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## TRAIL-induced kinases activation and apoptosis

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*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*

2013

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Azijli, K. (2013). *TRAIL-induced kinases activation and apoptosis: towards improved death receptor targeted therapy for lung cancer*. [s.n.].

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## **Summary of the Thesis of Kaamar Aziji**

### **“TRAIL-induced kinases activation and apoptosis: towards improved death receptor targeted therapy for lung cancer”**

#### **SUMMARIZING DISCUSSION AND FUTURE PERSPECTIVES**

Lung cancer is one of the deadliest diseases amongst the different types of cancer and the incidence is expected to rise globally. It is the second most common cancer in both men and women in the western world and is often detected at an advanced stage of disease. NSCLC represents around 80% of all lung cancers [1]. The current treatment of NSCLC patients has reached a plateau since efficacy of therapy is limited by resistance to conventional chemo- and radiotherapy. New therapies are therefore urgently needed to improve lung cancer survival. Genetic analyses of NSCLC has identified mutations in EGFR, p53, KRAS, BRAF, ERBB2, MET, STK11, PIK3CA and PARK2 genes. These mutations have led to new strategies, aiming at mutated protein targets, for example activating mutations of EGFR [1;2]. Targeting of the apoptotic machinery represents another novel approach and aims to selectively kill cancer cells by apoptosis activation while sparing normal cells. In this thesis the main focus was on exploring the use of the TRAIL receptor apoptotic pathway as a target for the treatment for NSCLC.

#### **NON-CANONICAL SIGNALING BY TRAIL IN NSCLC CELLS**

TRAIL is an interesting agent that induces apoptosis through the extrinsic pathway in tumor cells by binding to the death receptors TRAIL-R1 or TRAIL-R2. It has shown strong activity in vitro and in vivo either alone or as part of combination regimes [3]. Currently, different TRAIL receptor agonists have been developed such as recombinant (rh)TRAIL and receptor selective antibodies that are evaluated in phase II/III clinical trials in a wide variety of tumor types, including NSCLC [4]. The combined use of these agonists with other treatments has often been found to synergistically enhance anti-tumor activity or to overcome TRAIL resistance in preclinical studies, strategies that are currently explored in the clinic as well [5]. However, besides activating canonical caspase-dependent apoptosis via the TRAIL-specific death receptors, the same receptors can activate non-canonical cell survival or proliferation pathways in tumor cells, preventing effective therapy [6]. Recent studies even reported on metastases promoting activity of TRAIL as also outlined below. An overview of the currently known non-canonical TRAIL signals and their functional consequences in various tumor types is provided in Chapter 2. These include the activation of I $\kappa$ B/ NF- $\kappa$ B, MAPKs p38, JNK, ERK1/2, MAP3K, TAK1, PKC, PI3K/Akt and Src. The signals that stimulate invasive behavior of tumor cells, including those found in this thesis and as reported by others, such as Src, NF- $\kappa$ B and K-RAS are also discussed. In this thesis part of the work was dedicated to examine the possible counterproductive effects of TRAIL stimulation in NSCLC and to unravel underlying mechanisms of non-canonical kinases signaling.

In Chapter 3 we evaluated the kinetics of MAPK p38 and JNK upon TRAIL exposure in two different NSCLC cell lines and examined the effect on apoptosis activation. These kinases are known to be activated directly by TRAIL through the formation of a secondary complex consisting of the serine/threonine kinase RIP1, caspase-8 and FADD [7]. TRAIL treatment induced the phosphorylation of p38 and JNK1/2/3 only in sensitive H460 cells and not in the resistant A549 cell line. The use of chemical inhibitors and siRNAs indicated that JNK activation counteracted TRAIL-induced apoptosis, whereas activation of p38 stimulated apoptosis. RIP1 was cleaved following TRAIL treatment concomitantly with detectable JNK phosphorylation. Further examination of the role of RIP1 by short hairpin (sh)RNA-dependent knockdown showed that p38 can be phosphorylated in both RIP1-dependent and -independent manner, whereas JNK phosphorylation occurred independently of RIP1. The exact molecular mechanisms of p38 and JNK activation by TRAIL that occurs independently of RIP1 is yet unclear and remains to be unraveled. Immunoprecipitation experiments to determine the protein components that are responsible for the activation of these kinases will give us clues how to target these pathways more upstream. Inhibition of kinases that suppress apoptosis is expected to facilitate the elimination of cancer cells when combined with TRAIL receptor agonists. In addition, the activation of JNK by TRAIL occurred in a caspase-8-dependent manner, which was not the case for p38. Mcl-1 was shown to be a downstream target of both p38 and JNK and silencing of Mcl-1 with siRNA strongly augmented TRAIL-induced apoptosis in H460 cells. Taken together, our results suggest a model in which TRAIL-induced activation of p38 and JNK have counteracting effects on Mcl-1 expression leading to pro- or anti-apoptotic effects, respectively. The role of p38 and JNK in TRAIL signaling appears tumor-type dependent. In prostate cancer cells, for example, activation of both kinases prevented TRAIL-induced apoptosis [8]. JNK, on the other hand enhanced the apoptotic effect of TRAIL in human lymphoid cell lines [9]. This illustrates that caution is warranted when developing combination therapies with TRAIL receptor agonists and kinase inhibitors. Pre-selection of suitable patients groups and tumor type-dependent approaches will be required for optimal results in a clinical setting. Since Mcl-1 appears to be the main target of p38 and JNK, the combined use of TRAIL with a Mcl-1 inhibitor/antagonist, such as Gosypoll (AT-101) and GX15-070 could also provide an effective strategy. In colon, breast, and pancreatic cancer cells inhibition of Mcl-1 with these inhibitors increased TRAIL-induced apoptosis [10-12]. In NSCLC cells this remains to be formally demonstrated, although our data has shown that Mcl-1 knockdown sensitizes for TRAIL-dependent apoptosis (Chapter 3). Gosypoll and GX15-070 are both being evaluated in clinical trials ([clinicaltrials.gov](http://clinicaltrials.gov)) and combined use with TRAIL might be interesting to explore in subsequent trials.

Recently, various different TRAIL receptor complexes have been identified that trigger alternative forms of cell death, including the necroptosome and ripoptosome [13]. Currently, the more precise mechanisms that trigger the various kinases pathways are elusive. Further research is required to determine the molecular mechanisms triggering and regulating non-canonical TRAIL signalling, which will also provide new clues for optimal use of TRAIL receptor agonists for the treatment of cancer.

An unexpected feature of TRAIL is the induction of migration and invasion in tumor cells, which has been shown previously in colon, pancreatic ductal adenocarcinoma, and cholangiocarcinoma cancer cells [14-16]. In Chapter 4 and as published [17], we describe TRAIL induced migration and invasion in NSCLC cells in a panel of NSCLC cell lines with different sensitivities for TRAIL. Migratory and invasive properties of TRAIL were observed in the resistant NSCLC cells, demonstrating possible pro-tumorigenic activity of TRAIL in this cancer type for the first time. Using a kinomic approach with PepChip peptide arrays we were able to identify kinases that were activated in TRAIL resistant A549 cells and not in sensitive H460 cells. Following validation of the hits, the Src-STAT3 pathway directly activated by TRAIL was found to be responsible for invasive behavior. Activation of Src could be linked with RIP1 since knockdown of RIP1 or chemical inhibition with necrostatin-1 prevented activation. In literature, several other mechanisms have been described underlying TRAIL-induced migration/invasion. In apoptosis resistant cholangiocarcinoma cancer cells migration was found to depend on NF- $\kappa$ B [15], and in colon cancer cells K-Ras, Raf-1 and the ROCK/LIM kinase/cofilin pathway were involved [14]. It would be interesting to study if the RIP1-Src-STAT3 pathway is also involved in TRAIL-induced migration in other tumor types as well. The migratory effect of TRAIL impedes its clinical value since unwanted pro-tumorigenic effects may occur when administered in patients. We postulate that better knowledge of these mechanisms may provide markers for selecting patients that will likely benefit from TRAIL-based therapy and will provide a rationalized basis for combination therapies with TRAIL agonists. Thus, kinases and their phosphorylated active forms may provide biomarkers to predict the sensitivity and/or consequences of TRAIL treatment. When required, TRAIL receptor agonists could be combined with small molecules that inhibit mediators of migration/invasion, such as Src, STAT3 or RIP1 inhibitors. In this context, markers for predicting TRAIL sensitivity in general would be of great therapeutic value. However, thus far only a few possible suitable biomarkers have been identified, which have not been tested in the clinic for upfront patient selection as yet. For example, TRAIL receptor O-glycosylation was reported to be a good predictor for TRAIL sensitivity [18]. O-linked glycosylation involves the binding of glycosyl groups to threonine and serine side chains leading to improved ligand binding and receptor protein activation. In cancer tissue specific O-glycosyltransferases were found to be overexpressed when compared to normal tissue and interestingly, were correlated with TRAIL sensitivity. In NSCLC, melanoma and pancreatic cancer cells, the expression of O-glycosylation initiating enzyme GALNT14 was associated with TRAIL sensitivity [18]. Loss of cell surface expression of TRAIL-R1 and TRAIL-R2 by endocytosis was also found to be a biomarker for TRAIL resistance in breast cancer [19]. TRAIL sensitivity was recently reported to be predicted by a 71-gene signature as shown by a microarray study in a large number of different cell lines including NSCLC, leukemia, breast, renal, colon, melanoma, ovarian, prostate and CNS cancer [20]. Whether this gene signature will be of value for selecting patients for TRAIL therapy remains to be investigated. The next challenge will be to identify crucial targets and corresponding therapeutics from these biomarkers in order to determine the optimal TRAIL combination strategy. When possible samples obtained from both the primary tumor and metastatic lesions should be analyzed.

### **TRAIL COMBINATION APPROACHES FOR ENHANCING APOPTOSIS IN NSCLC**

As mentioned earlier, combining TRAIL with other anti-cancer treatments can greatly increase the anti-tumor effect of TRAIL. This is illustrated by several studies in a wide range of different tumor types, whereby drugs like, proteasome inhibitors, HDAC and topoisomerase inhibitors increased TRAIL sensitivity or even sensitized resistant cells to TRAIL-induced apoptosis [21-25]. In Chapters 5 and 6 we describe the combination of TRAIL with two different anti-cancer agents; 17-AAG, a Hsp90 inhibitor, and trifluorothymidine (TFT), a thymidylate synthase

inhibitor in NSCLC cells [26;27]. Both drugs showed synergy with TRAIL in a panel of NSCLC cells. Hsp90 is upregulated in many tumors and is thought to play an essential role in maintaining the malignant transformation of cancer cells, including by interaction and stabilization of several key signalling proteins such as Akt, ErbB2, c-Met, and Raf-1. We found that 17-AAG increased TRAIL-induced apoptosis, mainly through the extrinsic pathway in A549 and H460 cells. Combined treatment resulted in cleavage of RIP1 and down-regulation of Akt and ERK. Inhibition of Akt activity by the chemical inhibitor LY294002 resulted in a significant increase in TRAIL-induced apoptosis. These results indicate that 17-AAG stimulates TRAIL-induced apoptosis in NSCLC via the extrinsic apoptotic pathway through down-regulation of Akt. As RIP1 is a client protein of Hsp90 and it was decreased in A549 cells, it is also likely to prevent RIP1-dependent non-canonical signaling and subsequent counterproductive effects. The inhibition of migration/invasion by 17-AAG has already been shown in glioma and breast cancer cells [28-30]. Even though the combination of 17-AAG and TRAIL proved to be quite effective in eradicating NSCLC cells, additional preclinical studies in mouse models will be required to further determine the value of the combination for possible translation to the clinic. Especially, because single agent studies with 17-AAG have shown limited activity in clinical trials [31], combination of this drug and TRAIL might be beneficial for cancer patients.

The other combination treatment being explored in this thesis was TRAIL together with TFT. TFT is part of the novel oral formulation TAS-102, which is currently being evaluated in phase II clinical trials (clinicaltrials.gov). In this formulation, TFT is combined with a thymidine phosphorylase inhibitor (TPI) to increase the bioavailability of TFT. TFT interferes with thymidylate production and its triphosphate form is incorporated into the DNA, causing DNA damage. In Chapter 6, the combination TFT/TRAIL showed synergistic cytotoxicity in a panel of NSCLC cell lines, i.e. A549, H292, H322 and H460. The treatment affected cell cycle progression, with TRAIL inducing a G1 arrest and TFT a G2/M arrest. TFT activated Chk2 and reduced Cdc25c levels known to cause G2/M arrest. TRAIL-induced caspase-dependent apoptosis was enhanced by TFT, whereas TFT alone mainly induced caspase-independent cell death. Enhanced apoptosis correlated with the up-regulation of TRAIL-R2 surface expression, whereas TRAIL-R1 levels remained the same. TRAIL-R2 up-regulation was induced by p53, expression of which was also enhanced by TFT. Furthermore, the combination also caused a decrease in the expression of the anti-apoptotic proteins XIAP and FLIP<sub>L</sub>. These proteins inhibit apoptosis and their overexpression has been associated with TRAIL resistance [32]. Thus, TFT can enhance TRAIL-induced apoptosis in NSCLC cells by sensitizing the extrinsic apoptotic pathway. Our findings suggest a possible therapeutic benefit of combined use of TFT and TRAIL in NSCLC. TFT is degraded effectively by thymidine phosphorylase (TP), making its half-life very short, only 12 minutes after injection [33]. TP is often overexpressed in cancer, including NSCLC, and potentially plays a role in the stimulation of angiogenesis [34;35]. The exact mechanism of angiogenesis induction is unclear, but is postulated to be related to thymidine-derived sugars. TP catalyzes the conversion of thymidine (TdR) to thymine and deoxyribose-1-phosphate (dR-1-P), which can be converted to dR-5-P, glyceraldehyde-3-phosphate (G3P) or deoxyribose (dR). However, it was unclear which sugar accumulates in this reaction. In Chapter 7 [36], using liquid chromatography coupled to mass-spectrometry (LC-MS-MS) we found that in TP-overexpressing cell lines, dR-1-P and dR-5-P accumulated intracellularly at high levels and dR was secreted extensively by the cells. A specific inhibitor of TP completely blocked TdR conversion, and indeed no sugars were formed. When further analyzing the subcellular localization of these sugars in (3)H-TdR cultured cells, TdR-derived sugars accumulated in the cytoskeleton and to some extent in the cell membrane, while incorporation into the DNA was responsible for trapping in the nucleus. Further studies should focus on which exact metabolic pathway is involved in the induction of angiogenesis, which likely involves more indirect mechanisms. It would also be interesting to study whether TP-overexpressing cells are sensitive for TFT after TP inhibition.

## **CONCLUSION**

The TRAIL receptors have been convincingly demonstrated in preclinical models, including NSCLC, to be valid targets for the development of pro-apoptotic agonists. However, the differential sensitivity for apoptosis induction and even pro-tumorigenic effects in TRAIL resistant tumor cells requires a better understanding of the mechanisms that regulate TRAIL activity. Regardless of the more detailed molecular causes of the dichotomy in TRAIL signaling the combined use with standard anti-cancer drugs generally results in sensitization to TRAIL-induced apoptosis. Thus, possible unwanted effects of TRAIL treatment can be overcome by combination treatments, such as the ones we presented in this thesis. Elucidation of the molecular switches that determine the settings of the TRAIL pathway will help to develop targeted strategies to make the tumor cells apoptosis prone. Another challenge for the future is to identify biomarkers that allow the preselection of patients and determination of optimal TRAIL combination strategies in order to maximize therapeutic benefit.

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