RG7212 anti-TWEAK mAb inhibits tumor growth through inhibition of tumor cell proliferation and survival signaling and by enhancing the host antitumor immune response

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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.
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 anti-tumor 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, suggest RG7212 is a promising agent for use in combination therapies in patients with Fn14 positive tumors.

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) (3) (4, 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) (10) (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 cancer patients. TWEAK and Fn14 expression are negatively correlated with patient overall and disease-free survival in renal cell carcinoma (RCC) (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 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 non-canonical NF-κB pathways associated with tumor growth and survival. Here we demonstrate 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 pathological conditions in humans (21, 22).

In contrast to data demonstrating 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 interferon γ (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, though sharing complex pathway biology with these agents, is herein characterized as a pathway antagonist. RG7212 anti-tumor 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. Though 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 demonstrated in mice with agonistic anti-Fn14 antibodies, BIIB036 and PDL192 (Enavatuzumab, ABT-361) (12, 29, 30). These antibodies have distinct properties but each demonstrates 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)
In contrast, though 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) (33) (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 two 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 demonstrated 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.
pathway in non-tumor 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 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 anti-tumor 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 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 immune deficient and in transplanted tumors in immune competent 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 anti-tumor 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).

**Experimental Procedures**

Detailed experimental procedures are available in Supplemental Experimental Procedures.

**Cell lines**

Human U2OS, HCT116, ACHN, AsPC1, Calu-3, Caki-1, AsPC-1, Panc-1, SJSA, 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 (ATCC, Manassas, VA) and identities were confirmed via sequencing. HUVEC were purchased from Clonetics (San Diego, CA). Human NCI-H332M and murine tumor cell lines, B16BL6, B16F10, Pan02 and RENCA were obtained from the NCI Cell Repository (Frederick, MD).

**Mice**

Athymic nu/nu (nude) mice were purchased from Charles River Laboratories (Wilmington, MA), and SCID-beige mice were from Taconic Farms (Germantown, NY). C57BL6/J and Balb/c mice were obtained from Jackson Laboratory (Bar Harbor, ME).
Mice used in studies were approximately 6 – 8 weeks old. All procedures performed 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 Supplemental 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, Cambridge, MA). Dissociated cells and leukocytes were incubated with fluorescence-labeled antibodies followed by washes using PBS containing 0.5% BSA. Labeled cells were fixed in 1% formaldehyde solution and the differential cell populations were analyzed using the imaging flow cytometer ImageStreamX (Amnis Corporation, Seattle, WA). Antibodies are listed in Supplemental Information.

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

**Statistical Analysis**

In all studies, values are expressed as mean ± standard error of the mean (SEM) or box plot summaries, as indicated. Statistical analyses were performed by unpaired Student’s t test, or Mann-Whitney U test, or linear regressions, as indicated. Differences were considered statistically significant at p<0.05. Additional details found in the Supplemental 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 IgG1κ 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 were demonstrated (Supplementary Table S1). For simplicity, mAbs in this report are designated RG7212 and mRG7212.

RG7212 is fully cross-reactive, binding human, rat and murine TWEAK with high affinity (Fig. 1A and 1B) 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 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/week (rats; AUC 204mg*h/ml, Cmax 2670 μg/ml) and 50 mg/kg/week (monkeys; AUC 211mg*h/ml, Cmax 2110 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 demonstrated 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-2D and Supplementary Table S2). The antitumor effect of RG7212 is dose dependent after twice weekly administration. RG7212 treatment resulted in statistically significant TGI with bi-weekly dosing $\geq 3.75$ mg/kg (Fig. 2A). Together with the pharmacokinetic data from the same study (Fig. 1D), the estimated average steady state concentration (Css avg) and estimated trough concentration to achieve a 90% antitumor activity are 261 $\pm$ 88.2 $\mu$g/mL and 167 $\pm$ 56.8 $\mu$g/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 anti-tumor 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 five 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 suggests 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) (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., Hackensack, NJ) PULM009 (Supplementary Fig. S1F) and increased the activity of cisplatin from 34% TGI 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 ($\geq 20\%$, $p < 0.05$) or evidence of gross toxicity.

Exome sequencing data showed RG7212 was active in models from cells expressing either mutant or wild type \textit{BRAF, CDKN2A, KRAS, TP53 and TRAF2}. (Supplementary Fig. S1G). Mutations were not identified in TWEAK or Fn14 genes in responders or non-responders. All responsive models express wild type \textit{MAP3K14(NIK)} and a mutation (T763A) was found in non-responder AsPC1. All models assessed in vivo expressed wild type \textit{PIK3CA}. Lack of activity in models was not correlated with mutations in \textit{CD40, LTBR, TRAF3 or TAC1}, associated with constitutively activated NF-$\kappa$B (39). A \textit{TRAF2} mutation of uncertain functional significance was identified in responder Caki-1. Expression analysis suggests that downstream pathway expression and activation markers may 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-$\kappa$B genes and TWEAK- modulated genes. Additional candidate response prediction markers significantly elevated at baseline in responders were: \textit{IKBKGNEMO}, \textit{MAP3K14(NIK)}, \textit{STAT3}, \textit{STAT5B} and \textit{TRAF1} (Supplementary Fig. S2B). \textit{NIK} and \textit{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 non-canonical NF-κB activation (40), suggesting 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 human umbilical vein endothelial cells (HUVEC) 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, S3B, S3C, 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, cytokeratin-18 or caspase-3 cleavage or detection of cytoplasmic nucleosomes (Supplementary Fig. S3E, S3F, S3G, 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-κB signaling with induction of IκBa and NF-κB p65 phosphorylation (Fig. 3A and...
Supplementary Fig. S3I, S3J). 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-κB and significant, large fold-changes in TWEAK induced gene expression were observed (Fig. 3B, 3C, 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, 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 TNF receptor super family members, including Fn14, by activating NF-κB 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 pIκBα, enhanced by addition of the proteosome inhibitor MG132. TRAF1 and IAP are among several TWEAK induced proteins that are down modulated in tumors inhibited by RG7212 treatment in mice.

TWEAK-induced expression changes were assessed in Affymetrix gene arrays in time-course experiments in four 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-κB 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, Fig. 3C, Supplementary Table S3).

TWEAK-induces genes encoding critical protein regulators of NF-κB signaling: TRAF1, BIRC3, NFKB2, and NFKBIE with TRAF1 expression maximally induced up to 10-fold (Fig.3B, Supplementary Table S3) and > 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-κB and MAPK signaling (19). BIRC3 was increased up to 97-fold in gene array studies (Fig. 3B) and > 2.5-fold in 13/14 Fn14 expressing human tumor cell lines by qPCR (Supplementary Fig. S3M) NF-κB inhibitor-epsilon (NFKBIE), encoding an NF-κB inhibitory protein regulator of canonical signaling (40), was induced in by TWEAK in most tumor cell lines (Fig. 3B, 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, 3C, Supplementary Table S3). In vitro, TWEAK was demonstrated 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 timepoint 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 two genes, SORBS2 and PRICKLE, in > 1 cell line and ≥ 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 (Fig. 4A, 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 were demonstrated in mice. Dose-related decreases in serum and tumor levels of TWEAK (Fig. 4B, Supplemental Figure S4A) and levels 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, 4D and Supplementary Fig. S4B, S4C, S4D, S4E). Of the murine proteins, only MMP9 expression, but EGF or bFGF, are 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, cIAP-1 and c-IAP-2 (Figure 5A). Tumor shrinkage and decreases in pERK (Fig. 5B) and Ki67 were also observed in a melanoma patient after RG7212 treatment (36) supporting that proliferation is decreased in tumors with RG7212 treatment. Levels of TWEAK inducible- TRAF1 mRNA and protein (Supplemental Figure S4F and Fig. 5C) and -IAP proteins (Fig. 5A) were decreased in tumors from RG7212 treated mice. By quantitative IHC, TRAF1 protein was decreased > 50% in tumors in mice and in patient tumor biopsies in the Phase I study following RG7212 dosing (36).
Both murine \textit{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 non-canonical NF-κB signaling, respectively.

Contrary to reports of TWEAK inducing apoptosis in vitro, we observed evidence of apoptosis induction with a \textit{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 pro-survival, tumor promoting function for TWEAK. TWEAK was shown to dramatically increase the expression of inhibitors of apoptosis (Fig. 3B) including \textit{BIRC3} (19) and \textit{TNFAIP3} (43), encoding c-IAP-2 and A20 proteins, respectively.

\textbf{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 anti-tumor 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 demonstrated enhanced antitumor immune. 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 two additional models in SCID Beige mice. Nude mice have functional 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 immune competent 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 conducted with in C57BL6 mice with Pan02 tumors.
The efficacy of mRG7212 was evaluated in three groups of mice: depleted of CD4+, CD8+ or NK cells prior to RG7212 dosing and throughout the study (Fig. 6C). Depletion of NK cells and CD8+ T cells led to a complete loss of mRG7212 anti-tumor 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 melanoma patient where other pharmacodynamics changes presented here were observed (Fig. 6D). 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 demonstrated 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
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 may provide mechanisms of escape from an anti-tumor immune response suggesting RG7212 may represent a novel approach to overcoming these ligands. Antagonistic PD1 and PDL1 antibodies show evidence of antitumor activity in melanoma, kidney and NSCLC patients (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 chemoattractant 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 (TAMS), 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 versus 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 TWEAK blockade remolds the immune system over time in treated mice.

Consistent with decreased tumor growth and enhanced anti-tumor immune response in TWEAK knockout mice, RG7212 activity is dependent on immune cells. Immune cell redistribution with RG7212 treatment and depletion studies demonstrate that TWEAK plays a role in immune cell 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.

**Supplemental Information**
Supplemental Information includes Supplemental Experimental Procedures, 4 Supplemental Figures and 3 Supplemental Tables.

ACKNOWLEDGMENTS

We thank Susanne Arbogast, Antje-Christine Walz, Lisa Culton, Martin Weisser, Tobias Bergauer, Alvin Stern, Julie Hang and Ueli Gubler for help with experiments and Wei Xu for his careful review of the manuscript.

REFERENCES

Figure Legends

Figure 1. RG7212 properties. (A) High affinity RG7212 binding to recombinant mouse and human TWEAK (ELISA) (B) RG7212 binding and neutralizing properties. IC50 values in mouse, rat and human TWEAK binding ELISA, murine and human TWEAK:Fn14 blocking ELISA, TWEAK-stimulated IL8 secretion (ELISA) and U2OS and HUVEC proliferation (MTT). (C) Murine and human TWEAK have similar activity in human Fn14 expressing U2OS cells (5-day MTT assay) (D) RG7212 pharmacokinetics in ACHN-tumor bearing mice, where dose-dependent antitumor efficacy was observed. See Figure 2A. See Supplementary Methods for antibody generation and characterization (Table S1) and PK sampling protocol for modeling and efficacy summary Table S2.

Figure 2. RG7212 inhibits tumor growth in tumors with high level Fn14 expression. (A) RG7212 antitumor efficacy in ACHN xenografts. TGI is dose-related and statistically significant ($p \leq 0.004$) with $\geq 3.75$ mg/kg bi-weekly dosing. (B) Immunoblot and IHC show high levels of Fn14 protein in tumor cell lines that respond to RG7212, when grown as xenografts in mice. (C) TWEAK concentrations in conditioned media from tumor cell lines measured by ELISA (D) RG7212 is inactive in Fn14-non-expressing 22Rv1 prostate cancer model. Statistical analysis of xenograft data was determined by the rank sum test and One Way Anova and a post-hoc Bonferroni t-test. 10 mice per
group. P values are comparisons of RG7212 to vehicle treated mice. See also Supplementary Figure S1 and Table S2.

Figure 3. TWEAK induces proliferation, survival and immune signaling and gene expression. (A) RG7212 blocks TWEAK induced signaling. (B) Most highly TWEAK-induced genes across 4 Affymetrix gene arrays promote survival: TNFAIP3, VCAM1, BIRC3, TNFRSF9; regulate NF-κB activation: NFKB2, TRAF1, TNFAIP3, BIRC3, NFKBIE; leukocyte recruitment: CSF2, IL8, CCL2; proliferation: HBEGF. Data shown for models with in vivo efficacy data, ACHN, MDA-MB-436 and Pan02 and for U2OS that proliferates in response to TWEAK in vitro. Expression changes with 8h TWEAK stimulation shown; changes at 1h, 4h, 8h and 24h in Supplementary Table S1. *BIRC3 mRNA 97-Fold increase with TWEAK stimulation in U2OS cells was not plotted to maintain scale. (C) Heatmap representation of TWEAK induced cytokines by qPCR in Fn14 expressing cells. Values shown represent highest fold change with 4, 8 or 24h TWEAK stimulation. All changes are significant by p < 0.05. Uncolored squares represent no statistical increase. No changes in any genes in non-expressing Fn14 line 22Rv1 by qPCR or in B16BL6 by Affymetrix gene array with TWEAK stimulation (data not shown). 5-fold increase in CXCL3 in ACHN not quite significant p=0.06. Not included: Panc-1 only increases: < 1.5-fold in CSF1 and IL8 and H322M only increase: 2.5-fold in CCL5 (all changes p < 0.05). (D) TRAF1 and c-IAP-2 proteins initially degraded then increased in TWEAK stimulated cells. Immunoblots: Caki-1 and ACHN cell lysates from TWEAK (100ng/ml) stimulated cells. TWEAK increases NIK and
pIKBα; pretreatment with MG132 (25 μM) for 1 hr at 37°C leads to accumulation of both, but does not lead to increases in TRAF1 or cIAP2. See also Supplementary Fig. S3C, S3J, S3B, and Table S3.

Figure 4. RG7212-induced pharmacodynamic changes in tumor-bearing mice (A) RG7212 treatment decreases expression of TWEAK induced genes. Fold changes in ACHN tumor cell mRNA with TWEAK stimulation (blue). Fold changes in mRNA with RG7212 treatment 24h after first dose (red). *All mRNA changes in ACHN cells or xenografts except PDL1 induced in MDA-MB-436 in vitro with TWEAK stimulation and CXCL3 mRNA decreased in Caki-1 xenografts after RG7212 treatment. All p- and fold change- values in Supplemental Table S3. (B) Levels of human TWEAK are decreased in Caki-1 xenograft tumors and serum from RG7212 treated mice. Decrease observed within 4h in tumors and is maintained with repeat dosing in serum. Free TWEAK was quantitated by ELISA. Murine TWEAK was below the limit of detection in serum and decreased in tumors with RG7212 treatment (data not shown). (C) RG7212 treatment decreases serum levels of human CCL2 protein* 3.5-fold 24h (p = 0.002) and 18-fold 72h (p = 0.0002) following the first RG7212 dose in ACHN tumor bearing mice.** (D) MMP9 decreased in serum from ACHN, Caki-1 or Calu-3 tumor bearing mice 24h after first dose (p < 0.05)**. *Myriad RBM assays. Sera were collected 4, 8, 24 and 72h following the first dose and 6h following the doses day 7, day 14 or day 21. N= 5 per time point. Multiplexed ELISA (Myriad RBM, Human inflammation MAP™ and Rodent MAP version 2.0). Multiplexed ELISA (Myriad RBM, Human inflammation MAP™ and
Rodent MAP version 2.0). ** Difference between serum from RG7212 and vehicle treated mice. Data are mean ± SEM n=5 mice/serum samples per time point. Statistical analysis by Mann-Whitney U test. See also Supplemental Figure S4A-S4F for other serum and tumor PD effects.

Figure 5. RG7212 blocks TWEAK-induced signaling in tumor-bearing mice and Phase I patient. A) RG7212 decreases pERK, pAKT, c-IAP-1 and c-IAP-2 proteins in tumor lysates by immunoblotting. (B) pERK IHC from melanoma patient biopsy collected 7 days following first dose. (C) Immunoblots showing TRAF1 protein is decreased in Caki-1 tumors from RG7212-treated mice throughout the course of treatment. IHC showing TRAF1 decreased following second RG7212 dose. See also BIRC3 and TRAF1 mRNA induced prior to protein increases with TWEAK stimulation, Supplementary Table S3, Fig. S3L-S3M. (D) RG7212 inhibits non-canonical NF-κB signaling in ACHN tumors. Levels of activated NFκB2 (tumor p52 are decreased 24 and 72 hours following the first dose of RG7212.

Figure 6. Apoptosis induction and immune modulation are components of the mechanism of action of RG7212. (A) Apoptosis induced in tumors from RG7212 treated mice: PARP cleavage by immunoblotting with total anti-PARP antibody (ACHN tumor lysates, day 21 of study). PARP cleavage detected throughout treatment course (RG7212 20 mg/kg twice weekly) in Caki-1 lysates prepared from tumors collected from treated mice. Cleaved caspase-3 quantitated in specimens by IHC with imaging. N=5. (B) RG7212 treatment alters immune cell distribution. Immune cell populations in blood,
spleen, and Pan02 tumors quantitated using ImageStream® Flow cytometry. mRG7212 treatment increases CD3+ T cells in blood and spleen, decreases blood and increases tumor macrophages (CD11b+/F4/80+) and increases total tumor leukocytes (CD45+). Each dot is sample from one mouse. (C) Antitumor efficacy of mRG7212 is dependent on CD8+ T and NK cells and is increased with depletion of CD4+ T cells. mRG7212 efficacy with immune cell depletion study design. Tumor growth curves with RG7212 treatment with specific immune cell depletion. (D) RG7212 increases tumor T cell infiltration. Representative CD3 IHC from Phase I patient biopsies.
Figure 1.

A

**Human TWEAK**
- Control IgG
- BioLegend anti-human TWEAK
- RG7212

**Mouse TWEAK**
- Control IgG
- BioLegend anti-human TWEAK
- RG7212

B

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C

**TWEAK Stimulation of U2OS Proliferation**
- Mouse TWEAK
- Human TWEAK

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* AUCss = AUC at steady state (AUC_{0-48hr} post 7th dose).
* Cavg ss computed by AUCss divided by dosing interval of 84-hr.
Figure 2.

A. Graph showing the mean tumor volume (mm³) over days post tumor cell implantation for different treatment groups: Vehicle, 1.875 mg/kg 2 x/wk, 3.75 mg/kg 2 x/wk, 7.5 mg/kg 2 x/wk, 15 mg/kg 2 x/wk, and 30 mg/kg 2 x/wk.

B. Image showing immunohistochemistry for endothelium and Fn14 in 22Rv1 and ACHN cells.

C. Bar graph showing TWEAK levels (pg/ml) in different cell lines.

D. Graph showing the mean tumor volume ± SEM (mm³) over days post tumor cell implantation for different treatments: Vehicle, RG712 20 mg/kg 2x/wk, and Docetaxel 30 mg/kg 1x/wk.
Figure 3.

(A) TWEEN experiments with U2OS, MDA-MB-231, and ACHN cells treated with TWEAK 100 ng/ml, RG712 10 µg/ml, pIκBα, pERK, pNF-κB p65 (ser536), and Actin at 15 min and 2h.

(B) Fold change in mRNA for various genes in ACHN, MDA436, Pan02, and U2OS cells treated with TWEEN.

(C) Heatmap showing expression levels of various genes in ACHN, Caki-1, Calu-3, Capan-2, MDA436, SJSA, SKMES1, U118MG, MDA231, and AsPC1 cells.

(D) Western blot analysis of c-IAP-2 and TRAF1 in ACHN and Caki-1 cells treated with TWEEN.
Figure 4.

A

B

C

D

- Figure 4.

- A: Graph showing the fold change in mRNA levels for various genes.

- B: Bar graph and line graph showing TWEAK levels in Caki-1 tumor lysates over time.

- C: Box plot showing serum human MCP-1 (CCL2) levels.

- D: Bar graph showing serum murine MMP-9 levels for different treatments.
Figure 5.

A

**ACHN**

Tumor Lysates (Day 21)

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Caki-1 Tumor Lysates

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B

![pERK IHC](pre-dose) ![pERK IHC](post-dose)

C

24h post 1st dose | 6h post day 3 dose | 24h post day 7 dose

- - + + | - - + + | - - + +

TRAF1

Actin

D

Time after 1st dose (h): 24 | 72

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Figure 6.

A

ACHN Tumor Lysates

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Caki-1 Tumor Lysates

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*Sample collected 6 hours post dose on specified day.

B

- Blood-CD3+ T cells
- Blood Macrophages (F4/80+ and CD11b+)
- Sen-CD3+ T cells

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Pan02 Tumor Macrophages

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Pan02 Tumor B cells

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Pan02 Tumor CD3+ T cells

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C

Days of expansion against cell lines acquired in expansion

Days of expansion against cell lines acquired in expansion

D

CD3 IHC

Pre-dose

Post-dose
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 Yin, Leopoldo Luistro, Hua Zhong, et al.

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Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.