Kinome profiling of non-canonical TRAIL signaling reveals RIP1–Src–STAT3-dependent invasion in resistant non-small cell lung cancer cells

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Summary

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) triggers apoptosis selectively in tumor cells through interaction with TRAIL-R1/DR4 or TRAIL-R2/DR5 and this process is considered a promising avenue for cancer treatment. TRAIL resistance, however, is frequently encountered and hampers anti-cancer activity. Here we show that whereas H460 non-small cell lung cancer (NSCLC) cells display canonical TRAIL-dependent apoptosis, A549 and SW1573 NSCLC cells are TRAIL resistant and display pro-tumorigenic activity, in particular invasion, following TRAIL treatment. We exploit this situation to contrast TRAIL effects on the kinome of apoptosis-sensitive cells to that of NSCLC cells in which non-canonical effects predominate, employing peptide arrays displaying 1024 different kinase pseudosubstrates more or less comprehensively covering the human kinome. We observed that failure of a therapeutic response to TRAIL coincides with the activation of a non-canonical TRAIL-induced signaling pathway involving, amongst others, Src, STAT3, FAK, ERK and Akt. The use of selective TRAIL variants against TRAIL-R1 or TRAIL-R2 subsequently showed that this non-canonical migration and invasion is mediated through TRAIL-R2. Short-hairpin-mediated silencing of RIP1 kinase prevented TRAIL-induced Src and STAT3 phosphorylation and reduced TRAIL-induced migration and invasion of A549 cells. Inhibition of Src or STAT3 by shRNA or chemical inhibitors including dasatinib and 5,15-diphenylporphyrin blocked TRAIL-induced invasion. FAK, AKT and ERK were activated in a RIP1-independent way and inhibition of AKT sensitized A549 cells to TRAIL-induced apoptosis. We thus identified RIP1-dependent and -independent non-canonical TRAIL kinase cascades in which Src and AKT are instrumental and could be exploited as co-targets in TRAIL therapy for NSCLC.

Key words: TRAIL, Migration, NSCLC, RIP1, Kinome profiling, Src inhibitors

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (Field and Duffy, 2008). Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases. Although several attempts have been made to develop effective treatment strategies to combat lung cancer, still overall 5-year prognosis is less than 15% in NSCLC (Jemal et al., 2010). Despite recent successes in sub-populations of NSCLC patients using targeted agents, such as inhibitors of epidermal growth factor signaling (EGF) in patients with mutated EGF receptors, new therapies are urgently needed for improving treatment (Herbst et al., 2008). An attractive approach for the treatment of NSCLC is provided through the TRAIL receptors.

TRAIL receptors are attractive targets as their stimulation selectively mediates apoptosis activation in tumor cells while sparing normal cells (Gonzalvez and Ashkenazi, 2010). Apoptosis activation is mediated via two of five known TRAIL receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), whereas TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) are thought to encode non-functional receptors, also named decoy receptors. A fifth receptor is osteoprotegerin that acts as a soluble receptor. Apoptosis activation occurs upon ligand-induced TRAIL-R1 or TRAIL-R2 clustering leading to the assembly of the death-inducing signaling complex (DISC). In the DISC, Fas-associated protein with death domain (FADD) binds procaspase 8 or 10 in order to produce active caspase 8 subsequently leading to effector caspases activation and apoptosis (Gonzalvez and Ashkenazi, 2010).

Recombinant TRAIL preparations and TRAIL receptor specific agonistic antibodies have been produced that potently eradicate tumor cells of different origins, including NSCLC, and showed efficacy in various preclinical models (Stegehuis et al., 2010). Anti-cancer action of TRAIL receptor targeted agents

when applied as single agents, however, is frequently hampered by resistance in tumor cells. For example, approximately half of the NSCLC tumor cells are resistant to the apoptosis-inducing effect of TRAIL unless applied in combination with various conventional or biological agents resulting in sensitive cells (Stegehuis et al., 2010). Thus, although currently clinical testing of TRAIL receptor targeted agents shows only moderate efficacy, there is optimism that combination strategies with other therapeutics will overcome resistance and increase therapeutic benefit.

In contrast to its apoptosis-inducing activity, TRAIL has been reported to activate non-apoptotic signaling. In resistant tumor cells TRAIL receptors have been reported to activate pro-inflammatory (NF-κB), pro-survival (PI3K/AKT) and proliferation [mitogen-activated protein kinase (MAPK)] pathways (Newsom-Davis et al., 2009). Activation of these non-apoptotic signaling pathways involves the recruitment of a number of proteins to the receptors, such as receptor-interacting protein 1 (RIP1), TNF receptor associated factor 2 (TRAF2), TNF receptor type 1-associated death domain (TRADD) and NEMO/IKK-γ, thus forming secondary intracellular complexes (Jin and El-Deiry, 2006; Varfolomeev et al., 2005). The precise compositions and sequence of formation of the multiprotein complexes in which these factors reside are currently elusive. However, the death domain containing kinase RIP1 was shown to play a pivotal role in the activation of NF-κB, JNK, p38 and ERK1/2 by TRAIL, and in the activation of necroptosis by TNF (Declercq et al., 2009; Meylan and Tschopp, 2005). More recently TRAIL has also been implicated in the promotion of tumor cell invasion in pancreatic ductal adenocarcinoma and colorectal cancer models (Hoogwater et al., 2010; Trauzold et al., 2006), further illustrating pro-tumorigenic/non-canonical activity of TRAIL in resistant tumor cells. Clearly, the dual activity of TRAIL can hamper its clinical activity and delineating these unwanted effects is essential for developing strategies to revert them into pro-apoptotic signals. Together, these considerations prompted us for a systematic evaluation of the nature of these non-canonical TRAIL-dependent pathways.

In the present study we have examined the possible pro-tumorigenic effects of TRAIL on NSCLC cells. Particularly we found that TRAIL induces migration and invasion of TRAIL-resistant NSCLC cells. A kinome profiling strategy was used to dissect underlying molecular mechanisms and the identified kinases were further examined for involvement in non-canonical TRAIL signaling. Collectively, our results identified a novel TRAIL-induced Src-STAT3-dependent tumor cell migratory pathway and imply enhanced therapeutic benefit of TRAIL therapy when combined with Src and PI3K/Akt inhibition.

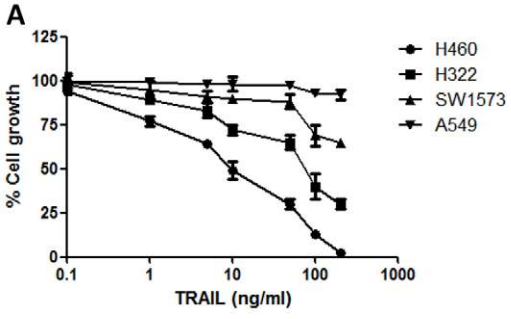
Results

TRAIL induces a spectrum from pro-apoptotic to pro-invasive responses in different NSCLC cells

TRAIL resistance of cancer cells hampers therapeutic exploitation of the TRAIL apoptotic pathway and may even provoke counterproductive effects. Also in vitro, different NSCLC cell lines differ markedly in their sensitivity to TRAIL as judged by the capacity of a 24 h TRAIL treatment to diminish cell viability as measured in MTT (tetrazolium dye) assays (Fig. 1A,B). Whereas H460 cells are highly sensitive for TRAIL [50% inhibition of cell growth (IC_{50})≈10 ng/ml], H322 cells require higher concentrations to respond with cell death to TRAIL treatment (IC_{50}≈81 ng/ml), and SW1573 and A549 cells seem incapable of inducing an apoptotic response to TRAIL (IC_{50}>200 ng/ml). Importantly, resistance to cell death is associated with TRAIL-induced pro-invasive effects in NSCLC. In wound healing assays, TRAIL treatment resulted in two- to threefold increased migratory activity of A549 cells when compared to untreated cells (Fig. 2A,B). Also, SW1573 cells showed an increase in migration activity in this assay upon TRAIL challenge, whereas TRAIL failed to induce migration in H322 and H460 cells in line with apoptosis activation in these cells. MTT assays performed in parallel showed that the enhanced 'wound healing' in A549 cells is not due to a TRAIL-mediated increase in proliferation (Fig 2E). Consistently, a decreased potential to respond to TRAIL stimulation with apoptosis is associated with increased invasion as detected in Matrigel-Transwell assays. TRAIL-apoptosis-resistant A549 cells exhibited approximately a twofold increase in invasive capacity following TRAIL stimulation when compared to untreated cells (Fig. 2C,D). Accordingly, SW1573 displayed enhanced invasion following TRAIL treatment, whereas H322 cells were not affected and H460 cells showed reduced invasion – correlating with the pro-apoptotic activity of TRAIL. We conclude that using this panel of NSCLC cell lines we can study a spectrum of TRAIL responses, ranging from full therapeutic sensitivity (H460 cells) to a cell line that almost exclusively displays non-canonical pro-oncogenic effects following TRAIL treatment (A549 cells), and we decided to exploit this situation to obtain fundamental insight into the molecular basis of the differential TRAIL response. Experiments were therefore initiated to characterize the signal transduction events involved.

Kinome profiling of TRAIL signaling in resistant and sensitive cells

The apparent dichotomy between canonical pro-apoptotic TRAIL responses in H460 cells and non-canonical pro-invasive TRAIL responses in A549 cells allowed us to delineate the signal transduction elements involved in TRAIL signaling. We resorted to using kinome profiling employing peptide arrays exhibiting 960



B

Cell line	IC_{50} (ng/ml)
H460	10.15 ± 2.11
H322	80.76 ± 10.29
SW1573	>200
A549	>200

Fig. 1. Cytotoxic effects of TRAIL in different NSCLC cell lines. (**A**) Cell growth was measured using an MTT assay following treatment with increasing concentrations of TRAIL for 24 h. (**B**) Calculated IC_{50} values for TRAIL in the different NSCLC cells.

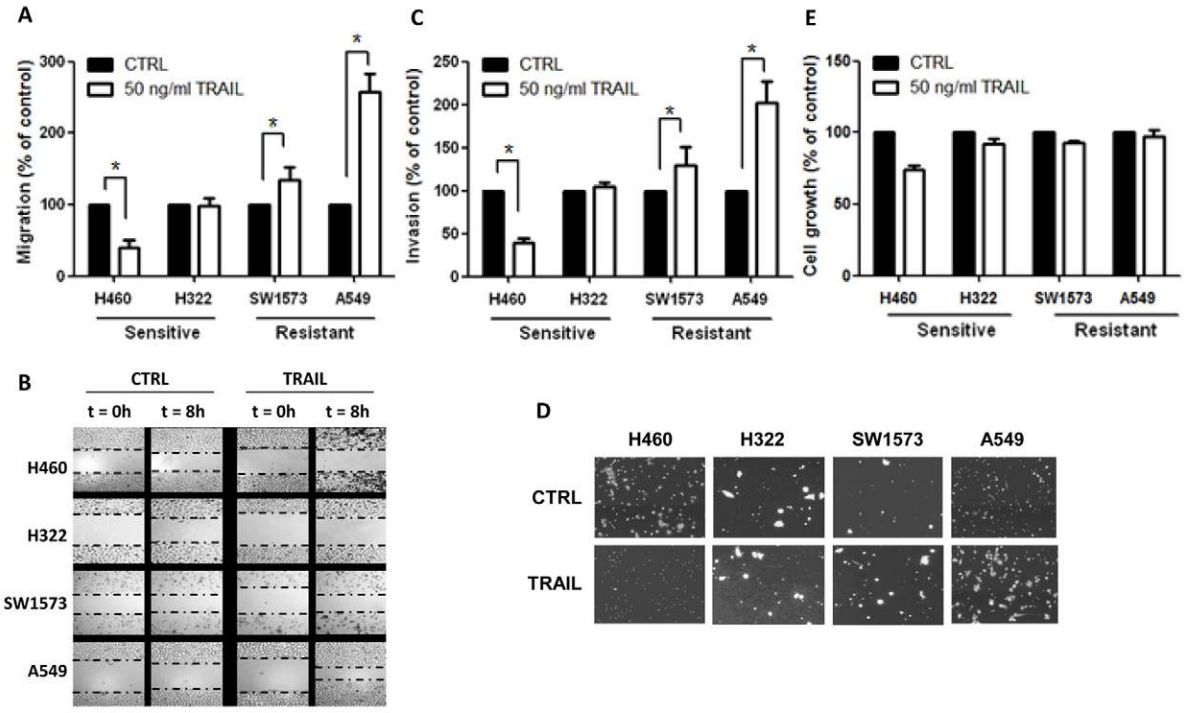


Fig. 2. TRAIL induces migration and invasion in resistant NSCLC cells. (**A**) Migration of H460, H322, SW1573 and A549 cells treated for 15 min with TRAIL. (**B**) The wound closure was measured after 8 h, as shown in a representative wound-healing experiment. (**C**) Effect of TRAIL on the invasiveness of NSCLC cell lines assessed with Matrigel-Transwell assays for the determination of invasiveness as shown in D. (**E**) Cell growth determined by MTT assays of cells treated with 50 ng/ml TRAIL for 15 min and further culturing for 8 h with refreshed medium.

different kinase pseudosubstrates that provide comprehensive descriptions of the cellular kinome. This allowed us to contrast TRAIL-induced kinomic changes in the canonically signaling H460 cells to those in the non-canonical signaling A549 cells. To this end, cells were treated for 15 min either with vehicle control or with 50 ng/ml TRAIL in order to detect early kinase activation events. The obtained kinome profiles of treated and untreated cells of each cell line revealed substantial differences between TRAIL-induced kinase activity in A549 and H460 cells (for a selection see Table 1). Importantly, a signal transduction cascade emerged in which A549 cells respond to TRAIL by activation of a proximal tyrosine kinase activity associated with several signaling events like increased JAK2 signaling to STAT3 and enhanced PI3K activity led to activation of the Akt/mTOR/S6 kinase signaling pathway. There is also increased activity of Rac, Rho and Src signaling able to enhance FAK activity and phosphorylation to other cytoskeleton remodeling proteins (e.g. cortactin, vimentin; see Table 1) and increased activity of the Raf/MEK/ERK signaling cassette.

Non-canonical TRAIL-dependent kinase activities are reflected in altered levels of target protein phosphorylation

The kinome profiling assay measures kinase activities, but within a cell kinase activities are counterbalanced by the enzymatic activity of phosphatases. Hence it is important to establish if the differences in kinase activities as determined by kinome profiling are reflected in altered levels of phosphorylation of target proteins. In accordance with the PepChip results, western blot experiments revealed that already within 5 minutes after TRAIL treatment Src and ERK were phosphorylated, whereas Akt, STAT3 and FAK phosphorylation were detectable at 10 to 15 min post-treatment in A549 cells (Fig. 3A). However, not all identified possible targets or upstream kinases could be corroborated. For example, TRAIL-induced VAV2 and JAK2 phosphorylation was not observed by western blotting, while we did not find evidence of activation of ROCK (not shown). Basal and TRAIL-induced phosphorylation of a number of kinases, including Src, Akt, ERK, FAK and STAT3, was variable in the other cell lines (Fig. 3B). In TRAIL-resistant SW1573 cells increases in phosphorylation of Src ERK, and FAK by TRAIL were seen. In H460 no detectable effects were observed and in intermediate TRAIL-sensitive H322 cells, ERK phosphorylation was slightly elevated after TRAIL exposure (Fig. 3B). Thus the non-canonical TRAIL signaling pathway identified by kinome profiling is reflected in meaningful changes in target protein phosphorylation.

Non-canonical TRAIL responses are mediated through the TRAIL-R2

Earlier we generated TRAIL variants that selectively engage either TRAIL-R1 or TRAIL-R2, named 4C7 and DHER, respectively (Reis et al., 2010; van der Sloot et al., 2006). We found these variants to be more potent than wild-type TRAIL in triggering apoptosis in a tumor cell line-dependent fashion. We now employed these variants to determine the TRAIL receptor mainly responsible for the induction of non-canonical pro-invasive properties of TRAIL. A549 cells express both TRAIL-R1 and -R2, whereas decoy receptors are hardly detectable on the

Table 1. Effects of TRAIL stimulation on kinase activity in TRAIL-sensitive H460 cells and TRAIL-resistant A549 cells

Peptide used for kinase reaction	Phosphorylation site in protein	Upstream kinase	Ratio H460	Ratio A549
KTRDQ**Y**LMWLT	p85-PI3K$_{Tyr580}$	TyrK	1.25	1.67*
LTIDR**Y**LAIVH	CCR2$_{Tyr139}$	JAK2	0.97	1.31*
GSAAP**Y**LKTKF	STAT3$_{Tyr705}$	JAK2/Src	1.09	1.75*
TEDQY**S**LVEDD	p85-PI3K$_{Ser608}$	p85-PI3K	0.88	14.64*
RLRTH**S**IESSG	MEK2$_{Ser80}$	Akt	0.68	1.29**
RPRSC**T**WPLPR	FKHR$_{Thr24}$	Akt	0.50	1.59*
RRRAA**S**MDSSS	AFX$_{Ser196}$	Akt	0.69	1.79**
RSRHS**S**YPAGT	Bcl2$_{Ser72}$	Akt	0.90	1.37**
ELRRM**S**DEFVD	Bcl2$_{Ser118}$	Akt	0.71	1.40*
RLRPL**S**YPQTV	Rac1	Akt	0.60	1.89*
RTRTD**S**YSAGQ	mTor$_{Ser2448}$	mTor	1.00	1.50*
RRRMA**S**MQRTG	p300EBP$_{Ser1834}$	p70S6K	1.77	1.52*
RRGDS**Y**DLKDF	Vav1$_{Tyr441}$	?	0.63	1.75***
GGDDI**Y**EDIIK	Vav2$_{Tyr172}$	?	0.88	2.82***
ASRPS**S**SRSYV	Vimentin$_{Ser26}$	PAK	0.67	1.79*
AEDST**Y**DEYEN	Cortactin$_{Tyr486}$	Src	0.54	2.30**
NVVPL**Y**DLLLE	Estrogen receptor	Src	1.16	1.42**
EDSTY**Y**KASKG	FAK$_{Tyr577}$	Src	1.50	1.29*
IESDI**Y**AEIPD	FAK2$_{Tyr402}$	FAK	1.13	1.71*
MEDYD**Y**VHLQG	p130Cas$_{Tyr666}$	FAK	0.63	2.44***
KTNLS**Y**YEYDK	BMX$_{Tyr40}$	FAK	1.31*	1.84*
IPRRT**T**QRIVA	CRMP2$_{Thr555}$	ROCK	0.60	2.58***
RDKYK**T**LRQIR	Moesin$_{Thr564}$	ROCK	1.25	2.72*
INRSA**S**EPSLH	Raf1$_{Ser621}$	Raf1	0.77	1.46*
DSMAN**S**FVGTR	MEK1$_{Ser222}$	Raf1	0.51	1.89**
VKRRP**S**PYEME	TAL1$_{Ser172}$	ERK	0.75	2.25***
PPVPA**T**PYEAF	SPIB$_{Thr56}$	ERK	1.25	1.74*
LKGPG**T**PAFPH	C/EBP$_{Thr74}$	ERK	1.37*	1.17*
KVEPA**S**PPYYS	PPAR$_{Ser112}$	ERK	0.94	1.58*
NLLPM**S**PEEFD	STAT$_{Ser727}$	ERK	0.65	1.49*

Peptide arrays were used on the selected signal-transduction-relevant undecapeptide kinase substrates listed. Values are ratios (15 min TRAIL/control) of STORM phosphoimager units (24 h exposure) of 12 replicates. *P<0.05; **P<0.01; ***P<0.001.

cell surface (Fig. 4A), in agreement with our previous findings (Voortman et al., 2007). As shown in Fig. 4B,C, migration and invasion of A549 cells is enhanced when stimulated with the TRAIL-R2 selective ligand (DHER), and not by TRAIL-R1 selective 4C7. Thus, the non-canonical migratory and invasive features of TRAIL in resistant NSCLC cells are predominantly mediated by TRAIL-R2. Furthermore, comparing DHER- with TRAIL-treated A549 cells showed a similar kinase phosphorylation profile (Fig. 4D).

The migratory and invasive effects of TRAIL are mediated by RIP1 and involve activation of Src

We next examined the role of RIP1 kinase in signaling pathways that mediate migration and invasion. RIP1, part of the non-apoptotic signaling complex, is known to be involved in TRAIL-induced kinase activation although its precise role is not well understood (Falschlehner et al., 2007; Varfolomeev et al., 2005). RIP1 was silenced using a selective shRNA in A549 and H460 cells and knockdown was confirmed by western blotting (Fig. 5A). A549-shRIP1 cells clearly showed a decrease in TRAIL-dependent migration in wound healing assays (Fig. 5B). The invasive effect of TRAIL was also abrogated in the absence of RIP1 (Fig. 5C). In contrast, there were no differences in TRAIL-dependent migration and invasion in H460-shRIP1 cells compared to control cells. RIP1 knockdown in A549 cells did not result in a detectable increase in TRAIL sensitivity (Fig. 5D).

The involvement of RIP1 in TRAIL-induced kinase activation was examined in more detail. As depicted in Fig. 5E, TRAIL-induced Src and STAT3 phosphorylation is abrogated in A549-shRIP1 cells. However, RIP1 depletion did not affect the phosphorylation of Akt, ERK and FAK following TRAIL treatment. In H460 cells phosphorylation of Src, Akt and ERK was not significantly altered (Fig. 5F). The abrogation of TRAIL-induced migration/invasion in A549-shRIP1 cells together with a lack of Src and STAT3 activation is suggestive of a role of these kinases in controlling the metastasis-prone events.

TRAIL-dependent activation of a RIP1-Src-STAT3 cascade mediates invasive behavior

To further investigate the role of Src in mediating TRAIL-induced migration/invasion, Src expression was silenced with shRNA in A549 cells. In these cells TRAIL-induced migration and invasion was completely inhibited (Fig. 6A,B). Knockdown of Src did not result in an increase in cell death after TRAIL treatment when compared to the empty vector control (Fig. 6C). Thus, the observed decrease in migration and invasion was not a consequence of apoptosis induction. Furthermore, three different Src inhibitors, PP2, dasatinib and saracatinib were employed at concentrations reported to be optimal for selective inhibition of Src as described previously by others (Johnson et al., 2005; Purnell et al., 2009). As shown in Fig. 6D,E, migration and invasion by TRAIL was completely repressed with each of the Src inhibitors. In addition, these inhibitors particularly when applied at a higher concentration did to some extent enhance TRAIL-induced apoptosis in A549 cells, most notably dasatinib (Fig. 6F). In SW1573 cells, Src inhibition by PP2 also repressed the migratory and invasive effects of TRAIL without significantly affecting apoptosis (Fig. 6G-I). Further delineation

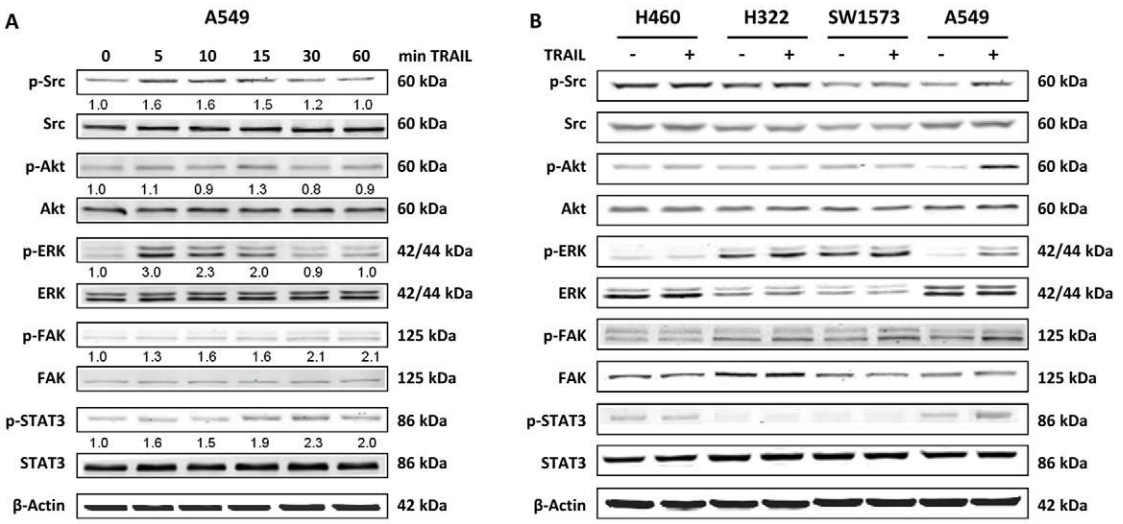


Fig. 3. TRAIL-induced kinase activation in NSCLC cells; confirming kinases identified by PepChip kinase arrays. (**A**) The indicated kinases were evaluated for TRAIL-induced phosphorylation in A549 cells over 60 min by western blotting, using antibodies detecting phosphorylated and total kinase. The numbers between the blots are the ratios of the intensity of bands for the phosphorylated form to the band of the total protein. The untreated control was set at 1.0. (**B**) Kinase activation in H460, H322, SW1573 and A549 cells after treatment with 50 ng/ml TRAIL for 15 min, determined by western blotting.

of the pathway in A549 cells, using the STAT3 inhibitor 5,15-diphenylporphyrin (5,15-DPP) and the RIP1 inhibitor necrostatin-1, showed attenuation of the migrating and invasive effects of TRAIL (Fig. 7A,B). Necrostatin-1 did not affect apoptosis sensitivity in resistant A549 cells (Fig. 7C), which is in line with shRIP1 knockdown results. Together these results implicate a TRAIL-induced RIP1-Src-STAT3 cascade to drive A549 NSCLC cell invasion.

TRAIL-induced kinase activation and effects on migration, invasion and apoptosis

Finally, we explored possible interactions between the identified TRAIL-induced kinase cascades. Knockdown of Src in A549 cells prevented, and even decreased, the phosphorylation of STAT3 after TRAIL application. Also TRAIL-induced FAK phosphorylation appeared to depend on Src, whereas p-Akt and p-ERK levels were not affected in A549-shSrc cells (Fig. 8A).

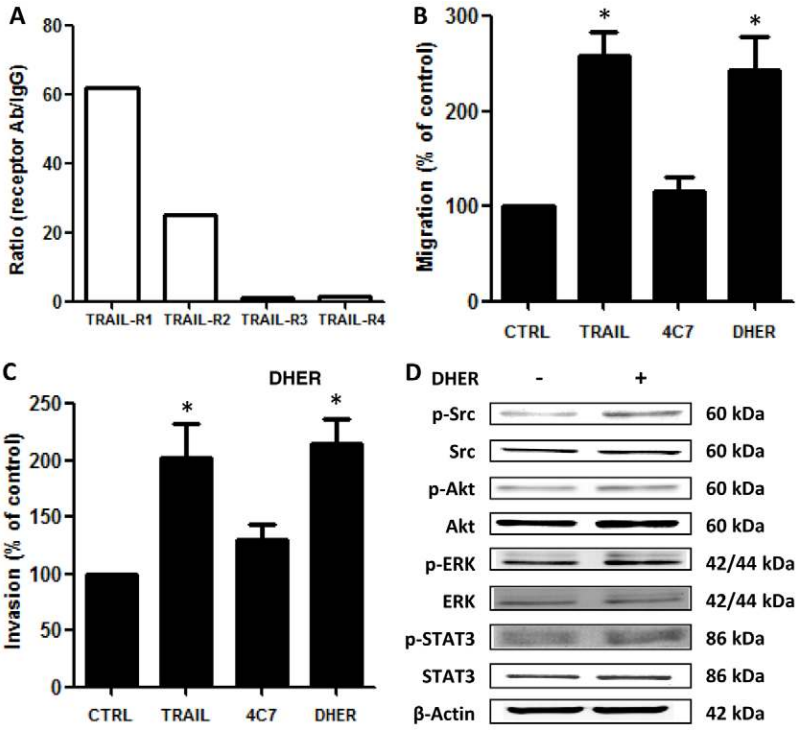


Fig. 4. TRAIL induces migration and invasion in A549 cells mainly through TRAIL-R2. (**A**) TRAIL receptor surface expression in A549 cells determined by FACS analysis. (**B**) Effect of TRAIL-R1 (4C7)- and TRAIL-R2 (DHER)-specific TRAIL variants on migration. (**C**) Effect of the TRAIL variants on invasion. Levels of untreated cells were set at 100%. Values are means ± s.d. of three experiments. *$P<0.05$, *t*-test compared with CTRL. (**D**) Western blots showing phosphorylation status of the indicated kinases in A549 cells after 15 min treatment with 50 ng/ml DHER.

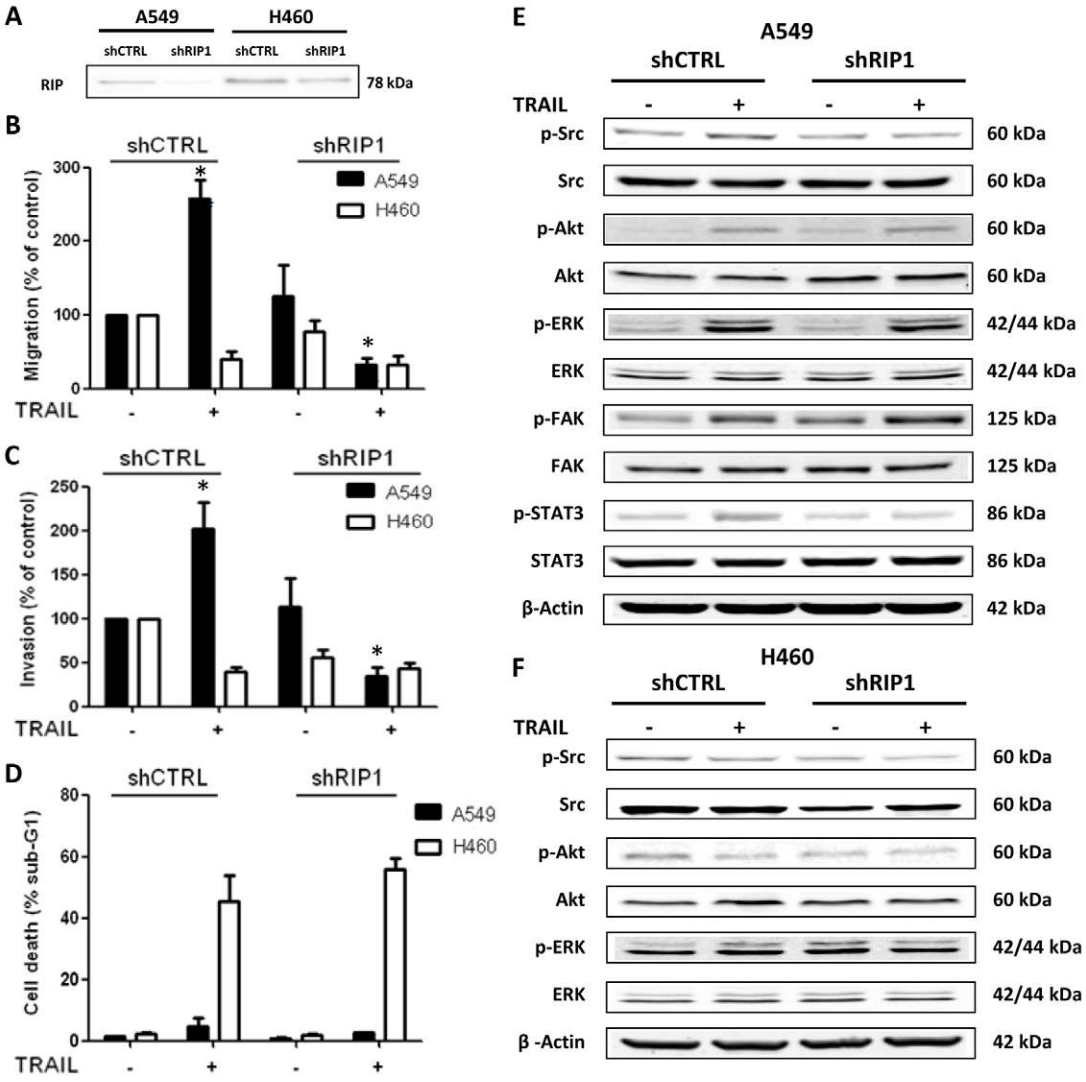


Fig. 5. TRAIL-induced migration and invasion, and Src-STAT3 activation is RIP1 dependent. (**A**) Expression of RIP1 was effectively silenced by a specific shRNA in H460 and A549 cells, as determined by western blotting. (**B**) TRAIL-dependent cell migration was determined in wound healing assays. (**C**) Invasiveness, as determined by Matrigel-Transwell assays. (**D**) The effect of RIP1 knockdown on TRAIL-induced apoptosis in A549 and H460 cells. (**E**,**F**) Western blots showing the effect of RIP1 knockdown on TRAIL-dependent kinase activation (50 ng/ml TRAIL for 15 min) in A549 cells (E) and H460 cells (F). Values are means ± s.d. of three experiments. *$P<0.05$, *t*-test, compared with each other.

On the other hand, inhibition of Akt by LY294002 and ERK by PD098059 also reduced migration and invasion induced by TRAIL (Fig. 8B). Interestingly, inhibition of Akt converted TRAIL resistant A549 cells into apoptosis sensitive cells. In conclusion, we identified novel parallel branches of TRAIL-induced kinase activation that mediate metastases-prone and/or pro-survival effects (see also Fig. 8C).

Discussion

The TRAIL receptor pathway is currently therapeutically exploited for selective activation of apoptosis in tumor cells. However, stimulation of the TRAIL receptors in preclinical models can also activate unwanted non-apoptotic/non-canonical signaling leading to proliferative, pro-survival and even pro-invasive effects. In the present study using a panel of TRAIL sensitive and resistant NSCLC cell lines we demonstrated non-canonical signaling in resistant cells that enhanced their migratory and invasive properties. Interestingly, whereas the NSCLC cells express both TRAIL-R1 and TRAIL-R2 on their cell surface we only found TRAIL-R2/DR5 to mediate these effects, thus illustrating that the TRAIL receptors have different signaling properties. Intriguing in this respect is an earlier described correlation of high TRAIL-R2 levels in advanced stage NSCLC patients with increased risk of death (Spierings et al., 2003). The underlying causes of differential signaling are still elusive and are subject of further studies.

Using peptide arrays exhibiting 1024 specific consensus sequences for protein kinases we identified substrates and corresponding kinases that are stimulated following TRAIL exposure in A549 cells contrasting findings in sensitive H460 cells. Subsequent confirmation of kinase phosphorylation by western blotting allowed us to identify several kinases that are activated by TRAIL in resistant A549 cells (see also Fig. 8C for a schematic overview). In particular activation of the Src-STAT3

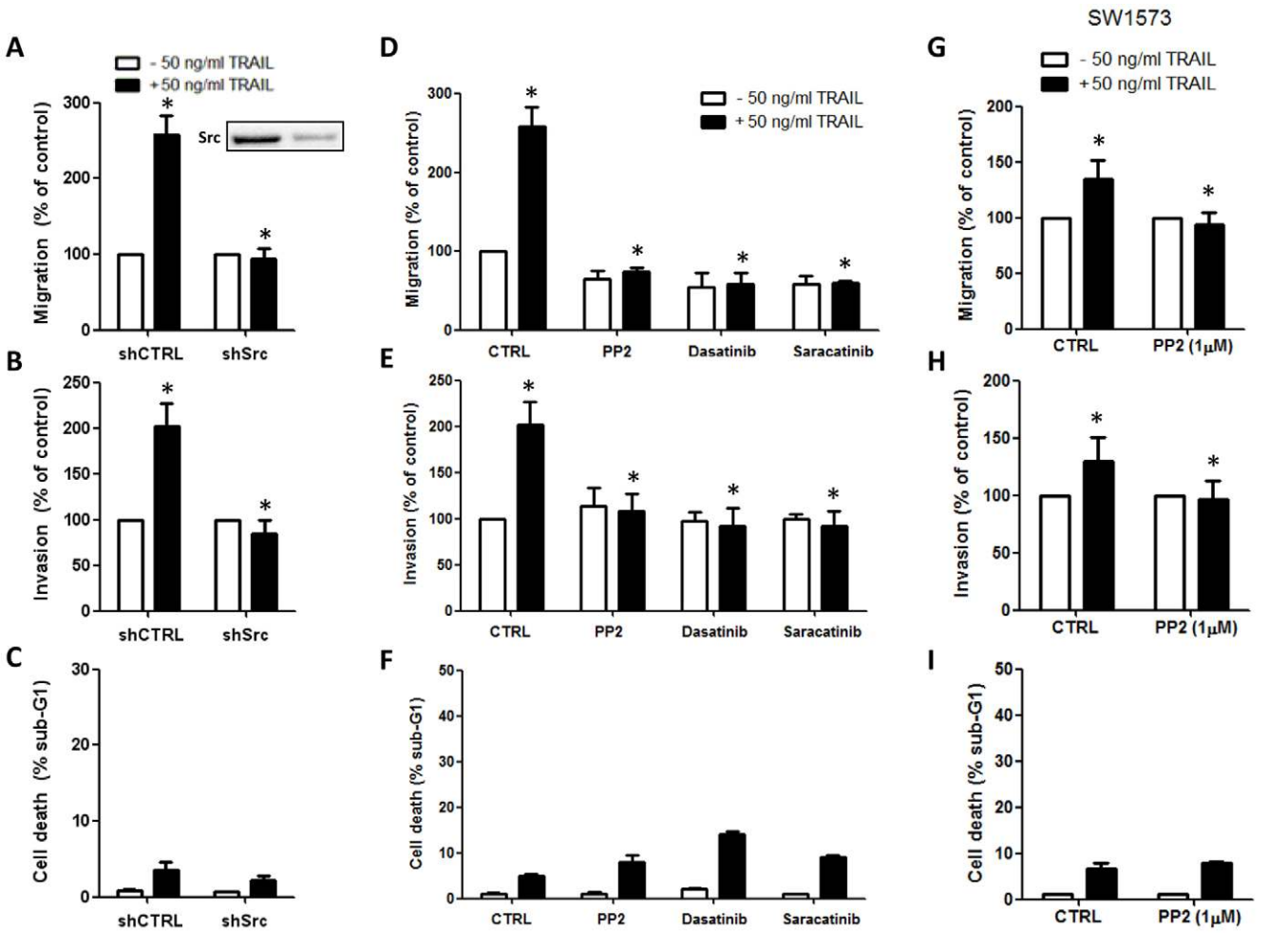


Fig. 6. Inhibition of Src blocks TRAIL-induced migration and invasion of A549 and SW1573 cells. (**A**) Src knockdown using specific shRNA (confirmed by western blotting in insert) blocks TRAIL-induced migration in wound healing assays. (**B**) Src knockdown inhibits TRAIL-induced invasion in Matrigel-Transwell chamber assays. (**C**) Silencing Src does not result in an increase in cell death after TRAIL treatment in A549 cells. (**D-H**) A549 cells were incubated with or without chemical inhibitors of Src, PP2 (1 μM), dasatinib (0.01 μM) or saracatinib (1 μM), to determine the effects on migration (D), invasion (E) and cell death (F) of treatment with 50 ng/ml TRAIL. (**G-I**) In SW1573 cells PP2 (1 μM) blocks TRAIL-induced migration (G), and inhibits the invasive effect of TRAIL (H) but has no effect on TRAIL-induced apoptosis (I). Values are means ± s.d. of three independent experiments. *$P<0.05$, *t*-test, compared with 50 ng/ml TRAIL.

axis was important for mediating TRAIL-induced migration and invasion. The non-receptor protein tyrosine kinase Src is a known activator of signal transduction pathways controlling various key cellular processes such as cell division, proliferation, survival and motility (Gallick, 2004). Elevated Src protein levels and/or kinase activity have been reported in 50-80% of lung cancer cases and correlates with poor patient survival (Mazurenko et al., 1992). Src is a known mediator of tumor cell migration and invasion as it leads to reorganization of the cytoskeleton and modulation of the cell adhesion system (Guarino, 2010). Downstream of Src we identified STAT3 as a mediator of migration. This is not unprecedented since recently Src-induced STAT3 activation has been implicated in the formation of podosome structures in primary murine cells facilitating cell migration (Mukhopadhyay et al., 2010). STAT3 inhibitors have also been reported to inhibit the migration of prostate cancer cells (Abdulghani et al., 2008). Mechanisms described by which STAT3 can promote cell migration include binding of STAT3 to βPIX leading to Rac1 activation (Teng et al., 2009) and direct phosphorylation of fascin, an actin-bundling protein, by STAT3 (Snyder et al., 2011). The precise downstream effectors of STAT3 signaling in TRAIL-induced migration/invasion in NSCLC cells remains to be explored. Another mechanism through which Src can stimulate invasion is via the activation of epithelial-mesenchymal transition (EMT) (Boyer et al., 2002). The acquisition of mesenchymal properties by epithelial cells characterized by for example loss of E-cadherin and gain of fibronectin expression has been found to stimulate tumor cell dispersion (Kalluri and Weinberg, 2009). However, we did not detect clear changes in the expression of these markers in response to TRAIL in NSCLC cells (not shown). Other Src-dependent signals that have been implicated in migration include activation of the Rho/ROCK/LIMK, Ras/MAPK, PI3K/Akt, FAK/paxillin/CAS pathways (Guarino, 2010). Although the kinome analyses identified possible ROCK activity after TRAIL exposure, we were not able to confirm ROCK

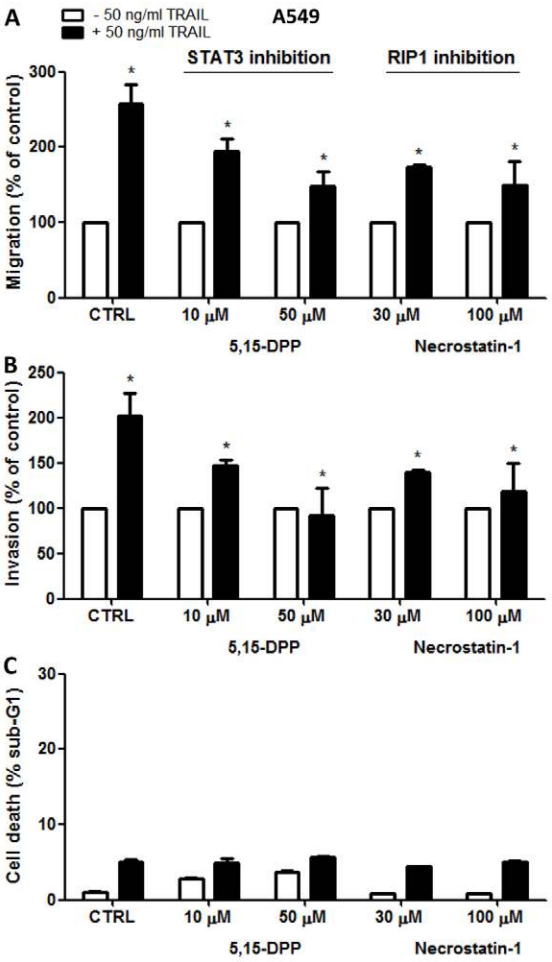


Fig. 7. Inhibition of STAT3 and RIP1 blocks migration/invasion induced by TRAIL in A549 cells. (**A**) A549 cells were incubated with or without chemical inhibitors of STAT3 (5,15-DPP) or RIP1 (necrostatin-1) and with or without TRAIL (50 ng/ml). Migration was determined after 8 h with the wound healing assay. (**B**,**C**) Invasion after 8 h (B) and cell death after 24 h (C) incubation were also determined. Values are means ± s.d. of three independent experiments. *$P<0.05$, *t*-test, compared with 50 ng/ml TRAIL.

activation in western blots and co-treatment with a ROCK inhibitor did not prevent TRAIL-induced invasion (not shown). On the other hand, knockdown of Src in A549 cells inhibited TRAIL-dependent FAK phosphorylation suggesting a possible role of this pathway as well. FAK is a non-receptor protein tyrosine kinase known to stimulate migration/invasion when in complex with Src (van Nimwegen and van de Water, 2007). Of note, in SW1573 cells TRAIL resulted in Src-dependent FAK phosphorylation, but not STAT3 phosphorylation. This may be related to the smaller stimulatory effect of TRAIL on migration/invasion in SW1573 cells (1.5-fold) when compared to A549 cells (2- to 3-fold). Inhibition of ERK did partially suppress invasive behavior of A549 cells, however, TRAIL-induced ERK phosphorylation was independent of Src as was also the case for Akt activation. Active Src has the ability to phosphorylate caspase-8 at tyrosine 380 resulting in blocking of its pro-apoptotic function (Cursi et al., 2006). However, we could not detect phosphorylated procaspase-8 (not shown) and shRNA-mediated knockdown or chemical inhibition of Src by dasatinib and saracatinib did prevent TRAIL-induced invasion, but failed to effectively sensitize for apoptosis in A549 cells, pointing to other mechanisms of resistance.

TRAIL stimulatory effects on tumor cell migration and invasion have been previously reported to be mediated by NF-κB in apoptosis resistant cholangiocarcinoma cancer cells (Ishimura et al., 2006), and by TRAIL-dependent upregulation of interleukin-8 and chemoattractant protein 1 in pancreatic ductal adenocarcinoma cells (Trauzold et al., 2006). A more recent study has shown that TRAIL-induced migration in colon cancer cells involves oncogenic K-Ras and Raf-1 that convert death receptors into invasion-inducing receptors by suppressing the ROCK/LIM kinase/cofilin pathway (Hoogwater et al., 2010). However, the K-Ras status not always predicts the outcome of death receptor signaling, since for example H460 NSCLC cells in our study are highly sensitive for TRAIL-induced apoptosis despite the presence of oncogenic K-Ras. Thus, in NSCLC cells TRAIL-induced Src activation appears to be the main route responsible for migration and invasion.

RIP1 is a serine threonine kinase belonging to the RIP family involved in promoting pro-survival, inflammatory and pro-apoptotic signals depending on the signal and tumor type (Meylan and Tschopp, 2005). RIP1 is part of the secondary complex and has been associated with non-apoptotic functions of TRAIL (Varfolomeev et al., 2005). To understand the role of RIP1 in our model shRNA-mediated knockdown as well as RIP1 inhibition by necrostatin-1 revealed prevention of TRAIL-induced Src-STAT3 activation and migration/invasion, corroborating the importance of this pathway. In contrast to a previous report we did not observe sensitization for TRAIL-induced apoptosis in A549 RIP1 knockdown cells (Song et al., 2007). Regarding Akt activation, in prostate adenocarcinoma cells TRAIL-dependent Src activation has been reported to result in activation of PI3K-Akt signaling (Song et al., 2010). We found in NSCLC cells TRAIL to activate a Src-independent mechanism responsible for Akt activation, and moreover, Akt and also ERK activation was independent of RIP1. The activation of Akt and ERK may be a more indirect consequence of TRAIL pathway activation. For example, in colorectal cancer cells TRAIL was found to activate EGFR and HER2 through Src family kinases (SFK) that in turn activated the cell surface protein A Disintegrin And Metalloproteinase-17 (ADAM-17) also known as Tumor Necrosis Factor Converting Enzyme (TACE) leading to cleavage and shedding of TGF-α. Subsequently, TGF-α activated the EGFR/HER2 pro-survival signaling pathways in an autocrine and paracrine manner (Van Schaeybroeck et al., 2008). Furthermore, we found TRAIL to be able to phosphorylate FAK in RIP1 knockdown NSCLC cells, in which TRAIL-induced Src activation was prevented. Thus, FAK can be activated in a RIP1-dependent and -independent way, indicating more complex interactions at the level of RIP1, Src and FAK. In this study we identified Akt as an important mediator of TRAIL resistance in NSCLC cells. This confirms earlier studies where the PI3K inhibitors wortmannin or LY-294002 and the Akt-inhibitor perifosine were shown to cooperate with rhTRAIL to induce apoptosis in NSCLC cells involving increased TRAIL-R2 expression and a reduction of c-FLIP levels (Elrod et al., 2007; Kandasamy and Srivastava, 2002).

Taken together, it can be concluded from our work that TRAIL non-canonical signaling in NSCLC involves a parallel activation of RIP1-dependent and -independent mechanisms that stimulate

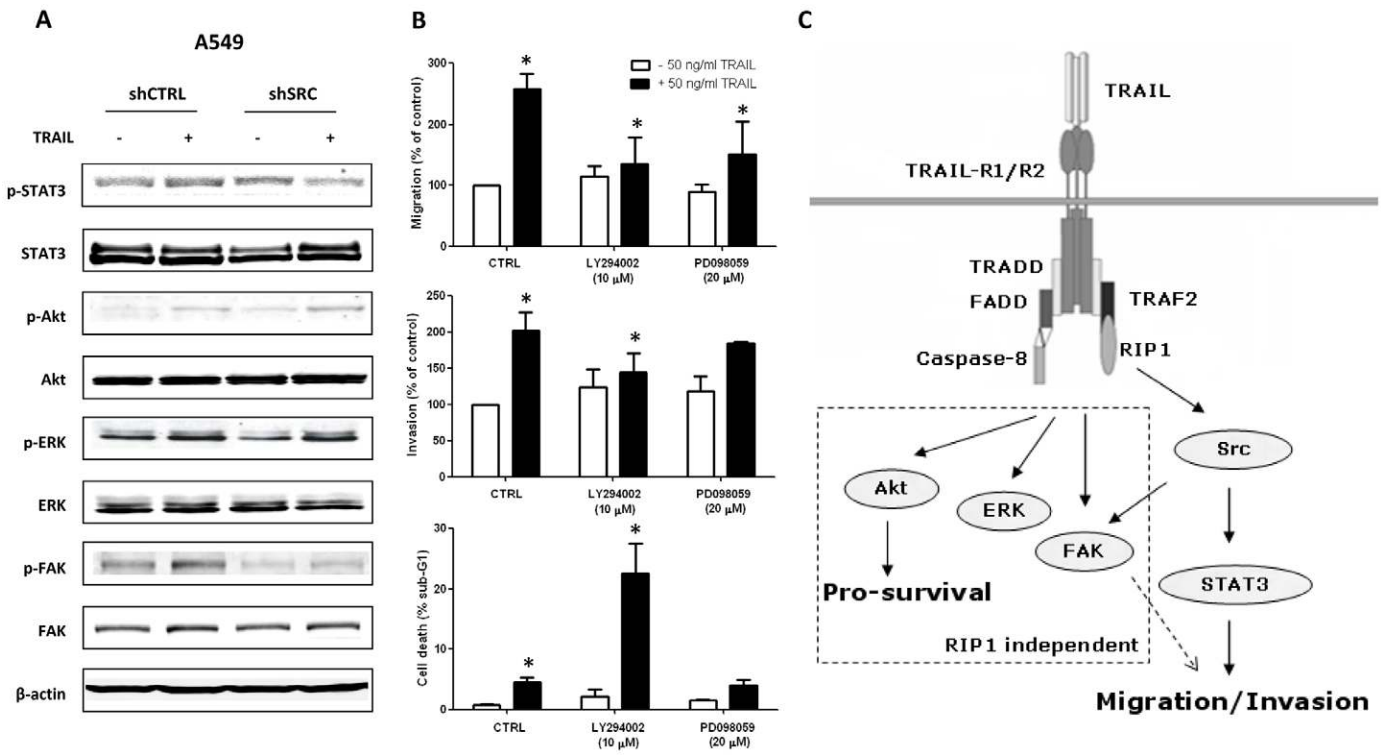


Fig. 8. The role of Src in TRAIL-induced kinase activation and the effect of Akt and ERK inhibition on migration, invasion and cell death. (**A**) Western blots showing the effect of Src knockdown on TRAIL-dependent activation of the indicated kinases. (**B**) Migration, invasion (at 8 h post-treatment) and cell death (24 h after treatment) of A549 cells with or without PI3K/Akt inhibition by LY294002 and ERK inhibition by PD098059. *$P<0.05$, *t*-test, compared with 50 ng/ml TRAIL. (**C**) Schematic representation of the identified kinases involved in non-canonical TRAIL signaling. RIP1-dependent and -independent mechanisms were found, leading to parallel activation of pro-survival and migration/invasion-promoting pathways.

both pro-survival (Akt) and migration/invasion (Src, STAT3) mechanisms. Although more work is required to evaluate the unwanted non-canonical effects in other models, our findings may indicate that care should be taken when using TRAIL receptor targeting agents for treating patients. Moreover, treatments with TRAIL agonistic agents in NSCLC may benefit from combined treatment with PI3K/Akt and Src inhibitors in order to potentiate antitumor activity and to prevent unwanted side effects.

Materials and Methods

Cell lines and chemicals

NSCLC cells, H460, H322, SW1573 and A549, obtained from ATCC in 2003, were cultured as monolayers in RPMI 1640 medium, supplemented with 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in a humidified 5% CO_2 atmosphere at 37℃. The cell lines were tested for their authenticity by short tandem repeat (STR) profiling DNA fingerprinting (Baseclear, Leiden, The Netherlands). PP2, PD098059 (Sigma-Aldrich, St Louis, MO, USA), dasatinib and saracatinib (both from LC Laboratories, Woburn, MA, USA) were dissolved in DMSO to 20 mM stock solutions. LY294002 (Sigma-Aldrich) was dissolved in DMSO to 10 mM stock solution.

MTT assay

A total of 10,000 cells were seeded in 96-well plates (Greiner Bio-One, Frickenhausen, Germany). The next day 100 μl medium with or without TRAIL was added with increasing concentrations to the cells. After 24 h incubation, the medium was discarded and 50 μl of a MTT solution [0.5 mg/ml (Sigma-Aldrich) in HBSS] was added and incubated at 37℃ for 1.5 h. The formazan crystals were dissolved using 150 μl dimethyl sulfoxide (DMSO) and absorbance was measured at 540 nm (Tecan, Männedorf, Switzerland). Results are presented as percentage of viable cells taking the control (untreated cells) as 100% survival. The concentration resulting in 50% inhibition of cell growth (IC_{50}) was derived from the growth inhibition curve.

Receptor cell surface expression

Analysis of TRAIL-receptor membrane expression was performed using a flow cytometer (Epics Elite, Coulter-Electronics, Hialeah, FL, USA). Adherent cells were harvested by treatment with trypsin and washed twice in PBS containing 1% BSA. Appropriate concentrations of antibodies dissolved in PBS/1% BSA were added to the cells. The following antibodies were used to determine TRAIL receptor membrane expression: TRAIL-R1 (HS101), TRAIL-R2 (HS201), TRAIL-R3 (HS301), TRAIL-R4 (HS402), all from Alexis. Mouse IgG (DAKO) was used as isotype control. Subsequently, cells were incubated for 30 min on ice, washed twice with cold PBS/1% BSA, and incubated with FITC-conjugated rabbit-antimouse (DAKO, Glostrup, Denmark) for 30 min on ice. After washing, the cells were analyzed by flow cytometry. Surface expression is shown as a ratio of the signal of the specific TRAIL-receptor antibody and the negative isotype control antibody.

Migration assays

Cell migration was determined using the wound healing assay as described previously (Bijnsdorp et al., 2011). In brief, NSCLC cells were seeded in 96-well plates and grown till confluence. A 96-well floating-pin transfer device with a pin diameter of 1.58 mm coming to a flat point at the tip with a diameter of 0.4 mm (VP Scientific VP-408FH) was used to make the scratches (Yarrow et al., 2004). TRAIL was added for 15 min and after medium refreshment migration was monitored. Indicated kinase inhibitors were incubated for 30 min, followed by 15 min incubation with TRAIL. Wounds were captured at 2.5× magnification with a microscope (DMIRB, Leica Microsystems, Wetzlar, Germany), and Q500MC software (Leica Microsystems) at 0 and 8 h. The wound width at 8 h was measured in four areas and compared with the initial width at the 0 h time point.

Invasion assays

The invasion assay was carried out by using Transwell chambers with fluorescence-blocking 8 μm pore polycarbonate filter inserts (no. 35-1152; HTS Fluoroblock Insert, Falcon, Becton Dickinson Labware, Bedford, MA) in 24-well plates (Bijnsdorp et al., 2011). The insert was coated overnight at RT with 100 μl Matrigel (50 ng/ml in PBS; Sigma-Aldrich). The cells were treated for 15 min with TRAIL. In each insert 200,000 H460 or A549 cells were seeded in serum free RPMI 1640 medium. For H322 and SW1573 800,000 cells were taken. In the

bottom compartment, medium containing 10% FCS was added. NSCLC cells were allowed to invade for 8 h. After 8 h 5 μM calcein-AM (Molecular Probes, Eugene, OR, USA) was added to the lower compartment for 30 min. Pictures were captured and fluorescently labeled cells were counted.

Kinome profiling arrays

The PepChip® kinome array (Pepscan Systems, Lelystad, The Netherlands) consisting of 1024 peptides with specific phosphorylation sites was used to evaluate the kinome after TRAIL treatment in NSCLC cells, similarly as described previously (Parikh et al., 2009; van Baal et al., 2006). Lysates generated from H460 and A549 cells treated with or without TRAIL (15 min) were analyzed in the assays.

PepChip® data analysis

A PepChip® contains 1024 peptides that are spotted in triplicate. Two slides were taken for one condition and experiments were performed in duplicate, resulting in 12 data points per condition. The spots were quantified using the ScanAlyze software and the mean intensity of the 12 data points representing a specific peptide was calculated. Spots deviating more then 2× standard-deviation were excluded. Peptides were considered to represent true phosphorylation events when the average phosphorylation minus 1.96 times the standard deviation of the 12 spots was higher than the value expected from the background distribution. A value of 1.96 was taken, which yields a *P*-value of at least 0.05. A list of peptides was generated by ranking the spots and curve-fitting analysis, resulting in an 'on' or 'off' call for each peptide used to create provisional signal transduction schemes as described earlier [van Baal et al., 2006].

Western blotting

Western blot analysis was performed as described before (Bijnsdorp et al., 2011). The following primary antibodies were used all from Cell Signaling Technology Inc. (Danvers, MA, USA): anti-p-Src (Tyr416; no. 2101), anti-Src (no. 2109), anti-p-Akt (Ser473; no. 9271), anti-Akt (no. 9272), anti-p-ERK 42/44 (Thr202/Tyr204; no. 9101), anti-ERK (no. 9102), anti-p-STAT3 (Ser727; no. 9134), anti-STAT3 (no. 9132) and RIP (no. 3493). β-Actin was from Sigma-Aldrich. The bands were analyzed and the activities of the kinases were determined by calculating the ratio between the phosphorylated form and the total kinase.

Gene silencing

For silencing the expression of genes pSUPER.retro was used similarly as described previously (Brummelkamp et al., 2002). Targeted short hairpin (sh) RNA sequences were inserted into the *Bgl*II and *Hin*dIII sites of the pSUPER.retro vector. All cloned shRNA sequences were verified by DNA sequencing. Retroviruses were packaged and introduced into cells as described previously (van Leuken et al., 2009). A549 and H460 cells were retrovirally infected with control pSUPER.retro or pSUPER.retro-shRIP1 (RIP1-targeting sequence no. 1, 5′-GAGCAGCAGTTGATAATGT-3′; RIP1-targeting sequence no. 2 5′-TACCACTAGTCTGACGGATAA-3′) or pSUPER.retro-shSrc (5′-GGACCTTCCTCGTGCGAGA-3′) for 24 h. Infected cells were selected with 2 μg/ml puromycin.

Cell death measurement

Cell death measurements were performed by FACS analysis as described previously (Janmaat et al., 2003). In brief, cells were seeded at a density of 400,000 cells/well in 6-well plates. After treatment, cells were trypsinized, resuspended in medium collected from the matching sample and centrifuged for 5 min at 1200 rpm. Subsequently, cells were stained with propidium iodide (Sigma-Aldrich) buffer (0.1 mg/ml with 0.1% RNAse A (Qiagen, Venlo, the Netherlands) in dark on ice. DNA content of the cells was analyzed by FACS (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) with an acquisition of 10,000 events. The sub-G_1 peak was used to determine the extent of cell death.

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