor cells lacking Fn14 expression (Supplementary Fig. S3K). In contrast, activation of NF-xB and significant, large fold-changes in TWEAK-induced gene expression were observed (Fig. 3B and C, Supplementary Table S3), suggesting that these are likely to be important drivers of tumor growth and maintenance in Fn14-expressing tumors. TRAF1- and IAP-encoding genes are TWEAK inducible (Fig. 3B and Supplementary Table S3 and Supplementary Fig. S3L and S3M), and levels of these proteins were decreased in tumors from RG7212-treated mice. TRAF and IAP complexes modulate signaling via TNF receptor superfamily members, including Fn14, by activating NF-xB signaling via ubiquitin ligase activity (19, 41). TRAF-1, -2, -3, and -5 have been shown to bind Fn14 (11). Ligand binding induces ubiquitin-mediated degradation of these proteins and activation of NIK. TWEAK stimulation of cells leads to rapid degradation of TRAF1 and c-IAP-2 proteins, transcription of both genes, and then increases in both TRAF1 and c-IAP-2 protein levels (Fig. 3D). TWEAK stimulation increased levels of NIK and pIKBx, enhanced by addition of the proteasome inhibitor MG132. TRAF1 and IAP are among several TWEAK-induced proteins that are downmodulated in tumors inhibited by RG7212 treatment in mice.

TWEAK-induced expression changes were assessed in Affymetrix gene arrays in time course experiments in 4 tumor cell lines (Supplementary Table S3) and by quantitative PCR (qPCR) in 14 tumor cell lines. Cell lines were selected using a Roche internal gene expression database to identify high and non-Fn14–expressing tumor lines, derived from histologically distinct tumor types. Data describe the biology of TWEAK and were used to monitor pathway inhibition in mice treated with RG7212. With respect to understanding the role of TWEAK in driving tumor growth, it is notable that the most strongly, consistently induced gene changes across a range of tumor cell lines regulate cellular proliferation, survival, NF-xB pathway activity, immune cell recruitment, and function and here we show that a number of these, at the mRNA and/or protein level, are decreased in mice treated with RG7212.

TWEAK induces multiple mitogens (Fig. 3B and C and Supplementary Table S3). TWEAK induces genes encoding critical protein regulators of NF-xB and MAPK signaling (19). TWEAK stimulation leads to modest increases in ERK and AKT phosphorylation but dramatic increases in NF-xB signaling with induction of IKBx and NF-xB p65 phosphorylation (Fig. 3A and Supplementary Fig. S3L and S3P). TWEAK signaling is blocked by RG7212 in tumor cells that respond to the antibody when grown as xenografts in vivo (Fig. 3A) and is not induced in tumor cells lacking Fn14 expression (Supplementary Fig. S3K). In contrast, activation of NF-xB and significant, large fold-changes in TWEAK-induced gene expression were observed (Fig. 3B and C, Supplementary Table S3), suggesting that these are likely to be important drivers of tumor growth and maintenance in Fn14-expressing tumors. TRAF1- and IAP-encoding genes are TWEAK inducible (Fig. 3B and Supplementary Table S3 and Supplementary Fig. S3L and S3M), and levels of these proteins were decreased in tumors from RG7212-treated mice. TRAF and IAP complexes modulate signaling via TNF receptor superfamily members, including Fn14, by activating NF-xB signaling via ubiquitin ligase activity (19, 41). TRAF-1, -2, -3, and -5 have been shown to bind Fn14 (11). Ligand binding induces ubiquitin-mediated degradation of these proteins and activation of NIK. TWEAK stimulation of cells leads to rapid degradation of TRAF1 and c-IAP-2 proteins, transcription of both genes, and then increases in both TRAF1 and c-IAP-2 protein levels (Fig. 3D). TWEAK stimulation increased levels of NIK and pIKBx, enhanced by addition of the proteasome inhibitor MG132. TRAF1 and IAP are among several TWEAK-induced proteins that are downmodulated in tumors inhibited by RG7212 treatment in mice.

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TWEAK induces multiple mitogens (Fig. 3B and C and Supplementary Table S3). TWEAK induces genes encoding critical protein regulators of NF-xB signaling: TRAF1, BIRC3, NFKB2, and NFKBIE with TRAF1 expression maximally induced up to 10-fold (Fig. 3B and Supplementary Table S3) and more than 2-fold in the majority of tumor cell lines tested (Supplementary Fig. S3L).

Cellular inhibitors of apoptosis, c-IAP-1 and -2 proteins (encoded by BIRC2 and BIRC3 genes), are required for TWEAK-induced activation of NF-xB and MAPK signaling (19). BIRC3 was increased up to 97-fold in gene array studies (Fig. 3B) and more than 2.5-fold in 13 of 14
Fn14-expressing human tumor cell lines by qPCR (Supplementary Fig. S3M) NF-kB inhibitor epsilon (NFKBIE), encoding an NF-kB inhibitory protein regulator of canonical signaling (40), was induced in by TWEAK in most tumor cell lines (Fig. 3B and Supplementary Fig. S3N). TWEAK induced gene expression shapes the tumor microenvironment and may promote escape from antitumor immune mechanisms TWEAK-induced gene changes include increases in expression of critical regulators of tumor immune cell composition and function (Fig. 3B and C and Supplementary Table S3). In vitro, TWEAK was shown in Affymetrix arrays (Supplementary Table S3) and by qPCR (Fig. 3C) to increase expression of cytokine and chemokine genes in tumor cell lines. Among these were CCL2, CXCL3 (mouse ortholog CXCL2), CCL5, CCL7, CSF1, CSF2, CCL20, IL8, and IL6.

In addition to these factors that modulate immune cell recruitment, TWEAK was shown to induce CD274 (PDL1, B7-H1), encoding the ligand for the inhibitory T-cell receptor, CD279 (programmed death-1, PD-1, PDCD1; Fig. 3C). TNFα, a suggested factor in TWEAK-induced apoptosis, in these expression studies across multiple cell lines was only minimally modulated at the first time point in U2OS, cells that do not die but proliferate in response to TWEAK. Across multiple cell lines in Affymetrix arrays, TWEAK decreased expression of only 2 genes, SORBS2 and PRICKLE, in more than 1 cell line and more than 2-fold (Supplementary Table S3).

TWEAK-induced effects are reversed in RG7212-treated mice Expression of a number of TWEAK-induced genes was significantly downregulated in tumors from RG7212-treated mice.
mice (Fig. 4A and Supplementary Table S3). Tumor mRNA levels of TWEAK-inducible genes: CCL2, CXCL3, CXCL11, CXCL10, PD-L1, IL8, TNFAIP3, TNFRSF9, and NFKBIE and mRNAs encoding genes not induced by TWEAK: CCR5, CXCL9, and PD1 were decreased 24 hours following the first dose of RG7212 (Fig. 4A). These genes encoding cytokines, chemokines, and receptors regulate immune cell numbers, distribution, and function. The roles of these regulators of immune cell distribution and function in mediating the antitumor activity of RG7212 are an active area of current investigation.

RG7212 modulation of several additional pharmacodynamic serum and tumor markers was shown in mice. Dose-related decreases in serum and tumor levels of TWEAK (Fig. 4B, Supplemental Figure S4A) and levels of human CCL2, IL8, and IL6 and murine EGF, bFGF, and MMP9 proteins were significantly decreased in the serum from RG7212-treated tumor-bearing mice (Fig. 4C and D and Supplementary Fig. S4B–S4E). Of the murine proteins decreased, only MMP9 expression is TWEAK inducible. Host tissue expression changes are intriguing as EGF and bFGF are known to be key tumor-promoting mitogens secreted by tumor-associated macrophages (42) and TWEAK induces, and RG7212 treatment reverses, expression of several macrophage recruiting chemokines.

Downregulation of TWEAK-induced signaling was shown in tumors from RG7212 treated mice including decreases in pERK, pAKT, c-IAP-1, and c-IAP-2 (Fig. 5A). Tumor shrinkage and decreases in pERK (Fig. 5B) and Ki67 were also observed in a patient with melanoma after RG7212 treatment (36), supporting that proliferation is decreased in tumors with RG7212 treatment. Levels of
TWEAK-inducible TRAF1 mRNA and protein (Fig. 5C and Supplemental Fig. S4F) and IAP proteins (Fig. 5A) were decreased in tumors from RG7212-treated mice. By quantitative immunohistochemistry (IHC), TRAF1 protein was decreased more than 50% in tumors in mice and in patient tumor biopsies in the phase I study following RG7212 dosing (36).

Both murine NFKBIE mRNA (Fig. 4A) and levels of activated NFKB2 (p52) protein (Fig. 5D) were decreased relative to control tumors showing RG7212 modulation of both canonical and noncanonical NF-κB signaling, respectively.

Contrary to reports of TWEAK-inducing apoptosis in vitro, we observed evidence of apoptosis induction with a TWEAK blockade by RG7212 in our tumor models. Apoptotic signaling, assessed using a cleaved caspase-3 (CC3)-specific IHC antibody and image quantitation and PARP cleavage with immunoblotting, was increased in tumors from RG7212-treated mice (Fig. 6A). Increased ACHN tumor CC3 staining was detected 8 and 24 hours following the first dose. No changes in CC3 or in PARP cleavage were detected in tumors from B20.4.1 or erlotinib-treated mice (data not shown). PARP cleavage was more dramatic in Caki-1 tumor lysates in a study where tumor regression was observed compared to that observed in the ACHN model, where treatment resulted in tumor stasis. These and gene array data are consistent with a prosurvival, tumor-promoting function for TWEAK. TWEAK was shown to dramatically increase the expression of inhibitors of apoptosis (Fig. 3B) including BIRC3 (19) and TNFAIP3 (43), encoding c-IAP-2 and A20 proteins, respectively.

RG7212 efficacy is dependent on immune cell function

The results of these investigations as well as the published data in TWEAK knockout mice suggest a role of TWEAK in modulating antitumor immune response. Preliminary, intriguing results showing that higher level immune function in tumor-bearing mice increases the level of antitumor activity of RG7212 (Supplementary Table S2), that treatment with RG7212 in immunocompetent mice alters immune cell distribution (Fig. 6B), and that specific
immune cell depletion dramatically alters the level of antitumor efficacy of RG7212 (Fig. 6C).

TWEAK knockout mice showed enhanced antitumor effects. In the MDA-MB-231 model, TGI was 91% in nude and 48% in SCID beige mice. This loss of antitumor activity was observed in 2 additional models in SCID beige mice. Nude mice have functional natural killer (NK) cells, lack thymic function, and have decreased numbers of T cells. Mice with both SCID and beige mutations lack T, B, and NK cells. Both strains have functional macrophages.

In C57BL6 immunocompetent mice with Pan02 tumors, at the end of RG7212 treatment, CD3+ T cells were significantly increased in blood and spleen and slightly, but not significantly increased in tumors (Fig. 6B). Monocytes/macrophages (CD11b+/F4/80+)) were significantly decreased in blood and significantly increased in tumors from mRG7212-treated mice. A significant increase in total leukocytes (CD45+) and no significant changes in B cells, NK cells, or monocytes (CD11b+/F4/80+) with RG7212 treatment were detected. Increases in tumor and spleen T-cell numbers were reported for TWEAK-knockout mouse studies (35). These data show the impact of RG7212 treatment on immune cell distribution after chronic treatment but may not reflect earlier changes coincident with dramatic cytokine and chemokine changes observed early in efficacy studies.

To examine the role of specific immune cells in antitumor efficacy of mRG7212, immune cell depletion experiments were carried out with in C57BL6 mice with Pan02 tumors. The efficacy of mRG7212 was evaluated in 3 groups of mice:
depleted of CD4\(^+\), CD8\(^+\), or NK cells before RG7212 dosing and throughout the study (Fig. 6C). Depletion of NK cells and CD8\(^+\) T cells led to a complete loss of mRG7212 antitumor efficacy, suggesting a role for TWEAK in suppressing the antitumor immune response through suppression of these immune cell populations. Conversely, depletion of CD4\(^+\) T cells appears to improve the antitumor efficacy of mRG7212. Clearly, antitumor activity with RG7212 is impacted by immune system components and multiple immunomodulatory factors are modulated with antibody treatment.

As in the mouse tumors, increased tumor T-cell infiltration (Fig. 6D) was observed after treatment in the patient with melanoma where other pharmacodynamics changes presented here were observed (Fig. 5B). Consistent with the expected changes in immune cell recruitment from the cytokine changes in mice, a decrease in tumor macrophages was observed the melanoma tumor (36).

Discussion

RG7212 has shown antitumor efficacy in multiple diverse tumor models in mice with efficacy associated with tumor Fn14 expression and pathway activation. Expression of Fn14 has been shown to be upregulated in many human cancers, and RG7212 efficacy is independent of TP53, BRAF, and KRAS mutational status, suggesting that RG7212 may provide broad clinical benefit in patients with TWEAK-driven tumors.

Our data show that TWEAK induces signaling and gene expression to promote tumor cell proliferation, survival, and NF-κB signaling and these effects are reversed in tumors from RG7212-treated mice. TWEAK also induces expression of a number of mitogens and cytokines and chemokines that interact with host cells within and beyond the tumor microenvironment.

TWEAK-induced factors modulate T-cell and monocyte recruitment, T-cell activation, and macrophage differentiation and these were downregulated in RG7212-treated mice. PDL1, CCL2, CXCL-9, -10, and -11 and -1 may provide mechanisms of escape from an antitumor immune response, suggesting that RG7212 may represent a novel approach to overcoming these ligands. Antagonistic PD1 and PD1L antibodies show evidence of antitumor activity in patients with melanoma, kidney, and NSCLCs (44). T-cell regulating CXCL-9, -10, and -11, CXCR3 ligands may play immunosuppressive roles in cancer (42). This has been shown for CXCL11, a chemotractant for T cells. In RCC specimens, its expression was correlated with increased tumor Foxp3\(^+\) T cells with expression characteristics of functional Tregs (45). TWEAK strongly induces CCL2 and CSF1 that recruit and induce differentiation of tumor-promoting macrophages, respectively. TAMS also impact T and NK cell tumor infiltration and activation to suppress adaptive immune responses (42).

Blocking TWEAK signaling in mice and humans alters the tumor immune cell composition including tumor macrophages that express both TWEAK and Fn14 (9). RG7212-induced changes in the microenvironment may influence both TWEAK signaling in macrophages, as well as their phenotype (cytotoxic M1 vs. tumor-promoting M2). Future studies will look at the evolution of the immune cell changes during treatment to define initiating events and to understand how a TWEAK blockade remodels the immune system over time in treated mice.

Consistent with decreased tumor growth and enhanced antitumor immune response in TWEAK knockout mice, RG7212 activity is dependent on immune cells. Immune cell redistribution with RG7212 treatment and depletion studies show that TWEAK plays a role in immune cell recruitment, defining the tumor immune cell content, and RG7212 antitumor efficacy is dependent on NK and CD8\(^+\) T-cell function and is diminished by CD4\(^+\) T cells.

Further studies are needed to assess the effects of RG7212 monotherapy and combination therapies on T-cell function, macrophage phenotype, and NK activation to specifically ask whether treatment enhances tumor cell killing by CD8\(^+\) cells and to characterize CD4\(^+\) cells and tumor macrophage populations.

Together our results suggest that TWEAK signaling promotes tumor growth and survival, establishes a tumor microenvironment that supports tumor growth, and interacts with the host immune system to suppress tumor destruction. RG7212 treatment reverses these effects and inhibits tumor growth in mice.

Preliminary results from the phase I study of RG7212 show decreased tumor cell proliferation and pathway activity plus immune cell changes in patients that are consistent with the mechanism of action for RG7212 emerging from these mice studies.

Disclosure of Potential Conflicts of Interest

T.A. Lin is employed as Research Leader/Principal Scientist in Hoffmann-La Roche, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: X. Yin, L. Luistro, K. Packman, J. Rosinski, M.E. Simcox

Development of methodology: X. Yin, L. Luistro, D. Biondi, J. Rosinski, W. Berkofsky-Fessler, S. Pant

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Yin, L. Luistro, M. Smith, T. Nevins, H. Hilton, T.-A. Lin, T. Truitt, D. Biondi, X. Wang, S. Pant, S. Vega-Harring


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Luistro

Study supervision: L. Luistro, K. Packman

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Xuefeng Yin, Leopoldo Luistro, Hua Zhong, et al.

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