Secretome Signature Identifies ADAM17 as Novel Target for Radiosensitization of Non–Small Cell Lung Cancer

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Intrinsic and acquired resistances to ionizing radiation represent a major challenge in the treatment of NSCLC. A recent phase III trial for patients with stage IIIA or IIIB NSCLC (RTOG 0617) demonstrated that addition of the EGFR-directed monoclonal antibody cetuximab to concurrent chemoradiation and consolidation treatment did not provide any added benefit. Thus, novel molecular targets for anticancer agents alone and in combination with radiotherapy are of high demand. The sheddase ADAM17 is associated with aggressive progression and poor prognosis in NSCLC and activates multiple ErbB- and non ErbB-related pathways. Here we demonstrate that genetic and pharmacological inhibition of ADAM17 sensitizes in vitro and in vivo NSCLC to ionizing radiation. The findings of this study suggest that clinically relevant ADAM17 inhibitors should be considered for the treatment of NSCLC in combination with radiotherapy.
Abstract

**Purpose:** Ionizing radiation (IR) induces intracellular signaling processes as part of a treatment-induced stress response. Here we investigate IR-induced ADAM17 activation and the role of ADAM17-shed factors for radiation resistance in non-small cell lung cancer.

**Experimental Design:** Large scale secretome profiling was performed using antibody arrays. Secretion kinetics of ADAM17 substrates was determined using ELISA across multiple *in vitro* and *in vivo* models of non-small cell lung cancer. Clonogenic survival and tumor xenograft assays were performed to determine radiosensitization by ADAM17 inhibition.

**Results:** Based on a large scale secretome screening, we investigated secretion of auto- or paracrine factors in non–small cell lung cancer in response to irradiation and discovered the ADAM17 network as crucial mediator of resistance to IR. Irradiation induced a dose-dependent increase of furin-mediated cleavage of the ADAM17 proform to active ADAM17, which resulted in enhanced ADAM17 activity *in vitro* and *in vivo*. Genetic or pharmacologic targeting of ADAM17 suppressed IR-induced shedding of secreted factors, downregulated ErbB signaling in otherwise cetuximab-resistant target cells and enhanced IR-induced cytotoxicity. The combined treatment modality of IR with the ADAM17 inhibitor TMI-005 resulted in a supra-additive antitumor response *in vivo* demonstrating the potential of ADAM17 targeting in combination with radiotherapy.

**Conclusions:** Radiotherapy activates ADAM17 in non–small cell lung cancer, which results in shedding of multiple survival factors, growth factor pathway activation and IR-induced treatment resistance. We provide a sound rationale for repositioning ADAM17 inhibitors as short-term adjuvants to improve the radiotherapy outcome of non–small cell lung cancer.
**Introduction**

Radiotherapy along with chemotherapy or surgery is the primary treatment regimen for locally advanced non-small cell lung cancer (NSCLC). However, the response rates and clinical outcomes are still disappointing, with the 5-year survival rate being only approximately 15% (1-3). Ionizing radiation (IR) triggers multiple intracellular signaling processes as part of IR-induced stress responses that lead to the secretion of various para- and autocrine factors into the tumor microenvironment (4-6). These secreted components generate *de novo* resistance mechanisms during the course of the treatment and at the same time represent interesting targets to sensitize for IR-induced cell killing (7-9). Several studies have shown that targeting tumor-derived factors such as VEGF, PDGFA, TGF-β, MMP-13, SDF-1 counteract growth of carcinoma cells and sensitize them to irradiation (10-14).

Growth and survival of NSCLC cells are often dependent on ectodomain shedding which includes the proteolytic cleavage of the extracellular part of membrane proteins primarily mediated by membrane-anchored metalloproteases, and results in release of various soluble growth factors and cytokines regulating cell proliferation and migration (15, 16). ADAMs (a disintegrin and metalloproteinase) are membrane-associated metalloproteinases with modular design and complex multi-domain structure (17). Most of the members of the ADAM family (13 out of 21 human ADAMs) have proteolytic activity, as well as domains with adhesive properties and a cytoplasmic domain involved in cell signaling (18). They are actively associated with the process of proteolytic ‘shedding’ of membrane-bound proteins and hence the rapid modulation of key signals in the tumor microenvironment. ADAM-mediated shedding is both constitutive and inducible, depending on upstream kinase activation, intracellular $Ca^{2+}$ levels, membrane lipid composition and other experimental and natural stimuli (19, 20).

Multiple resistance mechanisms in NSCLC have been linked to the epidermal growth factor receptor (EGFR), which has therefore been regarded as promising target for a combined
treatment modality. However, a recent phase III trial for patients with stage IIIA or IIIB NSCLC (RTOG 0617) demonstrated that solely addition of the EGFR-directed monoclonal antibody cetuximab to concurrent chemoradiation and consolidation treatment does not provide any added benefit for these patients (21). Thus, additional inhibition of the EGFR is insufficient, which might be due to the heterogenous expression status of other ErbB receptor family members in NSCLC or the mutational status of EGFR downstream signaling entities such as K-ras (22-24).

Increased ADAM17 expression in NSCLC is associated with aggressive progression and poor prognosis. ADAM17 regulates shedding of multiple key oncogenic growth factors, cytokines and adhesion molecules including ligands of ErbB family members (15, 16, 19, 25-28). Thus ADAM17 drives pleiotropic pathways and might therefore represent a more relevant target for a combined treatment modality in NSCLC.

Here we provide novel insights into activation of ADAM17 and subsequent ligand shedding in response to irradiation. We show for the first time that direct targeting of ADAM17 with a clinically relevant inhibitor sensitizes lung carcinoma cells in vitro and in vivo to IR and provide a sound rationale for positioning ADAM17 inhibitors as radiosensitizers to improve the treatment of NSCLC.

Materials and Methods

Cell culture, Compounds and Irradiation

The human NSCLC cell lines A549, NCI-H125, H460, Calu-3, Calu-6 and the human epidermoid carcinoma cell line A431 were cultured in RPMI1640 media supplemented with 10 % (v/v) fetal calf serum, 1 % (v/v) penicillin-streptomycin and 1 % (v/v) L-glutamine at 37°C in 5 % CO2. All cell culture media and supplements were obtained from Gibco (Life Technologies). All cell lines were authenticated through STR sequencing done by Microsynth AG. TMI-005 was obtained from Axon Medchem (Axon 1507). For in vitro experiments,
TMI-005 was dissolved in DMSO (10 mM stock) and further diluted with cell culture media. N-acetyl-L-cysteine (Sigma Aldrich; A8199) was dissolved in PBS (10 mM stock). The furin convertase inhibitor Dec-RVKR-CMK (Enzo Life Sciences; ALX-260-022) was dissolved in PBS (5 mM stock). Cetuximab (Erbitux) was obtained from Merck KGaA (5 mg/ml stock solution for infusion) and was further diluted to 100 nM for in vitro experiments. Irradiation was performed using an Xstrahl 200 kV X-Ray unit at 100 cGy/min. To produce conditioned media, fetal calf serum-supplemented (FCS) or FCS-deficient cell culture media was applied to proliferating cells 1 hour prior to sham-treatment or irradiation, collected 24 hours thereafter and filtered through a 0.45 µm filter.

**Cell proliferation and clonogenic cell survival assays**

The proliferative activity of tumor cells was assessed in 96-well plates with the colorimetric alamarBlue assay (Biosource International, Camarillo, CA). Clonogenic cell survival was determined by the ability of single cells to form colonies in vitro as described in (29). Dose enhancement factors (DEF) were calculated using α and β values for each survival curve. Survival data were fitted by weighted, stratified, linear regression to obtain the linear and quadratic parameters (29). The DEF values at 37 % and 10 % survival level were calculated using linear regression in IBM SPSS statistics 22. All assays were repeated as independent experiments at least thrice.

**Tumor xenograft in nude mice and application of treatment regimes**

A549 (4 x 10^6 cells) were injected subcutaneously on the back of 4- to 6-week old CD1 athymic nude mice. Tumor volumes were determined from caliper measurements of tumor length (L) and width (l) according to the formula (L x l^2)/2. Tumors were allowed to expand to a volume of 200 mm^3 (± 10 %) before treatment start. Tumors were sham-irradiated or irradiated using a customized shielding device with 3 × 1 Gy for 3 consecutive days. TMI-005
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(dissolved in 0.9 % NaCl, 0.5 % methylcellulose and 2 % Tween 80) was administered orally at 25 mg/kg twice daily with 10 hours apart for seven consecutive days. For combined treatment (sham)-irradiation was performed 1 hour after the first daily TMI-005 application on day 3 to day 5 of the TMI-005 schedule.

*In vivo* shedding experiments were performed on blood serum derived from locally irradiated A549-derived tumor xenografts. At a tumor volume of 400-500 mm³, tumors were sham-irradiated or irradiated with 10 Gy. IR-treated animals were sacrificed 48 hours after treatment. Tumors were excised and blood serum was collected from heart-punctured animals following euthanasia. All *in vivo* experiments were performed according to the guidelines for the welfare and use of animals of the Veterinäramt Kanton Zürich, Switzerland.

**Immunohistochemistry**

Immunohistological endpoints were analyzed on paraffin-embedded blocks for MIB1/Ki-67 (prediluted; Ventana-Roche, 790-4286), ADAM17 (1:200; Novus Biologicals, NBP2-15281), Amphiregulin (1:250; Atlas Antibodies HPA008720), ALCAM (1:150; Novocastra Lab, NCL-CD166), CD31 (1:10; DAKO, M0823) using a Discovery immunohistochemistry staining system (Ventana Medical Systems).

MIB1/Ki-67-positive tumor cells and amount of vessels (CD31) were counted in at least 4 randomly chosen visual fields (magnification 400x) in each xenograft (n=4 for each group). Viable areas of the whole tumor sections were quantified for specific ADAM17, ALCAM or Amphiregulin staining intensity. Each treatment group consisted of at least 4 animals and tumor sections were manually quantified for low, medium and high staining.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism (version 5). If not indicated otherwise, *in vitro* data were analyzed using the unpaired Student *t* test and data represent at
least three independently performed assays, with error bars indicating the standard deviation. In vivo treatment response was evaluated by two different statistical metrics. Area under the tumor volume curve (AUC) was analyzed by one-way ANOVA using the Tukey Test for pair-wise comparisons. Additionally, tumor growth delays were also calculated by Kaplan Meier analysis with Log-rank (Mantel-Cox) test for pair-wise comparisons. Immunohistochemical data were analyzed by one-way ANOVA on replicate tumor samples (n=4) and pair-wise analysis was performed using the Tukey Test. For all experiments, *P<0.05, **P<0.01, ***P<0.001.

Results

IR induces the secretion of multiple ADAM17 substrates

A semiquantitative differential large scale secretome analysis (>300 factors) was performed to identify auto- and paracrine factors that are secreted in response to IR. Using several growth factor-, chemo- and cytokine-specific antibody arrays, multiple, differentially secreted factors were identified in conditioned media (CM) derived from sham-irradiated and irradiated A549 lung adenocarcinoma cells, respectively (Fig. 1A and Supplementary Methods and Table 1). Detailed analysis of hits pointed towards a limited number of upstream effectors regulating IR-induced secretion of biologically active factors. Interestingly, several substrates of the metalloprotease ADAM17 were upregulated (see Supplementary Table 1 and Fig. 1A), in particular Amphiregulin and ALCAM. Of note, lactate dehydrogenase (LDH) activity, a control marker for unspecific cytolysis, was not increased in response to irradiation (Supplementary Fig. S1A). Thus, no general protein leakage could be observed, indicating specific, IR-mediated secretion of these ADAM17 substrates. Based on the increased abundance of ADAM17 substrates, we investigated regulation of ADAM17 in response to IR in detail.
To confirm IR-enhanced ADAM17 substrate secretion in additional cell lines (A549, H460, NCI-H125, CALU-3, CALU-6, all NSCLC and A431, vulval), ALCAM and Amphiregulin-directed ELISA were used to determine these ADAM17 substrates in a quantitative way in supernatants of irradiated tumor cells. Significant upregulation of both ADAM17 substrates could be detected in the NSCLC cell lines A549 and NCI-H125, and to different magnitudes in the other cell lines (Fig. 1B).

Enhanced secretion of these ADAM17 substrates could be due to IR-induced expression. However, only a marginal increase of Amphiregulin gene expression in A549 cells could be detected in response to IR, while no IR-induced ALCAM gene expression could be observed (Fig. 1C). Similar expression profiles could be determined in the additional lung carcinoma cell line NCI-H125 and at an early time point (4 hours) after irradiation (Supplementary Fig. S1B and S1C). Furthermore, protein levels of intracellular Amphiregulin and ALCAM in response to IR as determined in cell lysates did not change (Supplementary Fig. S1D).

To confirm these findings in vivo, we analyzed ALCAM secretion from irradiated A549-derived tumor xenografts in mice. Tumors and blood sera were harvested 48 hours after irradiation with 10 Gy. Ex vivo analysis of blood serum from mice with locally irradiated A549-derived tumor xenografts revealed significantly increased levels of ALCAM in comparison to ALCAM levels in blood serum from sham-irradiated mice (Fig. 1D). Unfortunately, Amphiregulin concentrations were below a minimal technically detectable concentration. Additionally, we also observed increased staining of both ALCAM and Amphiregulin in tumor sections derived from locally irradiated xenografts in comparison to sham-irradiated tumors (Fig. 1E and 1F). These results demonstrate that IR induces the secretion of multiple ADAM17 substrates across a panel of NSCLC cell lines and from A549-derived tumor xenografts.

**IR induces posttranslational modification and activation of ADAM17**
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To rule out the involvement of closely related ADAM10 in the regulatory process (30, 31), both ADAM17 and ADAM10 activities were determined in response to irradiation. Interestingly, irradiation only increased ADAM17 but not ADAM10 activity in a dose-dependent way (Fig. 2A). ADAM17 activity steadily increased over 30 hours in response to irradiation (Fig. 2B). As a control experiment, ADAM17 activity was determined in irradiated cells pretreated with the reactive oxygen scavenger N-acetyl cysteine (NAC). NAC completely attenuated IR-induced ADAM17 activity in both A549 and NCI-H125 cells while having no effect on its basal activity (Supplementary Fig. S2A). Significant induction of ADAM17 activity in response to irradiation was also observed in several other NSCLC cell lines. However, not all cell lines responded to IR with increased ADAM17 activity (Fig. 2C). Of note, gene expression of ADAM17 or ADAM10 did not increase in response to IR in A549 and NCI-H125 lung carcinoma cells at the 24 hour and at an early 4 hour time point, respectively (Fig. 2D and Supplementary Fig. S2B, S2C).

To acquire full activity, ADAM17 requires its conversion to the mature form lacking its inhibitory prodomain (18, 32). Reduced amounts of zymogen and concomitantly increased levels of the mature form of ADAM17 could be detected in response to increasing doses of IR (Fig. 2E). Likewise, densitometric analysis of the ADAM17 zymogen and active form of ADAM17 revealed that the overall levels of ADAM17 did not change in response to increasing doses of irradiation (Supplementary Fig. S2D). To probe for IR-induced ADAM17 expression and activity in vivo, A549-derived tumor xenografts were irradiated with a single dose of 10 Gy and tumors were harvested 48 hours after irradiation. Immunohistochemical staining of tumor sections did not reveal an IR-induced change of the ADAM17 expression pattern. However, a significant increase of ADAM17 activity was detected in tissue homogenates derived from irradiated tumors in comparison to ADAM17 activity in sham-irradiated tumors (Fig. 2F and 2G). These results indicate that posttranslational modification of ADAM17 confers resistance to irradiation.

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and not increased levels of ADAM17 gene expression accounts for IR-induced ADAM17 activity.

Targeting of ADAM17 activity sensitizes NSCLC cells for IR

Deregulated shedding of ADAM17 substrates might affect the response of NSCLC cells to IR. We therefore targeted ADAM17 with the ADAM17 inhibitor TMI-005 (Apratastat, 25 μM) and two different ADAM17-directed siRNAs (si-ADAM17#1, 2). Cellular pretreatment with TMI-005 and si-ADAM17#2 strongly downregulated ADAM17 activity, and both targeting approaches significantly decreased the proliferative activity of A549 cells (Fig. 3A). si-ADAM17#1 was overall less potent than si-ADAM17#2 to downregulate ADAM17 expression, its shedding and proliferative activity in A549 cells (Fig. 3A and Supplementary Fig. S3A-S3C). To quantify the effect of reduced ADAM17 activity on cellular radiosensitivity we determined clonogenicity of NSCLC cells in siRNA- and TMI-005-pretreated A549 and NCI-H125 cells in response to irradiation. Cellular pretreatment with TMI-005 and si-ADAM17#2, respectively sensitized both A549 and NCI-H125 cells to IR (Fig. 3B and 3C). The DEFs for TMI-005 at 37 % cell survival were 1.40±0.24 and 1.45±0.26 for A549 and NCI-H125 cells, respectively.

Several ligands of the EGFR are shed by ADAM17, which results in EGF-receptor tyrosine kinase (RTK) activation (33, 34). We therefore determined the potency of direct EGFR-targeting with the monoclonal antibody cetuximab in comparison to ADAM17-targeting as a radiosensitizing strategy. Cetuximab strongly abrogated short-term EGF-mediated activation of the EGF-RTK demonstrating its EGF-RTK-directed inhibitory activity (Supplementary Fig. S3D). Interestingly though cetuximab did not reduce short-term proliferative activity when used as single treatment modality (Fig. 3A) and did not affect clonogenicity on combined treatment with increasing doses of IR in A549 cells (Fig. 3D). Complementary clonogenic survival experiments were performed with recombinant Amphiregulin. While
recombinant Amphiregulin could activate the EGF-RTK, it did not rescue clonogenic survival of both ADAM17 wildtype and ADAM17 knockdown A549 cells in response to irradiation (Supplementary Fig. S3E and S3F).

**The proprotein convertase furin mediates IR-induced ADAM17 activation**

The proprotein convertase furin proteolytically activates ADAM17 and ADAM10 (35, 36). IR-induced ADAM17 conversion and activity was determined in A549 cells pretreated with the irreversible furin inhibitor decanoyl-RVKR-chloromethylketone (Dec-RVKR-CMK). Dec-RVKR-CMK counteracted IR-induced conversion of pro-ADAM17 to active ADAM17 and kept ADAM17 activity at basal level in irradiated cells (Fig. 4A and 4B). The furin inhibitor abrogated IR-enhanced ADAM17 activity also in NCI-H125 cells (Supplementary Fig. S4A). Furthermore Dec-RVKR-CMK sensitized A549 cells for IR and reduced clonogenicity in a supra-additive way (Fig. 4C). In order to detect a putative upstream IR-regulated mechanism, furin gene expression in response to IR was determined by qRT-PCR. Interestingly increased, IR-dose dependent furin gene expression was observed in A549 cells already 4 hours after irradiation (Fig. 4D).

To exclude the possibility that furin-inhibitor-based radiosensitization is due to reduced ADAM10 conversion, radiosensitization of A549 cells was also determined in cells pretreated with two different ADAM10-directed siRNAs. Knockdown of ADAM10 or pretreatment with the specific ADAM10 inhibitor GI254023 did only minimally reduce the proliferative activity of A549 cells and did not alter clonogenic survival in response to IR (Supplementary Figs. S4B, S4C and S4D). These results corroborate that IR specifically activates ADAM17, most probably due to IR-enhanced furin expression, and that targeting of ADAM17 enhances IR-induced cytotoxicity.

**ADAM17 mediates pro-survival ligand shedding and downstream signaling in response to IR**
To determine downstream effects of ADAM17 activity, ADAM17-mediated ligand shedding was analyzed in irradiated cells pretreated with either the ADAM17 inhibitor TMI-005 or the ADAM17-directed siRNA. Treatment of A549 and NCI-H125 cells with TMI-005 significantly reduced the basal and IR-induced concentrations of the secreted ADAM17-specific substrates ALCAM and Amphiregulin. Likewise, IR-induced ALCAM and Amphiregulin levels were downregulated in supernatants derived from A549 cells, pretreated with the ADAM17-directed ADAM17#2 siRNA (Fig. 5A and 5B).

The EGFR-directed monoclonal antibody cetuximab did not sensitize A549 cells for IR. Nevertheless we used the EGFR phosphorylation status at tyrosine 1173 (Y1173) as surrogate marker to probe for autocrine receptor activation in response to irradiation and ADAM17 inhibition. Enhanced EGFR phosphorylation was observed in cells 24 hours after irradiation. On the other hand the EGFR phosphorylation status was strongly reduced at the 24 hour time point in sham-irradiated and irradiated cells pretreated with TMI-005 but only minimally in cells pretreated with cetuximab (Fig. 5C). Enhanced EGFR phosphorylation over time was observed in control but not in ADAM17-directed siRNA knockdown cells comparing the EGFR phosphorylation status 1, 4 and 24 hours after irradiation (Fig. 5D). These results correlate with IR-enhanced conditioning of the media and accumulation of autocrine ligands.

To corroborate that ADAM17 regulates an autocrine loop, naïve A549 cells were stimulated with conditioned media (CM) derived from irradiated and sham-irradiated, control and ADAM17-directed siRNA knockdown cells, respectively (Fig. 5E). A partially enhanced EGFR phosphorylation status was observed in cells that were short-time incubated with CM derived from irradiated cells in comparison to cells that were short-time incubated with CM derived from sham-irradiated cells. Stimulation of naïve A549 cells with CM derived from sham-irradiated and irradiated, but ADAM17 knockdown cells resulted in lowest EGFR activation levels. Collectively, these results indicate that ADAM17 strongly regulates an
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autocrine growth factor loop and suggest that sustained and IR-activated ADAM17 substrate shedding might contribute to radiation resistance.

We next assessed the relevance of ADAM17 inhibition for radiation-induced cell killing and investigated different modes of cell death augmented by targeting ADAM17 in combination with IR. Microscopic evaluation of the cellular morphology of A549 cells in response to treatment with IR and ADAM17 inhibition revealed a flat and enlarged phenotype indicative for treatment-induced senescence (data not shown). A dose-dependent increase of senescence-associated β-galactosidase was determined in irradiated A549 cells. The amount of β-galactosidase-positive cells was only minimally increased in ADAM17 knockdown cells and in cells pretreated with TMI-005. Interestingly though, irradiation of TMI-005-pretreated or ADAM17 knockdown cells strongly increased the amount of β-galactosidase expressing cells (Fig. 5F and Supplementary Fig. S5A), indicating that ADAM17 increases the threshold for IR-induced senescence. The same senescent phenotype was observed in ADAM17-inhibited, irradiated NCI-H125 cells (Supplementary Fig. S5B). Inhibition of ADAM17 by either targeting approach did not enhance the overall minimal amount of IR-induced apoptosis in these NSCLC cells (Supplementary Fig. S5C). In contrast to ADAM17 targeting, cetuximab did not further promote the IR-induced senescence phenotype, which correlates with lack of radiosensitization by cetuximab at the level of the quantitative clonogenic cell survival assay (Fig. 5G, see also Fig. 3D).

Effect of TMI-005 and IR alone and in combination on the growth of A549-derived tumor xenografts

To determine the efficacy of ADAM17 inhibition in combination with IR in vivo, immunocompromised mice with A549-derived tumor xenografts were treated with the ADAM17 inhibitor TMI-005 (administered orally at 25 mg/kg twice daily for seven consecutive days, day 1-7) and locally irradiated during 3 consecutive days (1 Gy, days 3-5)
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(Fig. 6A). Treatment with TMI-005 or IR alone resulted in a minimal treatment response, whereas combined treatment exerted a strong supra-additive tumor growth delay. The combined treatment regimen of TMI-005 and IR resulted in a statistically significant reduction of the area under the tumor volume curve (AUC) in these NSCLC tumors (AUC control 26596±3986 vs. TMI-005 plus IR 13091±3196, P<0.001) (Fig. 6B). Furthermore, the absolute growth delay to triple the initial tumor volume (200 mm³) was most enhanced in response to the combined treatment modality when compared with the absolute tumor growth delay in response to TMI-005 or IR alone (13.0±2.1 days (TMI-005 plus IR) versus 3.76±2.18 days (TMI-005) and 3.09±2.17 days (IR) (P<0.001 for TMI-005 plus IR versus control and each monotherapy)), respectively, suggesting a positive interaction between TMI-005 and IR (Fig. 6C). Tumor tissue staining intensity of both ALCAM and Amphiregulin was significantly reduced on TMI-005 treatment, demonstrating the potency of the ADAM17 inhibitor (Fig. 6D and 6F). Irradiation alone only minimally reduced tumor proliferation, which might be due to the minimal treatment regimen and correlated with the small tumor growth delay induced by IR alone. In comparison to control tumors, the number of MIB-1-positive cells was significantly reduced to 55% and 57% in tumors treated with TMI-005 alone and in combination with IR, respectively ((control vs. TMI-005 plus IR, P<0.001) (Fig. 6E and 6F)). Furthermore no changes could be observed at the level of the tumor vasculature in response to treatment (Supplementary Fig. S6A).

Analysis of tissue morphology and integrity of co-irradiated skin adjacent to the tumor did not reveal any normal tissue toxicities in response to the different treatment modalities, and determination of body weight did not show any weight loss with TMI-005 during course of the treatment and follow up period. Furthermore, primary endothelial cells (HUVEC) and primary pulmonary fibroblasts were less sensitive to ADAM17 inhibition, and only tumor cells were sensitized to IR by TMI-005 (Supplementary Fig. S6B-S6D).
Collectively, these results indicate that combined treatment of TMI-005 and IR exerts a supra-additive tumor growth delay in these NSCLC cell-derived tumor xenografts and leads to better tumor control with minimal normal tissue toxicity.

**Discussion**

Increased expression of ADAM17 significantly correlates with enhanced aggressivity of NSCLC on the clinical level (27, 28), and ADAM17-shedded substrates are cytoprotective against different anti-tumor agents, as demonstrated in multiple tumor cell systems (4, 13, 37). Here, we identified that IR promotes furin-mediated activation of ADAM17 with increased shedding of ADAM17 substrates and thereby contributes to an IR-induced stress response in NSCLC. Subsequently, direct inhibition of ADAM17 lowered an intrinsic and treatment-induced threshold and strongly enhanced the efficacy of IR *in vitro* and *in vivo*. These results represent a promising rationale for the clinical assessment of a combined treatment modality of ADAM17 inhibitors with irradiation in NSCLC patients. We identified ADAM17 as direct target for radiosensitization based on our semiquantitative antibody array approach, screening for factors differentially secreted in response to irradiation. These factors were quantitatively validated by ELISA across a panel of NSCLC cell lines and could subsequently also be monitored in the blood serum derived from mice with locally irradiated tumors. As such, serum detection of ADAM17 substrates in irradiated tumor patients might also serve as a relevant, minimally invasive endpoint on the clinical level.

An increase of specific ADAM17 substrates after irradiation was previously detected on the preclinical and clinical level without investigating the underlying mechanism mediating radiation-induced secretion. For example, upregulated CXCL16 levels were observed in breast cancer cell lines in response to IR (38) and increased TGF-α levels were detected after irradiation in hormone-refractory prostate cancer patients (4). Increased ADAM17 substrate
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secretion in response to irradiation was also reported from lung and prostate carcinoma cells by Chung et al., investigating in vitro a signaling and radiosensitizing link between MEK1/2 inhibition and reduced epidermal growth factor ligand secretion (39). Importantly, we here demonstrated in vitro and in vivo, that IR-enhanced secretion of ADAM17 substrates is primarily driven by IR-dependent, posttranslational activation of ADAM17 and not by IR-increased expression of the respective ADAM17 substrates.

The exact regulatory steps required for ADAM17 activation or for any ADAM protease remain poorly understood and posttranslational modification by an either Furin- and/or MAP/ERK-kinase-upstream mechanism might be stimuli- and cell system-dependent. ADAM17 activity is also increased on cellular treatment with chemotherapeutic agents (40, 41) and ADAM17 activation by the antimetabolite 5-FU and the topoisomerase I inhibitor SN-38 primarily occurs in a Kras-mutated background and via the MAP/ERK kinase pathway. Our results demonstrate that irradiation promotes ADAM17 activation, but in a furin-mediated way and interestingly, in both Kras-wildtype (NCI-H125, CALU-3) and Kras-mutated NSCLC cell lines (A549, H460). More important we demonstrate the potency of direct pharmacological targeting of ADAM17 to sensitize for IR and independent of the Kras-status (A549, NCI-H125).

Inhibition of ADAM17 significantly reduces the secretion of multiple ADAM17 substrates. Insofar ADAM17 is a promising target as inhibition of its proteolytic activity not only affects a single receptor tyrosine kinase, e.g. the EGFR, and its downstream signal transduction cascades, but deregulates multiple auto- and paracrine-controlled processes. Only recently novel ADAM17-directed inhibitory antibodies have been developed and demonstrated broad antitumor potency both in EGFR ligand-dependent and -independent tumor cells (42, 43). While EGFR-directed agents, also in combination with radiation therapy, are a promising treatment strategy, many tumors rapidly acquire resistances during the course of treatment. Alternative concepts suggest combination therapies of EGFR inhibition in combination with
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inhibitors for other HER family receptors (44, 45). A major advantage of targeting ADAM17 includes its broad spectrum of downregulated substrates for all different HER family and other RTKs, which might increase the potency of direct ADAM17 targeting as compared to the inhibition of individual receptors.

A recent phase III clinical trial for locally advanced NSCLC investigated both radiotherapy dose escalation and the combined use of cetuximab, which both failed to further improve treatment outcome (21). Lack of responsiveness to EGFR inhibition might be linked to the Kras mutational status, however the specific role of Kras in NSCLC for the sensitivity to EGFR inhibitors alone and in combination with radiotherapy is still controversial (46-49). We used the EGFR phosphorylation status as surrogate marker to determine shedding of ADAM17-mediated EGFR ligands. Interestingly, irradiation induced prolonged activation of EGF-RTK activity, which correlated with increased IR-mediated ADAM17 activation. EGF-RTK activity was strongly counteracted by pharmacologic inhibition of ADAM17 or in ADAM17 knockdown cells with subsequent downregulation of EGFR ligand shedding. In contrast the EGFR-directed antibody cetuximab only partially reduced prolonged, IR-dependent RTK activation and did not sensitize for IR. Thus, ADAM17 might be a superior target for radiosensitization due to the inhibition of multiple downstream processes, but also pharmacodynamically due to sustained inhibition of ligand shedding and thereby reducing an IR-induced, acquired resistance mechanism. Since NSCLC is also treated with radiochemotherapy regimens including classic chemotherapeutics e.g. cisplatinum and taxanes, it will be important to probe in detail inhibition of these pleiotropic effects by ADAM17 inhibition in combination with chemo-radiation for NSCLC.

IR-induced ADAM17 activity and subsequently shedding of ADAM17 ligands are part of prosurvival responses. Shed ligands, such as HER family receptor ligands, ALCAM and other chemokines, might directly increase the cellular capacity to cope with IR-induced DNA damage, may influence the migratory capacity of target cells, or may attract immune cells to
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the tumor microenvironment, respectively. At this stage we have not evaluated the relevance of the individual shed ligands for an increased treatment threshold acting in an auto- and paracrine way in vivo. Furthermore the relevance of differential ADAM17 activities, differentially expressed substrates and the absolute amounts and interplay between ligands, shed to different extents from different tumors cells will also require in-depth investigations.

Though, the supra-additive effect of ADAM17 inhibitors on clonogenic survival of irradiated cells in vitro, indicates a mechanism for radiosensitization that is at least in part based on an autocrine loop. Furthermore we cannot dissect the impact of basal and radiation-induced ligand shedding for the ADAM17 substrate-mediated treatment threshold. However, limited downregulation of radiation-induced shedding by the less potent ADAM-directed si-ADAM17#1 suggest that already minor changes of ADAM17 activity directly affect an ADAM17-regulated survival and treatment threshold.

Our in vivo results demonstrate the potency of the combined treatment modality of the ADAM17 inhibitor TMI-005 and IR. TMI-005 significantly reduced the tumor proliferative capacity and exerted a supra-additive tumor growth delay in combination with IR. TMI-005, also known as Apratastat is an orally bioavailable small-molecular inhibitor of ADAM17, which was initially designed for the treatment of rheumatoid arthritis (RA) as it inhibits TNF-α release (50, 51). Adequate ADAM17-inhibitory clinical dosing for TMI-005 was established in a long-term, randomized phase II study with up to 390 RA patients (52). Though, the clinical program with TMI-005 and other closely related derivatives were stopped due to lack of efficacy in RA, related to constitutive activation of the TNF-α receptor on immunological cells, but not due to toxicity reasons (50, 53). Based on its low toxicity profile and target relevance independent of TNF-α, TMI-005 and other new classes of ADAM17 inhibitors have thus a strong rational for repositioning in NSCLC as part of a combined treatment modality with radiotherapy.
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References

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Figure legends

Fig. 1. IR induces the secretion of multiple ADAM substrates in vitro and in vivo (A)
Screening was performed with multiple antibody arrays (>300 factors) against conditioned media derived from irradiated and sham-irradiated A549 cells 24 hours after irradiation (5 Gy). Scatter plots for angiogenic and secretory analytes are depicted. (B) ELISA-quantified levels of ALCAM and Amphiregulin in supernatants derived from a panel of NSCLC cells and A431 cells (24 hours after sham-irradiation or irradiation), unpaired Student’s t test. (C) qRT-PCR-determined gene expression of ALCAM and Amphiregulin 24 hours after irradiation in A549 cells, one-way ANOVA test. (D-F) A549-derived tumor xenografts were sham-irradiated or irradiated with 10 Gy. Tumors and blood sera were harvested 48 hours after irradiation. (D) ALCAM concentration in murine blood serum derived from sham-irradiated or locally irradiated A549 tumor xenografts and quantified by ELISA; n≥4, unpaired Student’s t test. (E) Quantification of immunohistochemical staining intensities for ALCAM and Amphiregulin in tumor sections derived from A549-derived tumor xenografts (n=5). Whole tumor sections were quantified for specific ALCAM or Amphiregulin staining intensity. (F) Representative images of immunohistochemical staining of ALCAM and Amphiregulin in response to treatment. Scale bar 100 μm. Data are represented as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001.

Fig. 2. IR induces posttranslational modification and activation of ADAM17 in vitro and in vivo (A) ADAM10 and ADAM17 activities in A549 cells determined 24 hours after...
irradiation with increasing doses of IR. (B) IR-induced ADAM17 activity in A549 cells determined at different time points after irradiation, unpaired Student’s t test. (C) IR-induced ADAM17 activity in a panel of NSCLC cell lines and A431 cells, 24 hours after irradiation, unpaired Student’s t test. (D) qRT-PCR-based quantification of ADAM10 and ADAM17 gene expression 24 hours after irradiation in A549 cells, one-way ANOVA. (E) Western blots of ADAM17 zymogen and active form in A549-derived cell lysates 24 hours after irradiation with increasing doses of IR. (F) ADAM17 activity quantified in tumor lysates derived from A549-tumor xenografts in response to sham irradiation or irradiation with 10 Gy. Tumors were harvested 48 hours after irradiation; n≥4, unpaired Student’s t test. (G) Representative images of ADAM17-immunohistochemical staining in response to sham-irradiation or irradiation with 10 Gy. Scale bar 100 μm. Data are represented as mean ± standard deviation. *P<0.05, **P<0.01.

Fig. 3. Targeting of ADAM17 activity reduces the proliferative activity of NSCLC cells and sensitizes for IR (A) Short term proliferative activity of A549 cells pretreated with the ADAM17 inhibitor TMI-005, cetuximab or transfected with ADAM17- or luciferase-directed siRNA. A549 (B) and NCI-H125 (C) cells were pretreated with TMI-005 (25 μM) for 1 hour or transfected with ADAM17- or luciferase-directed siRNA (A549 cells, middle panel), and respective clonogenic cell survival was determined 7-10 days after treatment with increasing doses of IR. (D) Clonogenic cell survival of A549 cells pretreated with cetuximab (100 nM) for 1 hour and irradiated with increasing doses of IR. Data are represented as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001. Significance was measured by the unpaired Student’s t test.

Fig. 4. IR-induced ADAM17 activity is abrogated by the furin inhibitor Dec-RVKR-CMK (A-C) A549 cells were preincubated for 1 hour with the furin inhibitor Dec-RVKR-
CMK (50 μM) followed by irradiation with the indicated doses of IR. (A) ADAM17 protein levels were investigated by western blotting, 24 hours after irradiation. Quantification of band intensities from 3 independent experiments is depicted in the right panel. (B) Cell lysates were probed for ADAM17 activity, 24 hours after irradiation. (C) Clonogenic cell survival of A549 cells was determined 7-10 days after treatment according to (A). Data are represented as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001. Significance was measured by the unpaired Student’s t test. (D) qRT-PCR-based quantification of furin gene expression 4 hours after irradiation in A549 cells; one-way ANOVA.

Fig. 5. ADAM17 mediates pro-survival ligand shedding and downstream signaling in response to IR (A-D) A549 or NCI-H125 cells were pretreated with TMI-005 (25 μM) for 1 hour or A549 cells were transfected with ADAM17- or luciferase-directed siRNA (middle panel), and irradiated with the indicated doses of IR. (A-B) Secretion of ALCAM (A) and Amphiregulin (B) into the cell culture media was measured by ELISA, 24 hours after irradiation. (C) EGFR phosphorylation status in control, TMI-005 or cetuximab- pretreated A549 cells. Levels of phospho-EGFR (Y1173), EGFR, ADAM17 and β-actin were analyzed in whole cell lysates 24 hours after irradiation by western blotting. (D) EGFR-phosphorylation status (Y1173) in cell lysates derived from si-luciferase- or si-ADAM17-transfected A549 cells 1, 4 and 24 hours after irradiation and analyzed by western blotting. (E) EGFR-phosphorylation status in naive A549 cells stimulated with conditioned media collected from sham-irradiated or irradiated (10 Gy), si-luciferase or si-ADAM17#2 transfected A549 cells 24 hours after IR. Naive A549 cells were incubated with conditioned media for 1 hour, followed by whole cell lysate analysis and western blotting with the respective antibodies. (F-G) Quantification of senescence-associated β-galactosidase staining of sham-irradiated and irradiated A549 cells pretreated with (F) TMI-005 (25 μM), (G) cetuximab (100 nM) or (F) transfected with ADAM17- or luciferase-directed siRNA, 3 days...
Radiotherapy-induced ADAM17 leads to treatment resistance after irradiation. Data are represented as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001. Significance was measured by the unpaired Student’s t test.

Fig. 6. Effect of TMI-005 and IR alone and in combination on the growth of A549-derived tumor xenografts (A) Schematic illustration of the in vivo experimental setup and treatment regimen. (B) Tumor growth delay of A549-derived tumor xenografts in response to combined treatment with TMI-005 (25 mg/kg, twice daily for 7 days) and IR (3 x 1 Gy); n≥6, data is represented as mean ± standard error of the mean, one-way ANOVA Tukey test. (C) Kaplan-Meier survival analysis for A549 tumors reaching 600 mm³ tumor volume after treatment with TMI-005 or IR or their combination; n≥6, Log-rank (Mantel-Cox) test for pair-wise comparisons. (D-E) Immunohistochemical staining intensities for ALCAM, Amphiregulin and Ki-67 on tumor tissue from A549-derived tumor xenografts treated with TMI-005 and IR alone or in combination, data is represented as mean ± standard deviation; one-way ANOVA on replicate tumor samples (n=4), ; pair-wise analysis using the Tukey test. (F) Representative images of immunohistochemical staining of ADAM17, ALCAM, Amphiregulin and Ki-67 in response to treatments. Scale bar 100 μm. **P<0.01, ***P<0.001.
Fig. 3

A

Relative proliferative activity

<table>
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<tr>
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B

Survival fraction

Survival fraction versus Ionizing Radiation (Gy)

<table>
<thead>
<tr>
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C

Survival fraction

Survival fraction versus Ionizing Radiation (Gy)

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D

Survival fraction

Survival fraction versus Ionizing Radiation (Gy)

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Fig. 6

A

Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7

2x TMI-005 2x TMI-005 2x TMI-005 + IR (1Gy) 2x TMI-005 + IR (1Gy) 2x TMI-005 2x TMI-005 2x TMI-006

1 hour after last treatment IHC

Tumor Volume ~200mm³

B

Days after treatment start

Tumor volume (mm³)

control (7) TMI-005 (6) IR (6) TMI-005 + IR (7)

C

Days after treatment start

Proportion of mice with tumors < 600 mm³

control TMI-005 IR TMI-005 + IR

25mg/kg 3x1 Gy 3x1 Gy

D

Days after treatment start

% of tumor section area

ALCAM

Amphiregulin

High Medium Low

% of tumor section area

control TMI-005 IR TMI-005 + IR

E

Days after treatment start

Ki67 positive cells per visual field

control TMI-005 IR TMI-005 + IR

*** ns ns ***

F

Control TMI-005 IR TMI-005 + IR

ADAM17

ALCAM

Amphiregulin

Ki67

ns *** ns *** ***

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Secretome Signature Identifies ADAM17 as Novel Target for Radiosensitization of Non-Small Cell Lung Cancer

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