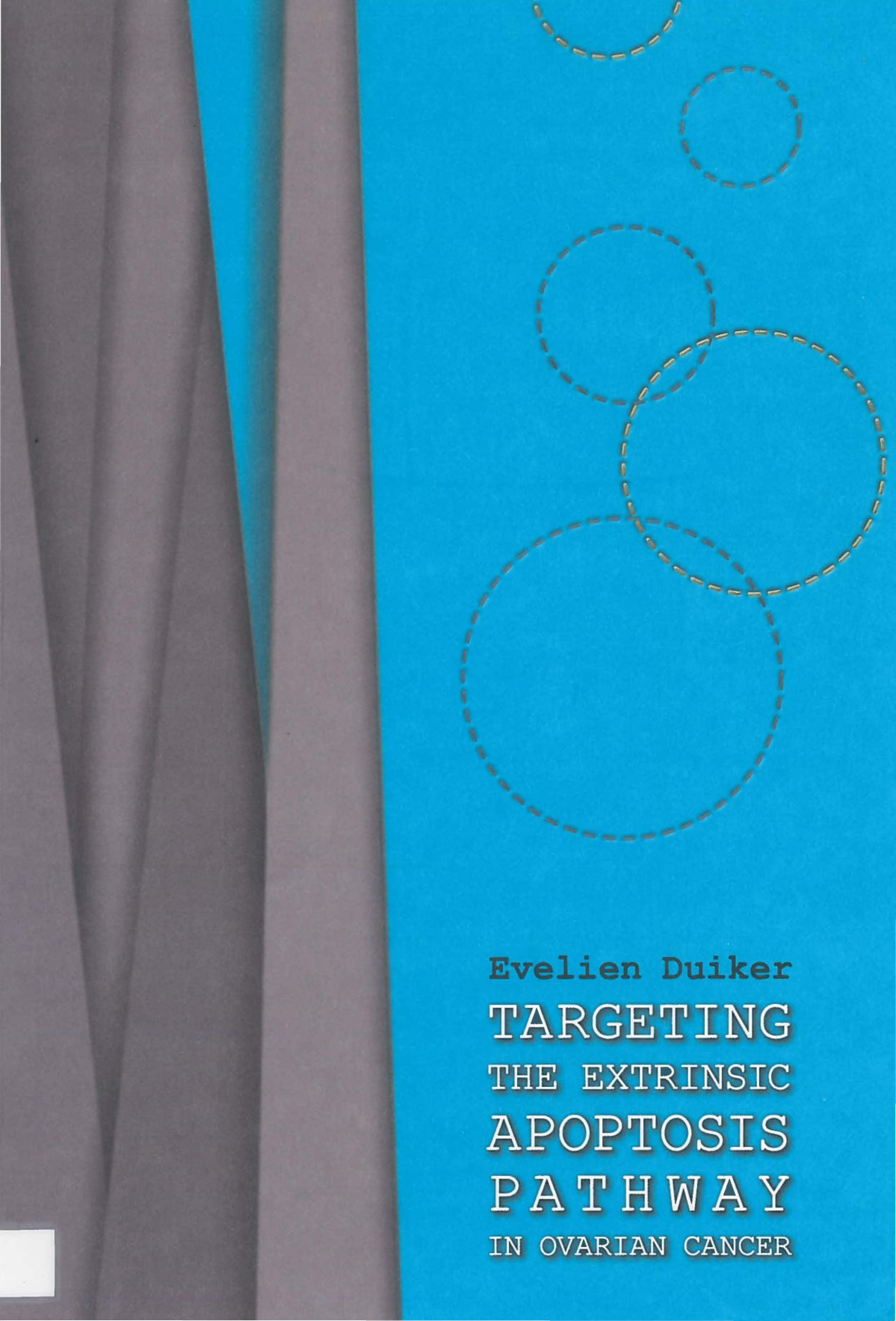

Evelien Duiker

TARGETING THE EXTRINSIC APOPTOSIS PATHWAY IN OVARIAN CANCER

TARGETING THE EXTRINSIC APOPTOSIS PATHWAY IN OVARIAN CANCER

STELLINGEN

1. De hotspot p53 mutatie op codon 273 kan samen gaan met een toegenomen gevoeligheid voor rhTRAIL. (Dit proefschrift)

2. Opregulatie van death receptor expressie in reactie op chemotherapievoorbehandeling hoeft niet betrokken te zijn bij sensitisering voor rhTRAIL. (Dit proefschrift)

3. C-FLIP expressie bij een meerderheid van de eierstoktumoren vormt een potentieel beperkende factor van succesvolle therapie met geneesmiddelen gericht op death receptoren. (Dit proefschrift)

4. Radioactief gelabeld rhTRAIL of TRAIL-receptor antilichamen kunnen bijdragen aan toekomstige tumortherapie op maat. (Dit proefschrift)

5. Intraperitoneale rhTRAIL therapie bij (intraperitoneale) tumoren leidt tot een hogere rhTRAIL blootsstelling van de tumor. (Dit proefschrift)

6. Omdat een preferentiële TRAIL-receptor voor apoptose inductie kan bestaan, hebben medicijnen gericht tegen DR4 of DR5 niet noodzakelijkerwijs hetzelfde effect. (British Journal of Haematology 2007; 4: 568-577)

7. Significante invloed van de experimentele condities op een genexpressieprofiel vraagt om voorzichtigheid bij het bepalen van functionele consequenties van een dergelijk profiel. (Proceedings of the National Academy of Sciences 2005; 23: 8287-8292)

8. Een betere overleving na medicamenteuze behandeling van vroege stadia van kanker wordt ondersteund door de evolutieleer. (Nat Reviews Cancer 2006; 6: 924-35)

9. In de Nederlandse dienstverlening is verantwoording afleggen een groter begrip geworden dan verantwoordelijkheid nemen. (NRC 24/11/07)

10. Rook-vrijheid is niet hetzelfde als rookvrij-heid.

11. Men zal een land nooit leren kennen als niet genoten wordt van zijn culinaire tradities. (Chinees spreekwoord)

12. Een Einstein-generatie die niet kan rekenen is een contradictio in ter ?

13. Wie niet door een bril heen kan kijken heeft een gebrekkig (in)zicht.

TARGETING THE EXTRINSIC APOPTOSIS PATHWAY IN OVARIAN CANCER

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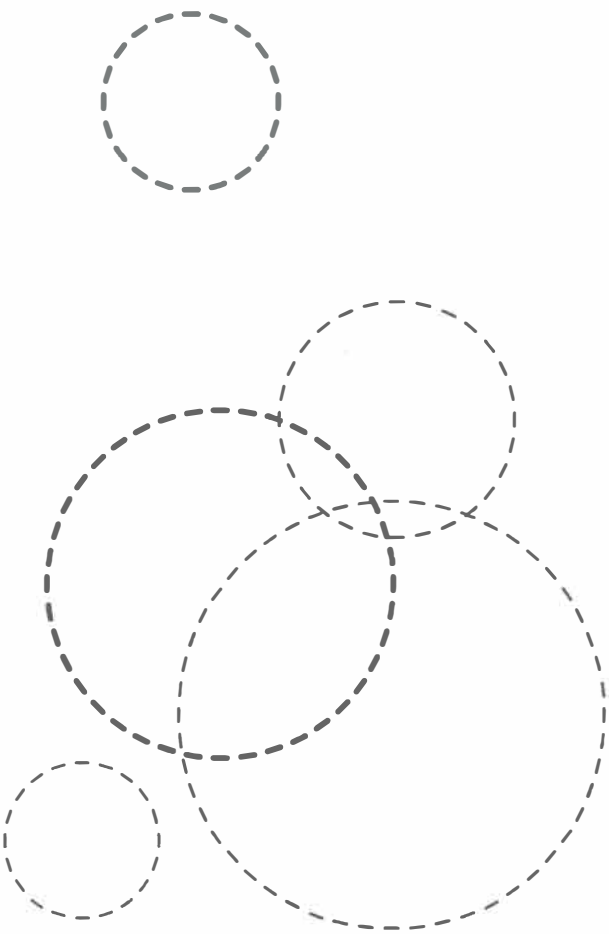
Aan mijn ouders

CONTENTS



CHAPTER 1

INTRODUCTION

INTRODUCTION

Ovarian cancer is the fifth leading cause of cancer death in European women, and it represents the most lethal gynecological malignancy (1). Although a distinctive genetic predisposition exists in approximately 5% of affected women, the cause of ovarian cancer remains unknown. Epithelial ovarian cancer, which represents the majority (90%) of cases, is suggested to arise from the ovarian surface epithelium (2). After initial invasion of adjacent tissues, epithelial ovarian cancer primarily disseminates intraperitoneally. Due to a lack of early symptoms and effective screening methods, it is this stage at which most patients are diagnosed with the disease. Treatment of advanced stage ovarian cancer consists of surgical debulking followed by platinum-based chemotherapy, which yields response rates of more than 80%, with clinical complete responses in 40-60% of the patients. However, due to intrinsic or acquired drug-resistance only 25% of patients with advanced-stage disease survive 5 years after initial diagnosis (3). Resistance to platinum-based therapies is attributed to numerous mechanisms, which can be broadly divided into decreased DNA damage upon treatment and increased cell survival, mainly through defects in apoptosis or proliferation programs (4). Apoptosis, the evolutionary conserved program for regulated cell death, can be induced through two alternative pathways, although crosstalk between both routes exists (5). The cell's intrinsic or mitochondrial pathway is activated after severe cellular stress and represents the main pathway for chemotherapy-induced apoptosis. In the cell's extrinsic or death receptor pathway, binding of death ligands to their cognate receptors at the cell surface sets the apoptosis cascade in motion. Defects in the intrinsic pathway result in a decreased response to drug-induced DNA damage and subsequent resistance to chemotherapy. Therefore, circumvention of this pathway by targeting the extrinsic pathway may provide a new strategy to combat malignancies from a different angle (6). The best characterized death ligands are tumor necrosis factor alpha (TNF-α), Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) which can induce apoptosis by binding to their receptors TNF receptor 1 (TNFR1), Fas and death receptor 4 (DR4) /death receptor 5 (DR5) respectively (7). Whereas strategies targeting TNFR1 and Fas are impeded because of severe toxicity upon systemic administration, the recombinant human (rh) form of TRAIL or apoptosis inducing antibodies targeting DR4 or DR5 have now reached the phase of clinical studies. This thesis explores the clinical potential and possible pitfalls of therapies with rhTRAIL or drugs targeted at its receptors, in particular in ovarian cancer.

In **chapter 2** a literature overview is presented on the current knowledge about important molecular pathways in ovarian cancer, with emphasis on clinically evaluated strategies targeting these pathways. In addition, novel approaches that enable identification of new pathways and prognostic markers of importance in ovarian cancer are discussed.

Chapter 3 focuses on the TRAIL pathway in general. A summary of the literature discussing its physiological function, the mechanisms that underlie sensitivity and resistance of cells to rhTRAIL and the development of novel agents directed at TRAIL receptors is provided.

The tumor suppressor gene p53 which plays a key role in the response to DNA damage is one of the most studied prognostic markers so far in ovarian cancer. A meta-analysis showing that aberrant p53-status results in reduced odds of surviving 5-years (8), confirms that alterations in key effectors of the intrinsic pathway greatly influence ovarian cancer outcome. Although new treatment modalities such as rhTRAIL can induce p53-independent apoptosis, p53 interacts with the extrinsic pathway, among others by increasing expression of death receptors (9,10). Therefore, the effect of mutant p53 on rhTRAIL sensitivity in an ovarian cancer cell line model is studied in **chapter 4**. A2780/m273, a cell line expressing p53 mutated at codon 273, is compared to its wild type p53 counterpart with respect to molecular determinants of rhTRAIL sensitivity. Furthermore, the mechanism of rhTRAIL resistance is established in a subline of A2780/m273 with acquired resistance to rhTRAIL.

Intrinsic or acquired resistance to agents targeting the TRAIL pathway is a potential problem in their clinical application (11). Not all ovarian cancer cell lines tested are sensitive to rhTRAIL as monotherapy. Fortunately, combinations of chemotherapy and drugs targeted at DR4 and DR5 show increased efficacy and can overcome resistance to either of the agents (12). Many studies compare cell lines with different genetic backgrounds for evaluation of sensitivity patterns, which hampers identification of causal factors involved in resistance and modulation. Therefore, in **chapter 5** the molecular determinants for rhTRAIL sensitivity and the mechanisms involved in synergy between rhTRAIL and cisplatin are investigated in an isogenic ovarian cancer cell line model of cisplatin resistance.

Exploration of the extrinsic apoptosis pathway in ovarian cancers is of interest given the possible use of agents targeting this pathway in cancer therapy and the physiological role death ligand/ receptor interaction plays in tumor-immune surveillance. In **chapter 6,** protein expression of key components of the extrinsic pathway TRAIL, DR4, DR5, Fas, FasL, caspase 8 and c-FLIP is determined by immunohistochemistry on a tissue microarray containing ovarian cancer tissue of 382 patients. It is analyzed whether these expression data correlate with clinicopathological characteristics, response to chemotherapy and survival.

Besides the biological behavior of new anti-cancer drugs, a major determinant of eventual clinical efficacy is their pharmacological behavior. Availability of radiolabeled variants of new agents offers the possibility of future molecular imaging in humans to study biodistribution and whole body pharmacokinetics. Moreover, imaging enables the illustration of efficient drug targeting. In **chapter 7** the development of radiolabeled rhTRAIL and radiolabeled antibodies directed at DR4 and DR5 for clinical application is described, including a biodistribution study in a human tumor bearing mouse model.

In preparation for the clinical assessment of death receptor targeted drugs in ovarian cancer patients, animal models that resemble the human situation of ovarian cancer spread are important intermediates. The application of intraperitoneal xenograft models of ovarian cancer is hampered by the difficulty to accurately monitor tumor growth, which complicates the definition of precise and ethically acceptable endpoints. Labeling of cancer cells with bioluminescent reporters enables non-invasive molecular imaging to visualize xenograft growth and evaluation of therapeutic interventions (13). **Chapter 8** describes the development of an intraperitoneally growing bioluminescent human ovarian cancer model in mice, in which the best route of rhTRAIL-administration, intravenous or intraperitoneal, is determined by means of a biodistribution study with radiolabeled rhTRAIL. In addition, the in vivo efficacy of single agent rhTRAIL, a variant of rhTRAIL designed to specifically target DR5 and cisplatin, as well as efficacy of combination therapy of either agent with cisplatin is evaluated in this model.

Finally, a summary of all results is given in **chapter 9**. This is followed by a discussion regarding the interpretation and implications of these results, along with a discussion of the future perspectives.

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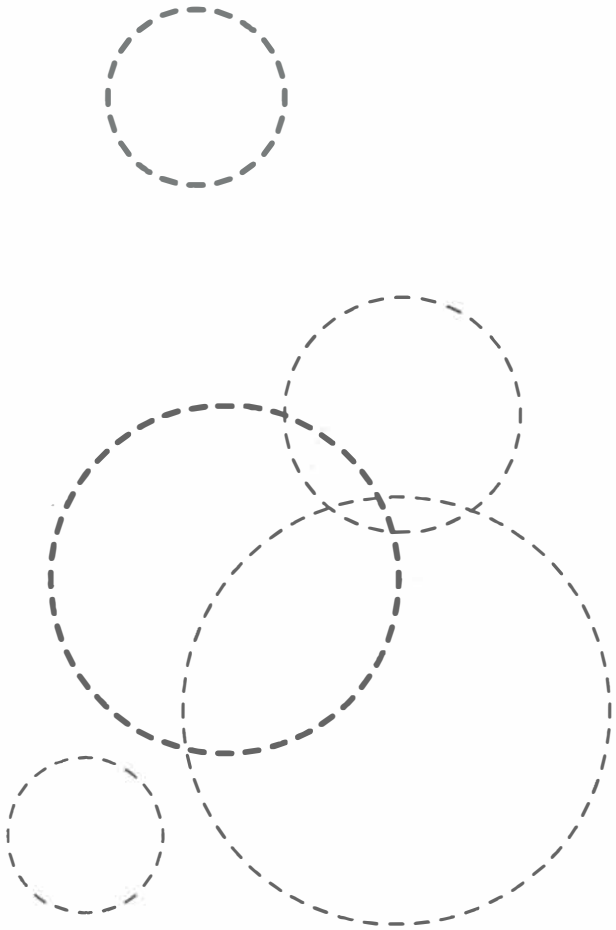

CHAPTER 2

MOLECULAR PROGNOSTIC MARKERS IN OVARIAN CANCER: TOWARD PATIENT-TAILORED THERAPY

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ABSTRACT

In ovarian cancer the ceiling seems to be reached with chemotherapeutics drugs. Therefore a paradigm shift is needed. Instead of treating all patients according to standard guidelines, individualized molecular targeted treatment should be aimed for. This means that molecular profiles of the distinct ovarian cancer subtypes should be established.

Until recently, most studies trying to identify molecular targets were single marker studies. The prognostic role of key components of apoptotic and prosurvival pathways as p53, EGFR and HER-2 has been extensively studied because resistance to chemotherapy is often caused by failure of tumor cells to go into apoptosis. However, it is more than likely that different ovarian cancer subtypes with extensive molecular heterogeneity exist. Therefore, exploration of the potential of specific tumor-targeted therapy, based on expression of a prognostic tumor profile, may be of interest. Recently, new profiling techniques, such as DNA and protein microarrays, have enabled high-throughput screening of tumors. In this review an overview of the current status of prognostic marker and molecular targeting research in ovarian cancer, including microarray studies, is presented.

INTRODUCTION

Ovarian cancer is the leading cause of death from gynecologic cancer and the fourth most common cause of cancer death in women. In 2005 it is estimated that 22,220 new ovarian cancer cases will be diagnosed in the United States and 16,210 deaths caused by ovarian cancer will occur (1). This high death rate is related to the difficulty in detecting ovarian cancer at an early stage and the lack of effective therapies for advanced disease. After surgery, for FIGO stages IC, IIB, III and IV disease, platinum-based chemotherapy is indicated. For patients with FIGO stages III/IV disease, this results in 25% survival at 5 years (2). Intrinsic, but especially acquired resistance to chemotherapeutic drugs is a major problem. The several newer first-line regimens studied hardly improved survival; therefore a paradigm shift is needed.

Within the current FIGO classification system, ovarian cancer subtypes with distinct clinical courses are likely to exist, but they have yet to be defined. Unraveling the molecular pathways of importance in the different subtypes may allow the development of more molecularly targeted therapies based on the biology of the disease. It is therefore of great importance to identify new prognostic molecular markers that will facilitate the classification of ovarian carcinomas into pathogenetic subtypes with distinct clinical courses.

However, research in ovarian cancer is hampered by the relatively low incidence of ovarian cancer; e.g., the incidence of ovarian cancer is about 9.5 times lower than the incidence of breast cancer (1). Furthermore, ovarian cancer research is complicated by the fact that approximately 36% of the patients with ovarian cancer is seventy years or older at the time of diagnosis (3). Because of comorbidity, elderly patients with ovarian cancer are often not treated according to standard guidelines.

In this review we will present an overview of the available data on molecular pathways of potential importance and describe ongoing initiatives to find new prognostic markers and new drug targets for ovarian cancer.

KEY-COMPONENTS OF MOLECULAR PATHWAYS; PUTATIVE PROGNOSTIC MARKERS

The classic clinicopathologic prognostic markers for ovarian cancer include age at diagnosis, FIGO stage, histiotype and grade of the disease, amount of residual tumor after primary debulking surgery and response to first-line chemotherapy. However, these prognostic markers are imperfect predictors of outcome and do not provide insight into the biologic mechanisms responsible for clinical tumor behavior.

Resistance to chemotherapy is considered to be especially due to failure of tumor cells to go into programmed cell death (apoptosis). Therefore, especially the role of apoptotic or prosurvival pathways in ovarian carcinogenesis has been extensively studied. Apoptosis can be executed through an "intrinsic" (mitochondria-dependent) and "extrinsic" (death receptor-dependent) pathway. The intrinsic apoptotic pathway is triggered by diverse stress signals, including withdrawal of growth factors. The extrinsic apoptotic pathway is initiated by activation of death receptors expressed on the cell membrane (4).

The prognostic value of various key components of apoptotic and prosurvival pathways has been frequently analyzed. The results of these studies are, however, contradictory. This is because of considerable methodological variability between the different prognostic studies. In addition, many studies were affected by small sample size, which may lead to overestimation or underestimation of the relevance of the molecular factor.

We have, therefore, performed meta-analyses of prognostic studies on the tumor suppressor gene p53 and the growth-factor receptors, EGFR, and HER-2 in ovarian cancer. These meta-analyses

showed that patients with aberrant p53, EGFR, or HER-2 tumor status had significantly lower odds of surviving 5 years (5). Agents targeting these molecular biologic factors, thus, may have therapeutic potential. Currently, several drugs targeted at these and other key components of apoptotic and prosurvival pathways are in various stages of development, and those that are clinically being assessed will now be discussed.

INTRINSIC APOPTOTIC PATHWAY

P53 is an important mediator of the intrinsic apoptotic pathway. The p53 network is involved in the cellular defense against various stress signals, such as DNA damage, oncogene or aberrant growth factor signaling and stress caused by ultraviolet radiation, hypoxia or chemotherapeutic drugs (6). Upon activation, the p53 protein will act as a tumor suppressor through effects on gene expression, resulting in cell cycle arrest, apoptosis, senescence or differentiation (7). For an overview of the intrinsic and extrinsic apoptotic pathways, see Figure 1. The involvement of a defective p53 pathway in carcinogenesis makes it a target for therapeutic intervention. Several strategies have been developed. The adenovirus dl1520 (ONYX-015) has been attenuated by deletion of its E1B 55-kd gene region, which encodes a protein product that is known to bind and inactivate p53, hereby preventing the p53-mediated activation of the cellular defense mechanism against viral infection. Deletion of this gene region should cause the virus only to replicate in p53-defective cells, leading to virus spread and subsequent cytolysis of the tumor cells (8,9). However, although there is evidence of therapeutic efficacy, the virus's mechanism of cellular selectivity is less clear (10). In a phase I trial with dl1520, including 16 ovarian cancer patients, it was demonstrated for the first time that a selective replication-competent virus can be safely administered intraperitoneally. Furthermore, there were signs of continuing viral replication in ovarian cancer cells after dl1520 administration (9). The adenovirus-5 E1A protein is able to reprogram transcription in tumor cells, thereby reversing the transformed phenotype of cancer cells and suppressing tumor growth (11). Several phase I studies with liposome-mediated E1A gene therapy have been performed (12,13). Recently, a phase I trial with intraperitoneal administration of an E1A-lipid complex in patients with recurrent epithelial ovarian cancer overexpressing HER-2 showed evidence of safety and gene transfer (14). SCH58500 (rAD/p53) is a replication-deficient adenovirus encoding human, recombinant, wild-type p53. The results of a phase I/II trial with SCH58500 in recurrent ovarian cancer showed safety, effective gene transfer and serum CA-125 reduction when combined with platinum-based chemotherapy (15). In a subsequent study the long-term follow-up was evaluated of the patients who had been enrolled into the phase I/II trial with SCH58500. The median survival was 12-13 months (16). However, a phase II/III trial, where patients with primary stage III ovarian cancer were randomized to receive either standard therapy or the same regimen combined with SCH58500 gene therapy, was prematurely closed due to lack of therapeutic effectiveness and increased treatment morbidity. The reasons for this failure of gene therapy in ovarian cancer are probably numerous and not exclusively limited to problems relating to gene therapy itself (17).

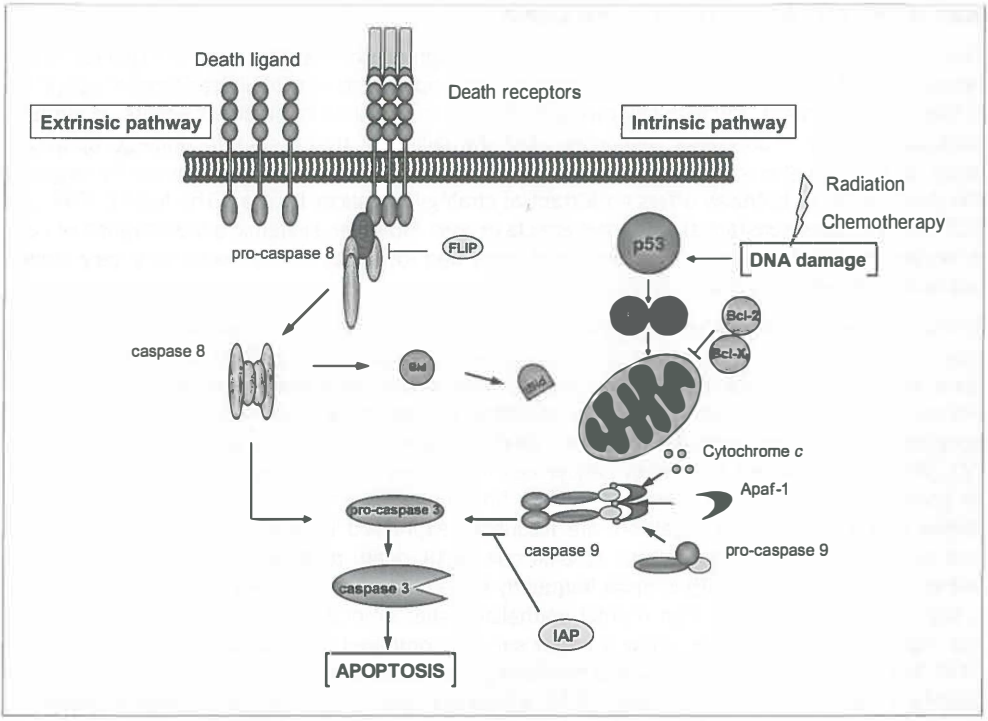

Figure 1. Apoptotic pathway. The death receptor-initiated apoptosis pathway is referred to as the extrinsic apoptosis pathway. The death ligand, TRAIL in this example, binds as a homotrimer to DR4 and DR5, which results in homotrimeriziation of the receptors. This leads to the assembly of a death-inducing signalling complex (DISC). At the DISC, the adaptor protein FADD (Fas- associated death domain) acts as a bridge between the death receptor complex and the initiator caspase 8. Upon recruitment by FADD, caspase 8 will be activated by autocleavage and activate downstream effector caspases such as caspase 3, 6 and 7. Cross-talk exists between the extrinsic pathway and the intrinsic or mitochondria-initiated apoptosis pathway through Bid, a BH3-only protein member of the Bcl-2 gene superfamily. Activated caspase 8 will cleave Bid, which then translocates to the mitochondria to induce cytochrome c release, forming the connection between the extrinsic and intrinsic pathway. The intrinsic pathway triggers apoptosis after DNA damage, hypoxia, starvation and many other kinds of severe cellular stress. When the intrinsic pathway is activated, pro-apoptotic members of the Bcl-2-gene family translocate to the mitochondria, causing subsequent release of cytochrome c and other mitochondrial factors into the cytosol. In the cytosol, cytochrome c binds in the presence of deoxyadenosine triphosphate (dATP) the adaptor protein Apaf-1 and pro-caspase 9, forming the apoptosome-signaling complex in which caspase 9 is activated and can activate on its turn the effector caspases 3, 6 and 7. *See appendix, page 184 for color figure.*

EXTRINSIC APOPTOTIC PATHWAY

The extrinsic apoptotic pathway can be executed through death receptors and their ligands, which belong to the tumor necrosis factor superfamily. They operate as essential mediators of apoptosis in the immune system, acting in cell-mediated toxicity in response to infectious agents, in immune homeostasis and in antitumor responses (18). As failure of tumor cells to undergo apoptosis leads to tumor progression and resistance to cancer therapy, induction of apoptosis by targeting the death receptor pathway offers an attractive strategy in cancer therapy. The ligands TNF and CD95L/FasL show substantial antitumor effects *in vitro*. However, systemic administration of FasL is limited by severe systemic side effects in mice and low-dose TNF showed only very limited antitumor efficacy in humans (19-21).

Tumor necrosis factor related apoptosis inducing ligand (TRAIL) is considered to be of special interest for clinical use because of its ability to induce apoptosis in several types of cancer cell lines and xenografts regardless of p53 status, without causing toxicity to normal cells (22-25). Administration of TRAIL to non-human primates proved to be safe (26). TRAIL can induce apoptosis through two agonistic receptors, death receptor 4 (DR4) and death receptor 5 (DR5) (27,28). *In vitro* studies in ovarian cancer cell lines show antitumor activity of TRAIL alone or in combination with chemotherapy (29-32). Immunohistochemical staining of ovarian tumors shows that TRAIL and its receptors are frequently expressed in ovarian cancer. Most primary and residual ovarian tumors express at least one TRAIL death receptor, while in residual tumors following chemotherapy, DR5 is more frequently expressed (33). Moreover, ovarian cancers show a higher TRAIL expression than normal epithelial tissue. Among the tumors studied, those with the highest TRAIL expression show a better survival compared to those with a lower expression (34). The results stated here show that combining chemotherapeutic drugs with biologic response modifiers, such as TRAIL or agonistic TRAIL antibodies, seems an attractive strategy to prevent or overcome drug resistance. A phase I study with recombinant human (rh)TRAIL has been started. Several phase I and II studies are currently ongoing with agonistic monoclonal antibodies targeting the death receptors DR4 and DR5, including a phase II study where HGS-ETR1, a monoclonal antibody (mAb) directed at DR4, is administered in combination with carboplatin and paclitaxel. A phase I study with a mAb against DR5 is ongoing (http://www.hgsi.com). The clinical studies that are presently accruing patients are summarized in Table 1.

Table 1. Ongoing clinical trials with novel molecular agents

Target	Novel agent	Phase of clinical development	Patient characteristics
DR4, DR5	RhTRAIL	Phase I	Solid tumors
DR4 mAbs	HGS-ETR1 with gemcitabine and cisplatin	Phase Ib	Solid tumors
	HGS-ETR1 with carboplatin and paclitaxel	Phase II	Solid tumors
DR5 mAbs	HGS-ETR2	Phase I	Solid tumors
	HGS-TR2J	Phase I	Solid tumors
EGFR TKIs	Gefitinib and docetaxel	Phase I	Advanced solid tumors
	Gefitinib and calcitriol with or without dexamethasone	Phase I	Advanced solid tumors

TKIs	Gefitinib (Iressa)	Phase II (pilot)	Advanced ovarian, fallopian tube, primary peritoneal or cervical cancer
	Erlotinib	Phase I/II	Advanced ovarian, non-small cell lung, or squamous cell carcinoma of the head and neck
mAbs	Erlotinib with carboplatin and paclitaxel	Phase I/II	Advanced ovarian, fallopian tube or primary peritoneal cancer
	Cetuximab (Erbitux)	Phase II	Persistent or recurrent ovarian or primary peritoneal cancer
	Cetuximab with carboplatin	Phase II	Recurrent ovarian or primary peritoneal cancer
HER-2			
mAbs	Trastuzumab with erlotinib and paclitaxel	Phase I	Advanced solid tumors
	Trastuzumab and imatinib mesylate	Phase I	Recurrent or metastatic HER-2 expressing cancer
VEGF			
mAb	Bevacizumab	Phase II	Ovarian or primary peritoneal cancer where doxil or topotecan has failed
	Bevacizumab and imatinib mesylate	Phase I/II	Advanced melanoma or other advanced cancers
Soluble decoy receptor	VEGF Trap	Phase I	Relapsed or refractory advanced solid tumors or non-Hodgkin's lymphoma
Integrin $\alpha_v\beta_3$			
mAb	Cilengitide	Phase I	Advanced solid tumors or lymphoma
Ras-Raf-MAPK and VEGF	Sorafenib (BAY 43-9006) with or without paclitaxel and carboplatin	Phase II	Recurrent ovarian, primary peritoneal or fallopian tube cancer
	Sorafenib and gemcitabine	Phase II	Recurrent or refractory ovarian or primary peritoneal cancer
	Sorafenib and bevacizumab	Phase I	Refractory, metastatic or unresectable solid tumors
Inhibitors of farnesyl transferase	R115777 with topotecan	Phase I	Advanced solid tumors
Proteasome	PS-341 (Bortezomib)	Phase I	Platinum and taxane resistant recurrent ovarian , primary peritoneal, and fallopian tube cancer
	Bortezomib	Phase II	Persistent or recurrent ovarian or primary peritoneal cancer
HSP90	17-AAG	Phase I	Unresectable solid tumors or relapsed lymphoma
	17-AAG with cisplatin and gemcitabine	Phase I	Advanced solid tumors
	17-AAG with docetaxel	Phase I	Metastatic or unresectable solid tumors
	17-AAG with paclitaxel	Phase I	Metastatic or unresectable solid tumors
	17-AAG with Bortezomib	Phase I	Advanced solid tumors

17-AAG, 17-allylamino-17-demethoxygeldanamycin.

PROSURVIVAL PATHWAYS

EGF receptor family

The EGFR family embodies four homologous receptors: the epidermal growth factor receptor (EGFR/HER-1/ErbB-1), HER-2 (ErbB-2), HER-3 (ErbB-3) and HER-4 (ErbB-4). The receptors consist of three main domains: a ligand-binding extracellular domain, a transmembrane segment and an intracellular tyrosine kinase domain (35). Activation of a monomeric receptor occurs by dimerization between two identical receptors (homodimerization) or between different receptors of the EGFR family (heterodimerization) (36). This results in activation of the tyrosine kinase domain with subsequent activation of interrelated intracellular signaling pathways, such as the (PI3K)-AKT pathway and the MAPK pathway. Activation of these pathways can lead to different cellular processes, such as cell division and migration, adhesion, differentiation and apoptosis. The eventual outcome depends on cellular context, the ligand causing dimerization and the receptor dimer formed (37). The normal function of HER signaling lies in the mediation of cell-cell interactions in organogenesis and adulthood (38). However, dysregulation of HER signaling is associated with malignant transformation. This dysregulation can occur through a number of mechanisms such as overexpression of a ligand, overexpression of a receptor, activating mutations leading to constitutively activated receptors and defective processing of receptors (37). Until now, only the EGFR and HER-2 receptors have been reported to be dysregulated in human cancer. A meta-analysis showed aberrant EGFR expression in 12-82% of ovarian cancer, whereas aberrant HER-2 expression was reported in 5-66% of ovarian carcinomas (5). Several strategies targeting EGFR and HER-2 have been developed. Mabs targeting the receptors extracellularly or small molecules targeting the intracellular tyrosine kinase domains (tyrosine kinase inhibitors, TKIs) have been developed and are in various stages of clinical testing in ovarian cancer. Those studies that are currently open are summarized in Table 1.

The TKI gefitinib, directed at the tyrosine kinase domain of EGFR, has been evaluated in three phase I trials, including 33 ovarian cancer patients. Of these 33 patients, three had stable disease during treatment with gefitinib (39). At this moment a phase II pilot study of gefitinib in patients with advanced ovarian cancer is recruiting patients (http://www.clinicaltrials.gov). Gefitinib has been approved in the United States in 2003 for the treatment of patients with non-small cell lung cancer who had failed two or more courses of chemotherapy. However, recently the gefitinib Survival Evaluation in Lung cancer (ISEL) showed no survival benefit for patients under treatment with gefitinib. It should be mentioned that, in non-small cell lung-cancer patients, it has been shown that only activating mutations in EGFR underlie responsiveness to gefitinib (40).

Another TKI directed at EGFR, erlotinib, was evaluated in a phase II trial in 34 heavily pretreated ovarian cancer patients. Three patients were reported to have a partial response, while 42% experienced stable disease. There was no relation between response to erlotinib and EGFR tumor expression (41). Various phase I/II trials are examining erlotinib in combination with chemotherapy (42). Preliminary results of a phase I study of erlotinib with docetaxel showed stable disease in ovarian cancer patients (43). The data of a recently closed phase II trial of erlotinib and carboplatin are being analyzed.

At this moment cetuximab, a mAb directed at the EGFR, is being evaluated in ovarian cancer in phase II clinical trials. A trial with cetuximab in advanced EGFR positive ovarian, fallopian tube or primary peritoneal cancer is no longer recruiting patients and its results are being evaluated. The results of a phase II clinical trial of EMD-7200, another mAb against EGFR, are currently under evaluation as well. Cetuximab is currently registered for the use in EGFR overexpressing, irinotecan refractory colon carcinomas.

The anti-HER-2 mAb trastuzumab (Herceptin), registered for the treatment of HER-2 positive metastatic breast cancer patients, has been designed to specifically antagonize the function of the HER-2 receptor in HER-2 positive tumors. A phase II trial with trastuzumab in patients

with recurrent or refractory ovarian or primary peritoneal cancer with overexpression of HER-2 showed a low rate of objective response (44). A phase II study of trastuzumab and paclitaxel in patients with HER-2 overexpressing relapsed or progressive breast and ovarian carcinomas has been completed. Furthermore, two phase I trials, one with interleukin-12 and trastuzumab and one with interleukin-12, trastuzumab and paclitaxel in patients with HER-2 overexpressing solid tumors have been completed as well. No results from these studies have been reported yet. Currently, a clinical trial with trastuzumab in combination with paclitaxel and interleukin-12 is ongoing. Another mAb targeting the dimerization domain of HER-2, pertuzumab, is currently being clinically evaluated. A trial with pertuzumab in ovarian cancer patients pretreated with one or more platinum-based chemotherapy regimens has completed enrollment.

Phosphatidylinositol 3-kinase-AKT pathway

The PI3K-AKT pathway represents one of the key pathways controlling survival, proliferation and growth in cells. A dysfunctional PI3K-AKT pathway is involved in several diseases, including cancer. PI3K can be activated by a diverse array of physiologic stimuli, including growth factor receptor signaling, several interleukins and stress, as well as by activated Ras proteins (45-48). Active PI3K is able to activate AKT at the cell membrane via a second messenger. AKT, also known as protein kinase B (PKB), exists in three closely related isoforms, AKT1, AKT2 and AKT3 (49). By phosphorylating numerous downstream proteins, activated AKT can control essential cellular processes. For an overview of the AKT and ERB/HER pathway, see Figure 2. AKT promotes cell survival through several mechanisms. It downregulates apoptosis through inactivation of several proapoptotic proteins (50-53). Increase of p53 degradation is executed by AKT through effects on MDM2, a negative p53 regulator (54,55). AKT promotes gene expression of several prosurvival genes via nuclear factor kappa B (NFkB) (56,57). Furthermore, AKT can exert effects on cell metabolism and growth through activation of the protein kinase "mammalian target of rapamycin" (mTOR) (58,59). AKT can also stimulate cell proliferation by effects on mediators of the cell cycle (55,60). Numerous other AKT targets involved in different cellular reactions have been recognized (61).

Dysregulation of several key components of the AKT pathway have been described in ovarian cancer. Alterations in the tumor suppressor PTEN, which is responsible for converting the AKT-activating second messenger to its inactive state, have been reported in ovarian cancer (62-65). Elevated AKT1 levels and amplification and overexpression of the isoform AKT2 were described (47,66,67). Amplification of subunits of PI3K have been characterized as well (68,69). Currently, several drugs targeting components of the PI3K-AKT pathway are in various preclinical stages of development.

Drugs targeting mTOR have entered clinical trials in breast cancer and renal cancer patients (70). No molecular agents targeting the PI3K-AKT pathway are presently being clinically assessed in ovarian cancer.

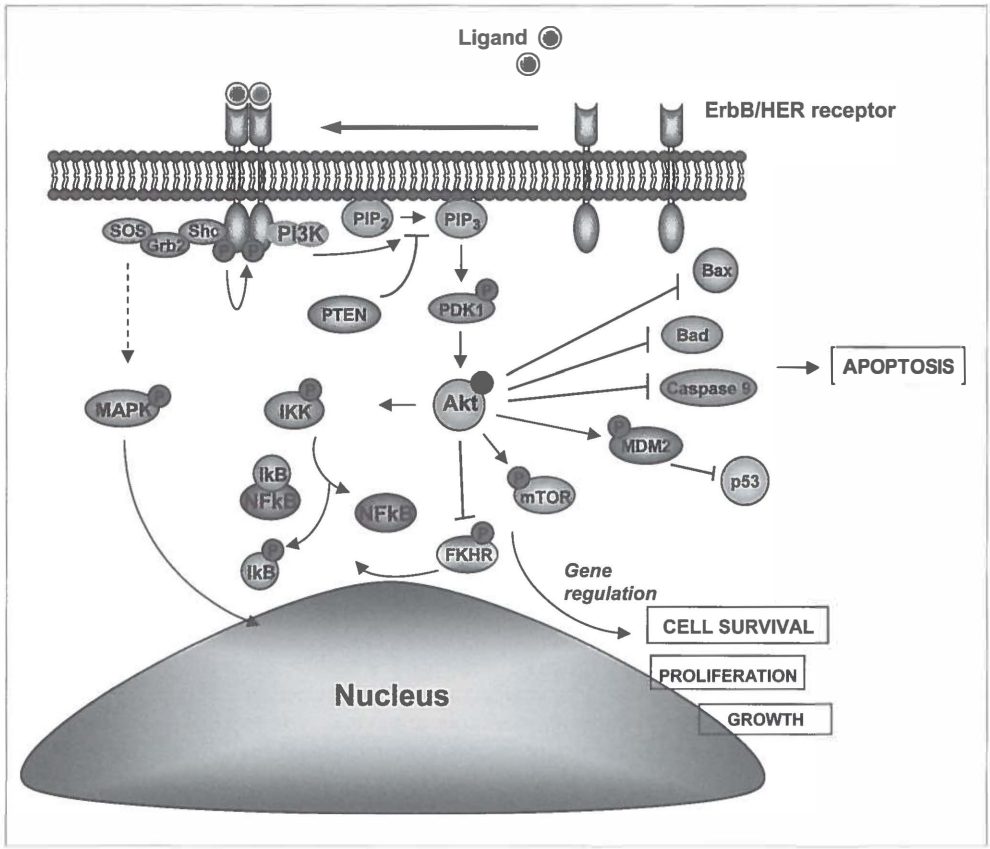

Figure 2. Prosurvival pathways; Erb/HER and PI3K-AKT. Dimerization between two growth factor receptors causes activation of the receptor tyrosine kinases (RTKs) with subsequent autophosphorylation. This leads to activation of the MAP-kinase pathway and of the PI3K-AKT pathway. Active PI3K converts phosphatidylinositol-4,5-biphosphate (PIP_2) to the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3), which is able to recruit pleckstrin-homology (PH) domain containing proteins, such as AKT to the cell membrane with subsequent activation of AKT. The tumor suppressor PTEN is responsible for converting the second messenger PIP_3 to its inactive state PIP_2. Active AKT is able to control essential cellular processes, such as apoptosis, survival, proliferation and growth by phosphorylating downstream proteins. P53 degradation is increased by phosphorylation of MDM2. AKT promotes gene expression of several prosurvival genes via nuclear factor kappa B (NFkB) and inhibits gene expression of several proapoptotic genes by preventing forkhead-related transcription factors (FKHR) to enter the nucleus. Furthermore, AKT can exert effects on cell metabolism and growth through activation of the protein kinase "mammalian target of rapamycin" (mTOR). AKT can also stimulate cell proliferation by effects on mediators of the cell cycle. Numerous other AKT targets involved in different cellular reactions have been recognized. *See appendix, page 185 for color figure.*

ANGIOGENESIS PATHWAYS

A solid tumor depends on angiogenesis to grow in size and form metastases. Whereas in normal tissue the endothelium is extremely quiescent and angiogenesis occurs as a tightly regulated process, the endothelium in tumors proliferates rapidly and contributes to active angiogenesis. Because of the specific properties of angiogenesis under malignant conditions, antiangiogenic agents may represent a promising anticancer strategy (71,72). More than 75 antiangiogenic drugs are in the process of being tested in clinical trials (71). Bevacizumab, a recombinant anti-VEGF mAb, has been approved by the Food and Drug Administration in February 2004 for the treatment of metastatic colon cancer. At American Society of Clinical Oncology (ASCO) 2005 two interim reports of phase II clinical trials of bevacizumab in recurrent ovarian or primary peritoneal cancer were presented. One trial with bevacizumab as monotherapy showed a median disease free interval of 6.5 months in 62 patients (73). Another report of a trial of bevacizumab combined with cyclophophosphamide described encouraging response rates in 29 patients (6 partial response (PR), 17 stable disease (SD), 6 progressive disease (PD)) and a progression free survival at 6 months of 47% of the patients (74). At ASCO 2005 the Gynecologic Oncology Group proposed a design for a phase III trial of combination therapy with or without bevacizumab in persistent or recurrent ovarian cancer or primary peritoneal cancer. Numerous agents, ranging from monoclonal antibodies, TKIs, soluble decoy receptors, matrix metalloproteinase inhibitors and others are currently being evaluated in clinical trials. A phase I trial with VEGF Trap, a soluble decoy receptor for VEGF, is now recruiting patients. Cilengitide, a mAb targeting the $\alpha_v\beta_3$ integrin receptor, which promotes angiogenesis upon binding the ligand basic fibroblast growth factor (bFGF), is currently being evaluated in a phase I trial. Several small molecule VEGF receptor TKIs have been developed.

MOLECULAR AGENTS TARGETING MULTIPLE TARGETS

Given the diversity of molecular alterations acquired during malignant transformation, there seems to be no single dominant neoplastic pathway in ovarian carcinomas. Therefore, it is unlikely that targeting a single step in ovarian carcinogenesis will be effective in treating this disease, especially when the putative target has many downstream effectors and redundant or overlapping pathways. To maximize anticancer efficacy, it seems necessary to intervene simultaneously in multiple pathways. Recently, agents that inhibit multiple targets have been developed and many are already being evaluated in clinical trials. One of the candidates is bortezomib, a reversible inhibitor of the proteasome. The proteasome is the multicatalytic protease complex that plays an essential role in the degradation of most intracellular proteins, including those controlling cell cycle progression and apoptosis (75). Bortezomib was approved by the Food and Drug Administration in 2003 for the treatment of multiple myeloma progressing on prior therapy. Clinical trials with bortezomib show promising results in numerous other malignancies (76). In ovarian cancer bortezomib is now being assessed as combination therapy with carboplatin in a phase I and as monotherapy in a phase II trial. There is increasing evidence that hypermethylation of tumor suppressor genes contributes to carcinogenesis. Therefore, another candidate is the DNA hypomethylating agent decitabine. Clinical trials with decitabine are ongoing. A phase I trial with decitabine and carboplatin in patients with advanced malignancies, including ovarian cancer, showed feasibility of the combination therapy (77). Inhibitors of farnesyl transferase (FTIs), the enzyme that catalyzes a key step in the addition of an aliphatic isoprenoid chain to a number of proteins (including Ras, Rho, PxF and laminins A and B), seemed interesting. However, clinical studies evaluating the FTIs BMS-214662 and L-778,123 as monotherapy or combined with chemotherapy in solid tumors have been stopped because of toxicity. Yet, numerous phase I/II studies with FTI R115777 are currently open. The small molecule BAY 43-9006, which shows both antiproliferative and antiangiogenic properties by targeting Raf kinase and the receptor tyrosine kinases VEGFR-2 and PDGFR, is due to enter phase III trials in 2005. In ovarian cancer patients, BAY 43-9006 combined with different chemotherapy regimes is currently being tested in two phase II clinical

trials. BAY 43-9006 has shown substantial antitumor effects in patients with metastatic renal cancer (78). Inhibitors of the chaperone protein Heat shock protein 90 (Hsp90) are presently emerging as targets in cancer therapy. Chaperone proteins are in charge of maintaining correct folding, function and stability of certain proteins. Hsp90 seems of special importance in malignant transformation because of its requirement in chaperoning proteins essential in cell signaling, proliferation and survival, such as AKT, HER-2 and c-Raf and in chaperoning mutated proteins such as mutant p53. The Hsp90- inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) is the first of a class of Hsp90 inhibitors that has reached clinical trials with successive promising results (79,80).

GENETIC, GENOMIC AND PROTEOMIC PROFILING TECHNIQUES

In ovarian cancer multiple oncogenic pathways are likely to exist. Until recently it was only possible to investigate single-pathway components instead of complete pathways.

However, endeavors such as the Human Genome Project and technical advances in molecular biology now allow for the comprehensive screening of tumors, at the genetic, genomic and proteomic levels (81). Examples of microarray-based technologies include DNA arrays for comparative genomic hybridization, single-nucleotide polymorphisms (SNPs) or expression analysis and (reverse-phase) protein arrays for protein profiling. Furthermore, tissue microarray technology enables high-throughput tissue analyses to evaluate and validate the clinical value of the results obtained by the microarray-based technologies.

Successful translation of knowledge obtained by these technologies may lead to more precise and patient-tailored therapies. Besides profiling the individual tumor to predict drug responsiveness, the interindividual variability in drug response should also be taken into account. This is studied by pharmacogenetics, the field that studies the influence of inherited genetic variability on treatment response (82-84).

DNA MICROARRAY STUDIES IN OVARIAN CANCER

Until now quite a large number of studies using DNA microarrays to determine gene expression profiles in ovarian cancer have been reported. The specific aims of the separate studies are summarized in Table 2.

Most microarray studies have tried to identify diagnostic markers by comparing ovarian carcinomas or ovarian cancer cell lines to normal ovarian epithelium. Furthermore, many microarray studies have tried to discover genes associated with classic clinical prognostic factors or drug resistance to pick up clues to treatment and prognosis.

Only four microarray studies have tried to identify prognostic factors and/or prognostic profiles for personalized prediction of disease outcome.

These microarray studies have provided long lists of genes associated with ovarian carcinogenesis; yet, the importance of these genes for the development and progression of ovarian cancer has still to be determined. Additionally, the cumulative results of diagnostic marker microarray studies revealed more than 150 potentially upregulated genes associated with ovarian cancer (85).

A way to assess the importance of the genes related to ovarian carcinogenesis is to try to identify pathways among the genes. Several microarray studies have suggested pathways to be involved in ovarian carcinogenesis, such as the FAK pathway, the FGF2 pathway, glucose/insulin metabolic pathway, BRCA1 or BRCA2-related pathways, the JAK/STAT pathway, and the IGF pathway (86-91). Samimi et al have identified pathways potentially involved in oxaliplatin resistance in ovarian cancer cell lines, such as the ribosome pathway, the Huntington disease pathway and the ATP synthesis pathway (92). Currently, the discovery of pathways involved in ovarian carcinogenesis

is eased by the availability of commercial pathway programs. Donninger et al discovered by importing the microarray data into such a pathway program that the FAK pathway, associated with tumor cell migration, spread and invasion, was activated in advanced stage papillary serous ovarian cancer (88). Still, the importance of these pathways in ovarian carcinogenesis has to be further determined.

Although the microarray studies in ovarian cancer have provided important information, they have limitations, such as small numbers of tumors analyzed (see Fig. 3), exclusive analysis of cell lines as opposed to primary tumors and normal epithelium, as well as a limited number of features on the microarrays used in the studies.

Table 2. DNA microarray studies in ovarian cancer

Aim of microarray study	Material	Number of studies	References
Diagnostic markers and/or carcinogenesis	Normal epithelial tissue or cell lines vs. cancer cell lines or carcinomas	22	85,86,88,89,117-135
Carcinogenesis	Carcinomas	3	87,136-138
Molecular determinants of classic clinicopathologic factors (stage, grade, histiotype, (sub)optimal cytoreduction, response to chemotherapy)	Carcinomas	12	90,91,94,121,139-147
Acquired drug resistance	Cancer cell line/tumor resistance model	9	92,139,148-155
Drug action and/or response to chemotherapy	Cancer cell lines or xenografts	7	156-163
Effect of overexpression of a particular gene on gene expression and/or drug response and/or metastasis	Cancer cell lines	5	164-168
Methylation alterations, genes, or profile associated with (progression free) survival	Carcinomas	4	93,132,169,170

Small sample size is particularly a severe problem for studies that eventually aim to use tumor gene expression profiling as a prognostic tool in ovarian cancer, as it affects the statistical reliability of the conclusions. Spentzos et al recently identified a prognostic 115-gene signature by profiling 68 ovarian carcinomas (93). Furthermore, a three-gene signature that classifies ovarian tumors according to their resistance to platinum-based chemotherapy was recently reported. This was achieved by profiling 24 ovarian carcinomas (of 5 responders and of 19 non-responders) with subsequent confirmation by reverse transcription polymerase chain reaction (RT-PCR) on 92 ovarian tumors (94). As is shown in Figure 3 more and larger microarray studies (including validation studies) have been performed in breast cancer to identify a prognostic profile (95-103). However, the value of the prognostic profiles is still questionable. In breast cancer the prognostic profiles of the different studies only had a few genes in common (98,101,104).

Ransohoff recently warned for inflated expectations about prognostic microarray studies, as prognostic profiles may actually be caused by chance and may not be reproducible. The solution is to assess reproducibility by applying the prognostic profile derived from a "training set" to an independent "validation set" consisting of samples that were not used in the training set (105). This requires enough frozen samples for both training and validation. In ovarian cancer obtaining sufficient samples will be especially hard because of the relatively low incidence rates (97,100,102). Furthermore, the clinical evidence of prognostic microarray studies (as well as prognostic studies on single markers) only reaches level 3 or 4 evidence (small/large retrospective studies) on a scale of 5 (low) to 1 (high) (106). The prognostic value of gene profiling in ovarian (and breast) cancer must be further evaluated in additional large prospective studies to reach level 2 or 1 clinical evidence as concluded by Spentzos et al and Helleman et al (93,94).

Interestingly, Paik et al have shown that it is possible to build a prognostic breast cancer predictor of 21 (16 cancer-related and 5 reference) genes prospectively selected from published literature, genomic databases and DNA microarray experiments on frozen samples. The expression of the 21 genes was determined by RT-PCR in paraffin samples from breast cancer patients enrolled in the National Surgical Adjuvant Breast and Bowel Project clinical trial B-14 (107). In this way a large (668) prospective prognostic study of level 2 clinical evidence could be performed.

Beside genomic microarray studies, genetics and proteomics studies in ovarian cancer are also giving promising results (108-115). Combining the results of genetics, genomics and proteomics may speed up translational research in ovarian cancer.

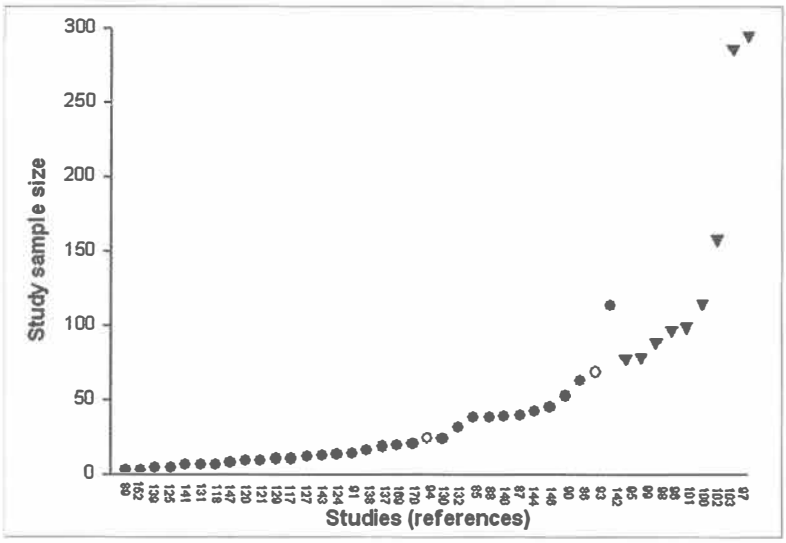

Figure 3. Number of tumor samples in (ovarian cancer) microarray studies.

Closed circles represent microarray studies in ovarian cancer, and the open circles represent ovarian cancer prognostic / predictive profile microarray studies. Triangles represent breast cancer prognostic profile microarray studies.

DISCUSSION

The ceiling seems to be reached with conventional chemotherapeutic drugs. It has become clear that ovarian cancer survival rates will not improve by treating all patients uniformly according to standard guidelines. There is a need of more patient-tailored therapy, where specific tumors will be treated with specific drugs. The present era of molecular targeting has generated excitement in the field of anticancer drug development. A large number of molecular targeted agents are currently in various stages of development and several are already being applied in anticancer therapy. However, no molecular targeted agents currently have a clinically proven role in ovarian cancer.

The "quick" translation of target identification and the subsequent development of molecular targeted agents are obstructed by the relative rarity of ovarian cancer.

This implies that clinically relevant answers will come only from the comparison of different experiments and the analysis of large numbers of patients.

For successful translational research in ovarian cancer, uniform methodological principles and the general availability of data are required. That way studies can be compared and a meta-analysis can be performed. In addition, a uniform format for display and storage of data will be helpful. For microarray studies a standard entitled MIAME – the Minimum Information About a Microarray Experiment – has been proposed to address this problem (http://www.mged.org/Workgroups/MIAME/miame.html) [116]. Efforts to standardize new techniques and set up large ovarian tumor banks will hopefully support patient-tailored therapy in ovarian cancer patients

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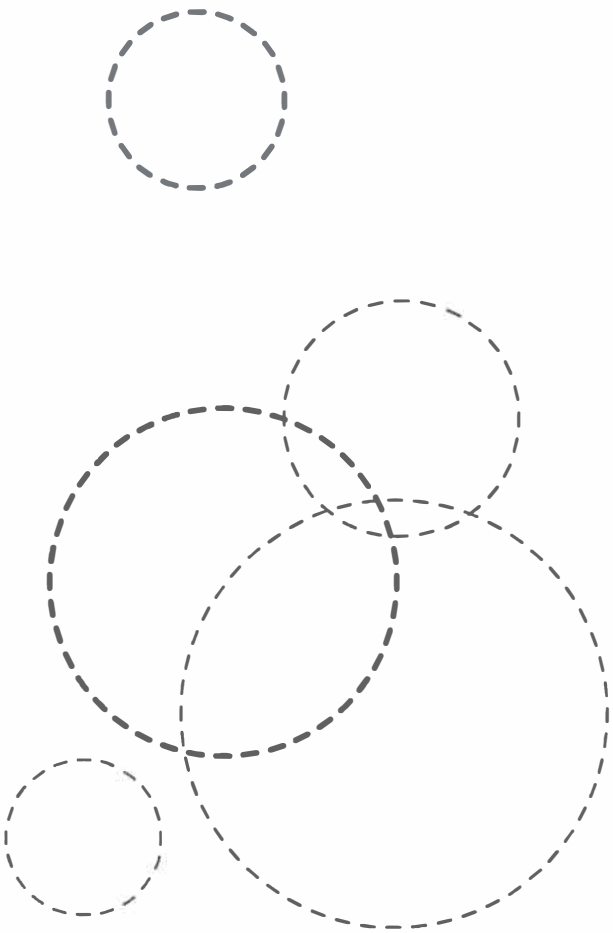

CHAPTER 3

THE CLINICAL TRAIL OF TRAIL

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ABSTRACT

The naturally occurring tumour necrosis factor related apoptosis-inducing ligand (TRAIL) induces apoptosis through two death receptors, death receptor 4 (DR4) and death receptor 5 (DR5) that are expressed on the cell membrane. Binding of the ligand to the death receptors leads to activation of the extrinsic apoptosis pathway. Chemotherapy on the other hand stimulates the intrinsic apoptosis pathway via activation of p53 in response to cellular damage. Many cancer cells have mutations in p53 causing resistance to chemotherapy-induced apoptosis. Concomitant signalling through the extrinsic pathway may overcome this resistance. Moreover, enthusiasm for TRAIL as an anticancer agent is based on the demonstration of rhTRAIL-induced selective cell death in tumour cells and not in normal cells. In this review we provide an overview of the TRAIL pathway, the physiological role of TRAIL and the factors regulating TRAIL sensitivity. We also discuss the clinical development of novel agents, i.e. rhTRAIL and agonistic antibodies that activate the death receptors.

INTRODUCTION

In recent years, the development of more selective, tumour-biology driven therapies that possess anti-tumour activity and might prevent or overcome resistance to chemo- and radiotherapy, has become a main field of interest within cancer research. Recombinant members of the Tumour Necrosis Factor (TNF) family, including Fas-ligand, TNFα and TNF related apoptosis inducing ligand (TRAIL), can induce apoptosis in preclinical models. Administration of Fas-ligand however is hampered by induced severe liver toxicity in preclinical studies (1). TNF is currently only administered for limb salvage by regional limb perfusion in soft tissue sarcoma treatment, since systemic use induces a sepsis-like syndrome (2,3). TRAIL is still considered a promising anticancer agent. In preclinical models it induces apoptosis in a wide range of tumour cells and xenografts, without causing toxicity to normal cells (4). In this review we will give an overview of current knowledge on TRAIL biology and the translation of this knowledge into clinical therapies based on TRAIL signalling.

TRAIL SIGNALLING PATHWAY

TRAIL was identified in 1995 based on its sequence homology to FasL/APO1L and TNF (5). TRAIL is a type II membrane protein, which can be cleaved from the cell surface to form a soluble ligand. Both full-length membrane expressed TRAIL and the soluble ligand can rapidly induce apoptosis in a wide variety of human cancer cell lines (5). TRAIL can bind to five different receptors: four membrane-bound and one soluble receptor (6,7). Two of these membrane receptors, death receptor 4 (DR4) and death receptor 5 (DR5), act as agonistic receptors, containing a cytoplasmic death domain through which TRAIL can transmit an apoptotic signal. Two other membrane receptors, decoy receptor 1 (DcR1) and decoy receptor 2 (DcR2), can also bind TRAIL, but may act as antagonistic receptors, lacking an intact death domain. In addition to these four receptors, a fifth soluble antagonistic receptor, osteoprotegerin (OPG) exists. The ability of OPG to act as an antagonistic receptor for TRAIL is disputed, because of its low affinity for TRAIL at 37°C (8). The existence of decoy receptors and their widespread expression on normal cells was initially seen as the explanation for protection of normal cells against TRAIL-induced apoptosis. Decoy receptor expression is, however, also present in cancer cells, without predicting sensitivity or resistance to recombinant human (rh)TRAIL (9). TRAIL binds as a homotrimer to DR4 and DR5, which results in trimerisation of the receptors (see figure 1). This leads to the assembly of a death-inducing signalling complex (DISC). At the DISC, the adaptor protein FADD (Fas associated death domain) acts as a bridge between the death receptor complex and the pro-domain of the initiator caspase 8. Dimerisation of caspase 8 molecules at the DISC leads to the formation of mature caspase 8, that is capable of activating downstream effector caspases such as caspase 3, 6 and 7, which execute apoptosis (10). This death receptor initiated apoptosis pathway is referred to as the extrinsic apoptosis pathway. Crosstalk exists between the extrinsic pathway and the intrinsic or mitochondria-initiated apoptosis pathway through Bid, a BH3-only protein member of the Bcl-2 gene superfamily, which can be activated by active caspase 8 to trigger mitochondrial perturbation. The intrinsic pathway triggers apoptosis after DNA damage by chemo- and radiotherapy, hypoxia, starvation and other kinds of severe cellular stress. When the intrinsic pathway is activated, pro-apoptotic members of the Bcl-2-gene family, such as Bax and Bak, translocate to the mitochondria, causing release of cytochrome c and other mitochondrial factors into the cytosol. In the cytosol, cytochrome c binds the adaptor protein Apaf-1 and pro-caspase 9 in the presence of dATP. This leads to the formation of the apoptosome signalling complex, in which caspase 9 is activated and can subsequently activate the effector caspases 3, 6 and 7 (7).

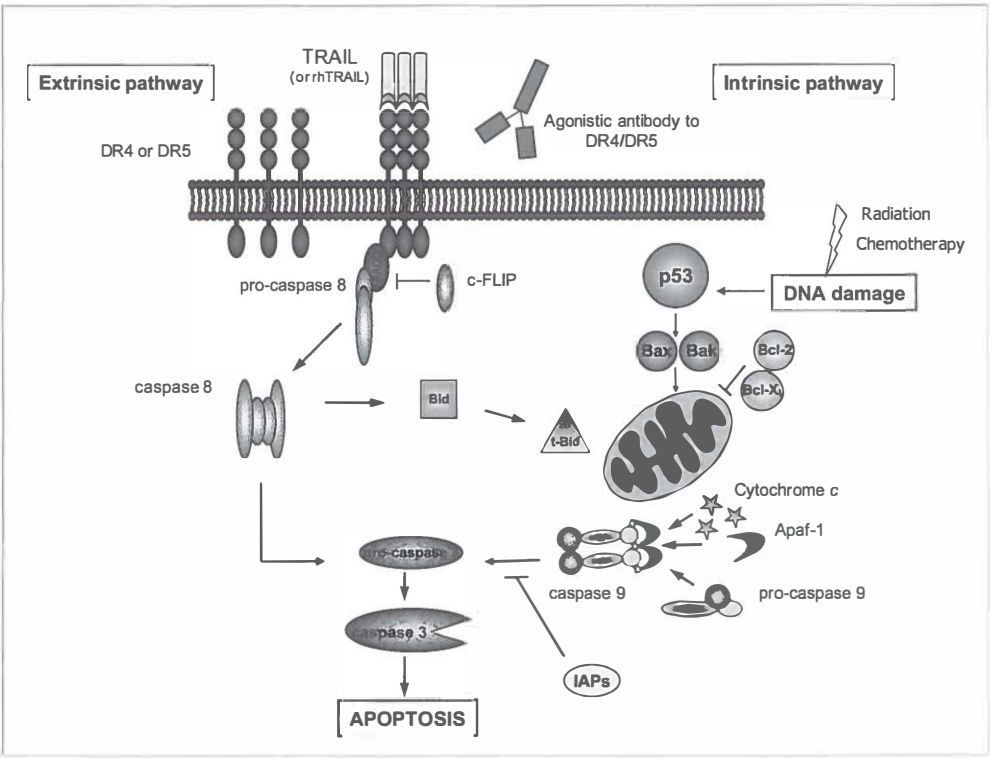

Figure 1. Apoptotic pathway.

The death receptor initiated apoptosis pathway is referred to as the extrinsic apoptosis pathway. TRAIL binds as a homotrimer to DR4 and DR5, which results in trimerisation of the receptors and subsequent assembly of a death-inducing signalling complex (DISC). At the DISC, the adaptor protein FADD acts as a bridge between the death receptor complex and the initiator caspase 8. Upon recruitment by FADD, caspase 8 will be activated and then activates downstream effector caspases such as caspase 3, 6 and 7. When the intrinsic apoptotic pathway is activated, pro-apoptotic members of the Bcl-2-gene family translocate to the mitochondria, causing subsequent release of cytochrome c and other mitochondrial factors into the cytosol. In the cytosol, cytochrome c binds the adaptor protein Apaf-1 and pro-caspase 9 in the presence of dATP. It hereby forms the apoptosome signalling complex, in which caspase 9 is activated and can activate subsequently the effector caspases 3, 6 and 7. Crosstalk exists between the extrinsic pathway and the intrinsic or mitochondria-initiated apoptosis pathway through Bid. Activated caspase 8 will cleave Bid, which then translocates to the mitochondria to induce cytochrome c release.

THE PHYSIOLOGICAL ROLE OF TRAIL

Although mice only express one receptor resembling DR4 and DR5 (11), mouse models have provided important information regarding the expression and physiological role of TRAIL in vivo. These models include TRAIL knockout mice, the use of neutralizing anti-mouse TRAIL monoclonal antibodies and soluble recombinant human DR5 (12). TRAIL-/- mice are viable and display no apparent haematological or reproductive defects (13). Expression patterns of TRAIL might reveal an indication to its natural function. While TRAIL mRNA and protein expression is found in a variety of cells and tissues (5,14), studies in mice and humans show that TRAIL is not expressed at the surface of freshly isolated T-cell, B-cells, monocytes, dendritic cells, natural killer (NK) cells or NKT-cells. Only a subset of mouse NK-cells expresses TRAIL at their surface. After stimulation with interferons most NK-cells, monocytes, peripheral T-cells, and dendritic cells express TRAIL at their surface, suggesting an important role of TRAIL in innate immune responses (12). In mice in which TRAIL is blocked with neutralizing antibodies and in TRAIL -/- mice an essential role of liver NK-cells in prevention against liver metastases was shown (13,15). Furthermore, TRAIL contributes to host immunosurveillance against primary tumour development. Neutralisation of TRAIL promoted tumour development in mice inoculated with the carcinogen methylchoranthrene (MCA) (15). This increased tumour promoting effect of MCA was also observed in TRAIL -/- mice (13). Moreover, the preferential emergence of TRAIL-sensitive fibrosarcoma cells in TRAIL-/- mice and IFNγ-deficient mice implies an immune selection pressure against TRAIL-sensitive cells during tumour development (15).

In addition to TRAIL's proposed role in tumour immune surveillance, various roles of TRAIL are described in autoimmunity. Most studies describe an inhibitory function of TRAIL on the development of experimentally induced autoimmune diseases in mouse models, such as rheumatoid arthritis, diabetes and experimental autoimmune encephalomyelitis. However, TRAIL may also be involved in acceleration of autoimmune diseases (16). Thus, TRAIL may exert dual functions depending on time, extent and location of TRAIL expression. A major task is to translate these results to the human situation and find therapeutic strategies involving TRAIL for the prevention or treatment of autoimmune diseases.

Besides studies performed in mice, several studies report increased serum levels *in vivo* of soluble TRAIL (sTRAIL) in patients with neoplastic, autoimmune and infectious diseases. In a human endotoxemia model sTRAIL levels increased 10-fold after administration of endotoxin (17). Patients with systemic lupus erythematosus display raised sTRAIL concentrations (18) and in human multiple sclerosis patients sTRAIL levels might serve as a potential response marker for IFN-β treatment (16). These results confirm a physiological role of TRAIL in various immune reactions in the human situation.

REGULATION OF TRAIL SENSITIVITY

Sensitivity to TRAIL-induced apoptosis can be modulated at several levels in the apoptosis signalling pathway. Numerous mechanisms to escape apoptosis induction have been described. These mechanisms account for intrinsic or acquired resistance to TRAIL, and since crosstalk exists between the extrinsic and intrinsic pathway some of them may be in part responsible for resistance to conventional therapies as well. Although it is highly likely that these mechanisms play a major role in the difference in sensitivity of tumour cells and normal cells to TRAIL, a uniform mechanism that can explain why normal cells are resistant to TRAIL-induced apoptosis has not yet been found.

Because of the physiological role of TRAIL in tumour surveillance, down-regulation or loss of agonistic TRAIL receptors might contribute to a malignant phenotype. Loss of heterozygosity (LOH) of region 8p, which is the region where the TRAIL receptors are mapped, is a frequent event in many cancers (19). This LOH may facilitate mutations or deletions in the TRAIL receptors,

leading to TRAIL resistance. In spite of this, deletions or mutations were found only in small numbers of non-small cell lung cancers, head and neck cancers, gastric cancers, non-Hodgkin lymphomas and breast cancers (10). Epigenetic changes such as promoter hypermethylation have been described to be responsible for loss of TRAIL receptor expression in neuroblastoma (20), small cell lung cancer (21) and ovarian cancer (22), but are not a common phenomenon.

Down-regulation of the initiator caspase 8 may be responsible for resistance to apoptotic signalling. Silencing of caspase 8 expression by DNA methylation as seen in neuroblastomas, primary neuroectodermal brain tumours, small cell lung cancer cell lines and retinoblastoma, correlated with resistance to rhTRAIL (23). Suppression of caspase 8 expression was shown to occur during the development of neuroblastoma metastases in vivo, and reconstitution of caspase 8 expression in deficient neuroblastoma cells suppressed metastases formation (24). This confirms the importance of TRAIL in preventing metastases.

Cellular FLICE-like inhibitory protein (c-FLIP) acts as an important intracellular inhibitor of TRAIL sensitivity. c-FLIP is structurally related to caspase 8 and can bind to FADD, but lacks enzymatic activity. It thus prevents apoptosis by blocking association of caspase 8 with the DISC. Down-regulation of c-FLIP renders cells sensitive to rhTRAIL. Besides acting as a specific inhibitor of apoptosis, c-FLIP is also involved in mediating growth signals through activation of the NFκB and ERK signalling pathways (25). Nonetheless, although the importance of c-FLIP as an anti-apoptotic protein is clear, a consistent correlation between c-FLIP expression and rhTRAIL resistance has not been established in cell line models.

Mitochondrial outer membrane permeabilisation (MOMP) and subsequent release of cytochrome c and other proteins, plays an essential role in the intrinsic apoptotic pathway. Although regulation and execution of MOMP can not be exclusively assigned to members of the Bcl-2 family, they act as key proteins in the control of MOMP (26,27). The Bcl-2 family consists of anti-apoptotic members, such as Bcl-2 or Bcl-X_L, which can block MOMP, and pro-apoptotic members. The latter can be divided into those that directly induce MOMP, such as Bax and Bak, and those that facilitate activation of Bax and Bax, such as Bim, Bad and PUMA (28). Altered expression of Bcl-2 family members occurs in several tumour types and can be associated with prognosis or treatment response (29). Overexpression of Bcl-2 or Bcl-X_L inhibits rhTRAIL-induced apoptosis in e.g. lung, prostate and pancreatic cancer cells, but was not able to block apoptosis in murine embryonic fibroblasts and Jurkat cells (10). Inactivation of either Bax or Bak does not seem to impair apoptosis greatly, but inactivation of both can dramatically impair apoptosis (30-32). In mismatch repair deficient colorectal tumours inactivation of Bax only was sufficient to cause resistance to rhTRAIL (33). These results show that in rhTRAIL-induced apoptosis, the importance of crosstalk between the extrinsic and intrinsic pathway varies between cell types.

The IAP-family of genes has an evolutionary conserved role in apoptosis regulation in animals ranging from insects to humans (34,35). IAPs are characterised by the presence of one to three baculovirus IAP repeat (BIR) domains, through which they can bind and inactivate caspases. Many IAPs also possess a C-terminal RING domain, that enables them to ubiquitylate themselves and other interacting proteins (36). XIAP is the most potent human IAP member, which can bind and inactivate caspase 3, 7 and 9 (34). cIAP-1 and cIAP-2 have been shown to bind caspase 7 and 9, but this does not seem to lead to inactivation of these caspases (37). Overexpression of members of the IAP family correlates with survival in many tumour types (38). Targeting IAPs in vitro sensitises many tumour cells to rhTRAIL.

TRAIL sensitivity can also be regulated by activation of pro-apoptotic pathways. The transcription factor NFκB can exert anti-apoptotic effects most likely through up-regulation of anti-apoptotic genes, such as c-FLIP, XIAP, cIAP-1, cIAP-2, Bcl-X_L. It is known that TNF regulates its own action by activation of NFκB. TRAIL is able to activate NFκB as well. This activation can be mediated through the agonistic receptors DR4 and DR5, as well as the antagonistic receptor

DcR2. Interestingly, TRAIL seems to activate NFκB only when its apoptosis inducing capacity is blocked (39), suggesting another role for NFκB in TRAIL signalling than for TNF. Constitutively active NFκB can prevent rhTRAIL-induced apoptosis in some cases and blocking or interfering with NFκB can sensitise cells to rhTRAIL (10). Several newly developed drugs, such as proteasome inhibitors and 17-allylamino-17-demethoxygeldanamycin (17-AAG) (40), can augment rhTRAIL-induced apoptosis by inhibition of NFκB.

The PI3K-AKT pathway is one of the key pathways controlling survival, proliferation and growth. It can promote tumour cell survival through direct interference with apoptosis through inactivation of Bax, Bad and caspase 9, up-regulation of c-FLIP (41) and stabilisation of XIAP (42). Furthermore, it can activate NFκB and increase p53 degradation. Several components of the AKT pathway are deregulated in a wide range of cancers (43) and contribute to resistance to chemotherapy and radiation. Numerous reports describe an inhibitory role of AKT in TRAIL signalling. Down-regulation of constitutively active AKT reversed resistance to rhTRAIL (7).

RHTRAIL AND AGONISTIC ANTIBODIES TARGETING THE TRAIL PATHWAY

The unravelling of the TRAIL pathway has resulted in many preclinical studies that have confirmed the potential of rhTRAIL for the treatment of cancer. RhTRAIL induces marked anti-tumour effects in a broad range of tumour cell lines (44). Substantial anti-tumour activity without systemic toxicity is demonstrated in mouse xenograft models treated with rhTRAIL as single agent (44,45). However, the identification of key factors involved in the regulation of sensitivity to TRAIL has shown that this regulation is highly complex and that tumour cells may present with intrinsic or acquired resistance to rhTRAIL. Fortunately, numerous studies have shown that combinations of rhTRAIL and chemotherapy or radiotherapy show synergistic effects in several human tumour types and can overcome resistance to either of the agents (4). These data have provided a solid ground for the exploration of the clinical applicability of agents targeting the TRAIL pathway.

The safety profile of rhTRAIL has been evaluated in non-human primates, because of the homology in sequence identity of TRAIL and TRAIL receptors. An 84-99% extra-cellular protein sequence identity is shared between humans and cynomolgus monkeys and a 97-99% extra-cellular sequence identity exists between humans and chimpanzees. No toxicity has been observed in cynomolgus monkeys and chimpanzees after administration of rhTRAIL (44-46). Fas-ligand and earlier recombinant versions of TRAIL could not proceed to the clinic due to liver toxicity. These early variants of TRAIL contained an exogenous sequence tag, for example polyhistidine. Moreover, they were not optimized for zinc content, which is crucial for stability and biologic activity. The tagged TRAIL versions therefore had a tendency to form insoluble aggregates. In preclinical studies they caused apoptosis in normal cells, including hepatocytes, possibly as a result of high-order multi-merisation of death receptors. In contrast, administration of the optimized version of rhTRAIL in non-human primates did not induce hepatotoxic effects (46). Pharmacokinetic studies with rhTRAIL show a half-life of 21-31 minutes in non-human primates. Furthermore, the rhTRAIL clearance is highly correlated to glomerular filtration rate in various species, suggesting that rhTRAIL is primarily eliminated through the kidneys (45). Based on these results, a phase I study with rhTRAIL (Genentech, San Fransisco, CA, USA) has been initiated.

DR4 and DR5 can also be targeted using agonistic antibodies. Upon binding to the receptor these antibodies activate the apoptotic pathway. The potential advantage of this approach is the specific binding to the target receptor: the antibodies bind selectively and with high affinity to their cognate receptor. Furthermore, the half-life of these antibodies is longer than the half-life of rhTRAIL. Three fully human monoclonal antibodies are under development, one directed at DR4 and two directed at DR5. HGS-ETR1 (mapatumumab; Human Genome Sciences, Rockville, MD, USA) is an agonistic antibody to DR4, whereas HGS-ETR2 and HGS-TR2J (both Human Genome

Sciences) are agonistic antibodies to DR5. Compared to HGS-ETR2, HGS-TR2J seems to be a more potent DR5 antibody, because it is more effective at inducing tumour regression in xenograft models. Whereas HGS-ETR2 needs cross-linking reagents for exertion of its apoptotic activity, HGS-TR2J induces apoptosis independently of cross-linking reagents, and is therefore capable of directly activating the apoptotic pathway (47). Whether the difference between both antibodies will have any implications with regard to efficacy and toxicity profile in humans will become apparent in the clinical studies with these agents.

In preclinical studies HGS-ETR1 inhibited tumour growth and induced apoptosis in a broad range of tumour cell lines and human tumour mouse xenograft models. Preliminary data from two clinical phase I studies have been reported (48,49). These dose-escalation studies have been conducted in patients with advanced solid malignancies and non-Hodgkin's lymphoma. They received doses of HGS-ETR1 varying from 0.01 to 20 mg/kg every 28 days. An alternate dose level of 10 mg/kg every 14 days is still being evaluated. HGS-ETR1 has been well tolerated so far and the maximum tolerated dose has not been reached. Stable disease was the best response observed. The mean terminal elimination half-life was approximately 17 days.

Data from three phase II studies with single agent HGS-ETR1 in patients with colorectal cancer, non-small cell lung cancer and non-Hodgkin's lymphoma have recently been presented. In a phase II study in patients with relapsed or refractory non-Hodgkin's lymphoma, the efficacy, safety and tolerability of HGS-ETR1 as single agent was evaluated (50). Forty patients were enrolled in one of two treatment groups, receiving either 3 mg/kg every 21 days (n=8) or 10 mg/kg every 21 days (n=32). Tumour responses (one complete and two partial responses) were seen in three patients (8%), who were all diagnosed with follicular lymphoma. Stable disease was observed in 12 patients (30%). The antibody was well tolerated, with minimal toxicity observed. Another phase II study was conducted in 32 heavily pre-treated patients with relapsed or refractory non-small cell lung cancer (51). Patients received 10 mg/kg of HGS-ETR1 every 21 days until disease progression. Stable disease was observed in nine patients (29%) with a median duration of 2.3 months. In a phase II study involving 38 patients with relapsed or refractory colorectal cancer HGS-ETR1 was administered at a dose of 20 mg/kg every 14 days during cycle 1 and 2, and at 10 mg/kg every 14 days in cycles 3-6 (52). The best response observed was stable disease in 12 patients (32%) for a median of 2.6 months.

Preclinical studies in which HGS-ETR1 was combined with chemotherapy showed an increased cytotoxic effect in human tumour cells and mouse xenograft models. Two phase Ib studies are currently ongoing to evaluate the safety and tolerability of HGS-ETR1 in combination with gemcitabine and cisplatin, and with paclitaxel and carboplatin (53,54). So far, these combinations have been well tolerated and the maximum tolerated dose has not yet been reached. Pharmacokinetic analyses show no signs of drug interaction.

HGS-ETR2 showed growth inhibitory effects as single agent in various human tumour xenografts derived from glioma, non-small cell lung cancer, colorectal cancer and breast cancer. This antibody was evaluated in two phase I studies in patients with advanced solid tumours. In the first study, 31 patients have been treated at the dose levels of 0.1 to 10.0 mg/kg every 14 days (55). HGS-ETR2 was well tolerated with minimal toxicity. Stable disease was observed in 10 patients (32.3%). In the second study, HGS-ETR2 was administered every 21 days at doses up to 20 mg/kg in 37 patients (56). At 20 mg/kg, four patients experienced dose-limiting toxicity. One patient in the 20 mg/kg cohort developed acute renal failure, sepsis and elevated AST (grade 4), ALT (grade 4) and bilirubin (grade 3) levels. The renal failure was considered possibly related and the liver function abnormalities probably related to the study drug. The sepsis was considered not related to HGS-ETR2. The patient died of renal failure. Three additional patients at this dose level developed dose-limiting toxicities, consisting of hyperamylasemia grade 3 (n = 2) and grade 4 elevations of AST and ALT. Administration of HGS-ETR2 was safe with minimal toxicity at the maximum tolerated dose of 10 mg/kg. Stable disease was the best response observed in 11 patients (29.7%).

Preclinical studies with HGS-TR2J showed regression or growth inhibition in human cancer cell lines and xenograft models, both as single agent and in combination with several chemotherapeutics. A phase I clinical trial is currently ongoing.

It has to be realised that all these reports on clinical studies are preliminary. Studies in mice and on human immune cells show a physiological role of TRAIL in innate immune responses. However, since many questions regarding the precise role of TRAIL in the human body remain unanswered, administration of external doses of rhTRAIL may lead to unforeseen immunological effects. Thus far, no immunological side effects have been reported following the administration of agonistic antibodies, but this may not be predictive for the effect of rhTRAIL, since the antibodies are only directed at one of the TRAIL receptors. In addition, because the mechanism of tumour selectivity by rhTRAIL has not been fully explained, possible toxicity to normal tissue needs to be monitored tightly, especially in combinatory regimens of rhTRAIL and other cancer agents.

POTENTIAL OF COMBINATION THERAPIES

Apart from the use of rhTRAIL and agonistic antibodies as monotherapy, combinations with traditional chemotherapeutics are particularly interesting. Moreover, new compounds that target proteins involved in the TRAIL signalling pathway are in development and they may enhance the effect of rhTRAIL. Belonging to this group of new agents are the histone-deacetylase (HDAC) inhibitors. HDAC inhibitors affect various cellular processes in tumour cells by activating transcription of target genes (57,58). They induce differentiation, growth arrest and/or apoptosis. HDAC-inhibitors can activate both the extrinsic and intrinsic apoptotic pathways. The combination of HDAC inhibitors and rhTRAIL results in a synergistic apoptotic response in cancer cell lines due to up-regulation of DR4, DR5 and pro-apoptotic members of the Bcl2 family, down-regulation of anti-apoptotic Bcl-2 family members, and activation of caspase 3, 9 and 8 (58). Phase I and II studies with several HDAC inhibitors are ongoing (57).

Other novel drugs that enhance apoptosis when combined with rhTRAIL in human tumour cells are proteasome inhibitors (59). The proteasome is responsible for the degradation of proteins. Proteasome inhibitors affect multiple processes leading to inhibition of tumour growth and apoptosis. The proteasome inhibitor bortezomib, that is registered for refractory multiple myeloma, inhibits NFκB activation, decreases levels of the anti-apoptotic protein c-FLIP, and induces cell surface expression of DR4 and DR5 in various cells. This may account for the sensitisation of tumour cells to rhTRAIL through proteasome inhibition (59).

Targeting of anti-apoptotic proteins that are involved in the TRAIL signalling pathway, such as Bcl-2 and the IAPs, is yet another approach to increase sensitivity for rhTRAIL (60). Bcl-2 can be down-regulated by anti-sense therapy and this strategy is in clinical development. An anti-sense oligonucleotide targeting XIAP, the predominant inhibitor of caspases 3, 7 and 9, is currently under investigation in a phase I clinical trial. Also, small molecule inhibitors directed at anti-apoptotic proteins are evaluated in preclinical studies (61).

The combination of rhTRAIL with therapeutics targeting other pathways may also be of interest. Inhibition of pro-survival routes, including the PI3K/AKT pathway, can be especially interesting. Mammalian target of rapamycin (mTOR) is a kinase that acts downstream of AKT, and is important for the regulation of cell growth and proliferation. Inhibition of mTOR by rapamycin or its analogues results in cell cycle arrest by prevention of progression from G1 to S phase in dividing cells. In phase I and II studies responses have been observed with a rapamycin analogue in several solid tumours. Preclinical data show an increased sensitivity to rhTRAIL-induced apoptosis when rhTRAIL is combined with rapamycin (62).

Other promising therapeutics targets are the heat shock proteins (HSP). These chaperone proteins are essential for the proper folding and assembly of proteins, their intracellular transportation and the proteolytic turnover of many of the key regulators of cell growth and survival. HSP90 plays

a crucial role in these processes and protects cancer cells from apoptosis through stabilisation of AKT and NFκB. Inhibition of HSP90 leads to cell cycle arrest and apoptosis. The HSP90 inhibitor 17-AAG is currently under clinical investigation in phase I trials. In the preclinical setting, combinations of HSP90 inhibitors with rhTRAIL or with HGS-ETR1 and -ETR2 show synergistic effects on apoptosis (63,64).

EGFR and HER2, members of the epidermal growth factor receptor family, are involved in proliferation, angiogenesis, invasion and survival of cancer cells. Trastuzumab, the antagonistic HER2 antibody, is used for breast cancer treatment. In preclinical studies, the combination of rhTRAIL and trastuzumab enhances apoptosis in HER2 overexpressing cancer cell lines (65). Trastuzumab down-regulates the HER2 receptor, resulting in decreased activation of the pro-survival AKT pathway, and increased sensitivity for rhTRAIL. RhTRAIL-induced apoptosis is also increased by inhibition of EGFR (66). However, because the effect of rhTRAIL on hepatocytes when administered in humans is yet unclear, caution should be exerted when combining rhTRAIL with drugs that may affect the liver.

CONCLUSION

Over a decade of TRAIL research, we have acquired considerable knowledge of the TRAIL signalling pathway and of crucial factors involved in its regulation. RhTRAIL has been shown to induce apoptosis in tumour cells and xenografts without inducing toxicity in normal cells. Furthermore, in the preclinical setting numerous combinations of classical chemotherapeutics and targeted drugs with rhTRAIL have shown even more potent anti-tumour effects. Phase I and II studies with rhTRAIL and agonistic antibodies to the TRAIL death receptors are ongoing. Besides providing insights in the efficacy and the possible adverse effects, clinical studies may also lead to an even more profound apprehension of the TRAIL-pathway and may give rise to true tumour-tailored therapy by combining knowledge on the TRAIL signalling pathway with clinical response data and tumour characteristics.

Conflict of interest statement:

E.G.E de Vries is study coordinator of a study with HGS-ETR1. The University Medical Center Groningen receives the study drug and financial support to perform this study.

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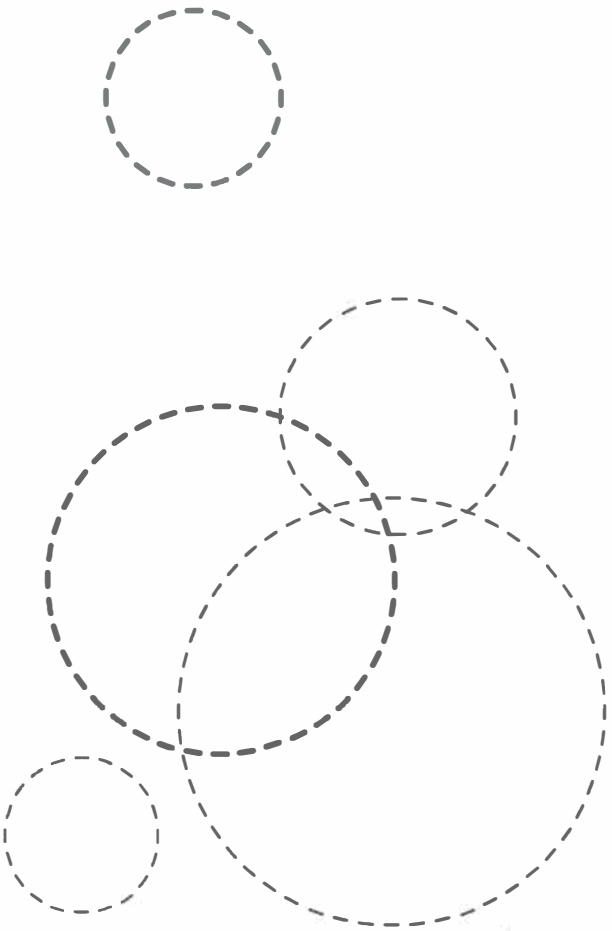

CHAPTER 4

INDUCTION OF TRAIL RESISTANCE IN MICROSATELLITE INSTABLE OVARIAN CANCER CELLS IS RELATED TO MUTATIONS IN *BAX*

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ABSTRACT

TNF-related-apoptosis-inducing-ligand (TRAIL) is a member of the TNF-family with clinical promise. Recombinant human TRAIL (rhTRAIL) is an inducer of apoptosis via binding to two death receptors DR4 and DR5. In the present study we investigated the effect of mutant p53 on rhTRAIL sensitivity in an isogenic model of the human A2780 ovarian carcinoma cell line stably transfected with a control vector (A2780/cmv) or a vector expressing mutant p53His273 (A2780/m273). A2780/m273 cells expressed elevated levels of caspase 8 protein and were highly sensitive to rhTRAIL as compared to A2780/cmv. Culturing A2780/m273 with increasing concentrations of rhTRAIL resulted in emergence of a >100-fold rhTRAIL-resistant subline (273TR). No difference in DR5 membrane expression or caspase 8 was observed between A2780/m273 and 273TR. Caspase 8 activation was detected in both A2780/m273 and 273TR following treatment with rhTRAIL, while Bid cleavage and caspase 9 activation were only observed in A2780/m273, suggesting a block at the mitochondrial level. Screening the expression of pro- and anti-apoptotic Bcl-2 family members showed no major differences in Bak, Bcl-2 or Bcl-X_L protein levels between A2780/m273 and 273TR. Bax expression, however, was lost in 273TR. Sequencing the *BAX* gene and the encoding mRNA demonstrated that A2780/cmv and A2780/m273 were *BAX* $^{+/-}$, while the rhTRAIL resistant cell line 273TR was *BAX* $^{-/-}$ due to a deletion of a G in the G(8) repeat within the protein encoding sequence of *BAX*. The microsatellite instability (MSI) of A2780/m273 and 273 TR suggests a similar mechanism for rhTRAIL resistance as has been observed in MSI-positive colon carcinoma cell lines. After culturing 273TR cells in the absence of rhTRAIL, the cells became partially sensitive to rhTRAIL, also reflected in caspase 9 activation but were still *BAX* $^{-/-}$. In conclusion, our results using an isogenic model indicate that mutant p53-expressing ovarian cancer cells can be more sensitive to rhTRAIL than wild type p53- expressing cells. Furthermore, induction of TRAIL resistance is partially due to a deletion in *BAX* resulting in a *BAX* $^{-/-}$ phenotype in these ovarian cancer cells.

INTRODUCTION

The occurrence of resistance to chemotherapy is a major problem in cancer treatment. First line chemotherapy for patients with advanced ovarian cancer consists of a platinum-based compound in combination with paclitaxel resulting in ~70% clinical responses. Clinical data show that the majority of these patients ultimately die of their disease suggesting the occurrence of intrinsic or acquired resistance to chemotherapy (1). In several studies, the overall survival of ovarian cancer patients was strongly related to mutations in tumor suppressor gene p53 (2-4). Mutations and deletions in p53 have been found in up to 60% of the ovarian cancers (2). The p53 protein is known to be involved in several cellular processes such as cell cycle, DNA repair, transcription and drug-induced apoptosis (5-7), and mutated p53 has been implicated in resistance to drug-induced apoptosis. New treatment modalities inducing apoptosis in a p53-independent manner are currently being explored.

Cytokines belonging to the TNF family such as TNF-related-apoptosis-inducing-ligand (TRAIL) can induce apoptosis in a wide variety of tumor cell lines (8). Binding of homotrimeric TRAIL to the pro-apoptotic TRAIL receptors DR4 and DR5 induces trimerization and subsequent clustering of the intracellular death domains of DR4 and DR5 (9,10). The death domain-containing adaptor protein FADD and caspase 8 are then recruited to the receptors, which results in caspase 8 activation followed by caspase 3 activation (the so-called extrinsic pathway). Depending on the caspase 8 level, an amplification of the apoptotic signal can be induced via the mitochondria (intrinsic pathway). Caspase 8 activation then leads to Bid cleavage and truncated Bid (tBid) stimulates Bax and Bak induced mitochondrial release of cytochrome c and Smac/DIABLO into the cytoplasm (11-14). Cytochrome c, APAF1 and caspase 9 form the apoptosome leading to caspase 9 activation, which in turn activates caspase 3 (15,16). Released Smac/DIABLO prevents binding of inhibitor of apoptosis proteins (IAPs) to caspase 3 or caspase 9 thus promoting caspase-mediated apoptosis (17,18). Bax is regarded as a key-player in TRAIL-induced apoptosis via the mitochondrial (intrinsic) pathway, as inactivation or deletion of Bax in colon carcinoma cells proved to be associated with resistance to TRAIL-mediated apoptosis (10,11,19). Furthermore, TRAIL exposure to mismatch repair (MMR) deficient colon cancer cells selected for cells with inactivated Bax due to a frameshift mutation (19).

The tumor suppressor p53 interacts with the death receptor pathway at several levels. First, p53 transactivates both the DR4 and DR5 gene promoters, which both contain p53 DNA-binding sites (20,21). Secondly, p53 interacts with the intrinsic (mitochondrial) pathway as a transcriptional activator of Bax or through a direct interaction with mitochondria (22).

In the present study, we used an isogenic model of the human A2780 ovarian carcinoma cell line stably transfected with a control vector (A2780/cmv) or with a mutant p53His273 expression vector (A2780/m273) to investigate the effect of mutant p53 on TRAIL sensitivity (23). Codon 273 is one of the most frequently mutated codons of p53 in ovarian cancer indicating the relevance of this isogenic model (2,24). To define proteins involved in TRAIL sensitivity in mutant p53-expressing ovarian cancer cells, the sensitivity of A2780/m273 to recombinant human TRAIL (rhTRAIL) and the mechanism of resistance to rhTRAIL in the acquired resistant subline of A2780/m273 were investigated at the molecular level.

MATERIALS AND METHODS

Chemicals

RPMI 1640 medium was obtained from Life Technologies (Breda, The Netherlands) and fetal calf serum (FCS) from Bodinco BV (Alkmaar, The Netherlands). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-solution and cycloheximide were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands). RhTRAIL was produced non-commercially in cooperation with IQ-Corporation (Groningen, The Netherlands) following a protocol described earlier (25). The TRAIL-receptor antibodies used for flow cytometry were obtained from R&D (R&D Systems Inc., Oxon, UK). Caspase 9 inhibitor zLEHD-fmk, caspase 8 inhibitor zIETD-fmk, and broad-spectrum caspase inhibitor zVAD-fmk were obtained from Calbiochem (Breda, The Netherlands).

Cell lines

The human ovarian cancer cell line A2780 containing wild-type p53 was obtained from Dr. R. Ozols, Philadelphia, PA, USA. Plasmids were kindly provided by Dr. A.J. Levine, Princeton University, NJ, USA. As described previously, A2780 was transfected by electroporation with G418-resistance gene-containing plasmids carrying no p53 (pC53-CMV) or p53 mutated at codon 273 (pC53-4.2N3) (23). Cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS and 1 mg/ml G418 in a humidified atmosphere with 5% CO_2 at 37°C. Presence of mutant p53 was confirmed by Western blotting as previously published (23).

To obtain a TRAIL-resistant subline, A2780/m273, cells were exposed to 1.0 μg/ml rhTRAIL and cultured for 14 days to select for resistant cells. After 14 days, the cells were continuously cultured in the presence of 2 μg/ml for 21 days and finally 5 μg/ml rhTRAIL for 21 days. After 2 months, the TRAIL-resistant cell line 273TR was established. 273TR cells were continuously cultured in the presence of rhTRAIL (2 μg/ml), but before being used in experiments, cells were cultured one week without rhTRAIL. To establish a revertant cell line 273TR was cultured without rhTRAIL and the sensitivity to rhTRAIL was determined every month. The newly established revertant cell line was named 273TAF.

Subcloning of cell lines was performed using the clonogenic assay according to Zijlstra et al. (26). Briefly, 200 single cells were plated in an agarose top layer. Visible colonies were picked with a glass-pipet and cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS and 1 mg/ml G418. An alternative sub-cloning approach was also used involving limiting dilution of cell lines in 2 volumes RPMI 1640 medium with 10% FCS and 1 volume conditioned RPMI 1640 medium with 10% FCS and 1 mg/ml G418. Cell suspension was diluted and 100 μl was added to each well of a 96-well plate to result in an average of 0.5 cells / well. After 14 days, culture wells with single colonies were harvested and transferred to a 24-wells plate, and the cells were propagated as described above.

Flow cytometry

A2780/cmv and A2780/m273 cells were washed and diluted in PBS (6.4 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 0.14 mM NaCl; 2.7 mM KCl; pH 7.4), 2% heat-inactivated FCS and 0.1% sodium azide. Approximately 0.5 x 10^6 cells were suspended in 50 μl PBS and incubated on ice for 30 min with PE conjugated mouse anti-human DR4, DR5, decoy receptor (DcR1) or DcR2 antibodies (R&D Systems Inc., Oxon, UK) at a final concentration of 5 μg/ml. After washing twice, cells were resuspended in 200 μl PBS/2% FCS/0.1% sodium azide and analyzed (10,000 cells) by flow cytometry (Epics Elite, Coulter-Electronics, Hialeah, FL, USA) with gate set on intact cells by forward/side scatter analysis. Membrane receptor expression was analyzed with Winlist and Winlist 32 software (Verity Software House, Inc., Topsham, ME) and is shown as mean fluorescent intensity (MFI) of all analyzed cells.

Cytotoxicity

For determining cytotoxicity the MTT-assay was used. The linear relationship of cell number to MTT formazan crystal formation and the exponential growth of cells in the wells were determined first. In a total volume of 200 µl, 1,250 cells for A2780/cmv, A2780/m273, 273TR and 273TAF were incubated. After a total incubation period of 96 h, 20 µl of MTT (5 mg/ml in PBS) was added for 3.5 h. Subsequently, plates were centrifuged and the supernatant aspirated. After dissolving the formazan crystals by adding dimethyl sulfoxide (Merck, Amsterdam, The Netherlands), plates were read immediately at 520 nm using a microtiter well spectrometer. Cell survival was expressed as a percentage relative to untreated cells.

SDS-polyacrylamide gel electrophoresis and Western blotting

Cells were treated with rhTRAIL for 3 h (unless indicated otherwise) and washed twice with ice-cold PBS. Cells were lysed with standard Western blot sample buffer (50 mM TrisHCl, pH 6.8, 4% SDS, 10% glycerol, 10% 2-ß-mercaptoethanol, 0.002% bromphenol blue) and boiled for 10 min. The Bradford assay was used to determine protein concentrations. In all experiments, lysates containing 15 or 20 µg protein were used. Total cell lysates were size fractionated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto activated polyvinylidene difluoride membranes (Millipore BV, Etten-Leur, The Netherlands). Western blot analyses have been performed at least three times and membranes were stained with Ponceau S to check for equal protein loading. Actin expression levels were used as a loading control.

Antibodies used included: mouse-anti-FADD and mouse-anti-XIAP from Transduction Laboratories (Lexington, KY), rabbit-anti-Bid, a gift from Dr. J. Borst (NKI, Amsterdam, The Netherlands), rabbit-anti-caspase 3, and rabbit-anti-caspase 9 from Pharmingen (Becton Dickinson, Erebodegem-Aalst, Belgium), goat-anti-DR4, mouse-anti-Bcl-2, rabbit-anti-BclX_L, rabbit-anti-Bax (N20), goat-anti-Bak (N20) from Santa Cruz Biotechnology (Santa Cruz, CA) rabbit-anti-DR5 and rabbit-anti-survivin from Oncogene Research Products (Calbiochem-Novabiochem, Germany), mouse anti-caspase 8 from Cell Signaling Technology (Leusden, The Netherlands), mouse-anti-caspase 10, rabbit-anti-cIAP-1 and rabbit-anti-cIAP-2 from R&D systems, mouse-anti-FLIP NF6 kindly provided by Dr. M. Peter (Chicago, IL), and mouse-anti-actin from ICN Biomedicals (Zoetermeer, The Netherlands). The secondary antibodies were labeled with horseradish peroxidase (all from DAKO, Glostrup, Denmark) and chemiluminesence was employed for detection using the BM Chemiluminescence Blotting kit or the Lumi-Light Plus Western blotting kit (Roche Diagnostics, Mannheim, Germany).

Apoptosis-Assay

For each cell line 5,000 cells were seeded in 96-well plates. Cells were not treated, treated with rhTRAIL (0.1 or 1.0 µg/ml) for 3 h at 37°C or pretreated for 1 h with cycloheximide (CHX) (5 µg/ml), zLEHD-fmk, zVAD-fmk or zIETD-fmk (20 µM) before rhTRAIL (0.1 or 1.0 µg/ml) was added and cells were incubated for an additional 3 h at 37°C. Apoptosis was identified by acridine orange staining of nuclear chromatin using fluorescence microscopy. Apoptosis was expressed as the percentage apoptotic cells in a total cell culture. Apoptosis experiments were performed at least three times.

BAX mutation analysis on DNA and RNA

DNA was isolated according to standard protocols. PCR amplifications were performed using previously published primer sequences (Le Blanc et al 2002) amplifying a 94-base pair DNA fragment encompassing the unstable G8 tract. PCR was carried out for 32 cycles, each cycle consisting of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C and extension for 1 min at 72 °C. PCR products were visualized on an 1.5 % agarose gel. For sequencing, PCR products were isolated with the Qiaquick PCR purification kit (Qiagen Inc., Chatsworth, CA, USA). A M13 universal primer sequence tail attached to the 5'-end of an amplification primer facilitated

sequencing of amplification products using the ABI Prism™ genetic analyzer (Applied Biosystems Product, Foster City CA, USA).

Total RNA was isolated by lysing cells in 500 µl guanidine thiocyanate buffer (4 M guanidine thiocyanate, 0.5% n-lauroyl sarcosine, 25 mM sodium citrate (pH 7.0), 0.1 M 2-β-mercaptoethanol). The quality of the samples was checked by agarose gel electrophoresis. Prior to cDNA synthesis, RNA was treated with DNase I (Roche Diagnostics, Almere, The Netherlands). cDNA was synthesized from 5 µg total RNA as described by the manufacturer's protocol (Life Technologies) using oligo dT primers and MMLV transcriptase. PCR amplifications were performed as described above.

MSI analysis

For MSI analysis, DNA was isolated from tumor cells and the HNPCC MSI kit was used (Roche, Basel, Switzerland), containing the five consensus markers as described previously (27). MSI analysis was performed on the ABI Prism 377 DNA sequencer using fluorescently labeled primers (Promega, Madison WI, USA).

RESULTS

Establishing a rhTRAIL resistant ovarian cancer cell line

The human A2780 ovarian cancer cell line, the stably mock transfected subline A2780/cmv, and A2780/m273, which is stably transfected with p53His273, were tested for rhTRAIL sensitivity using the MTT assay. Both A2780 and A2780/cmv were resistant to rhTRAIL, while A2780/m273 was highly sensitive to rhTRAIL (IC50 = 0.01 µg/ml). A2780/m273 was cultured in the presence of rhTRAIL (1 µg/ml) to establish a TRAIL resistant cell subline. Concentrations of rhTRAIL were stepwise increased up to 5 µg rhTRAIL/ml resulting in a more than 100-fold resistant TRAIL resistant cell subline (273TR) (Fig.1). Similar results were observed with an apoptosis assay as the MTT-based cell viability assay. Almost no apoptosis induction was found in cultures of A2780/cmv and 273TR cells, while rhTRAIL induced high levels of apoptosis in A2780/m273 (Fig.2). Stability of the TRAIL resistance phenotype was studied by culturing TRAIL resistant cells in the absence of rhTRAIL for several months. Resistance to rhTRAIL was partially reverted in 273TAF, as demonstrated with cell survival and apoptosis assays (Fig.1 and 2).

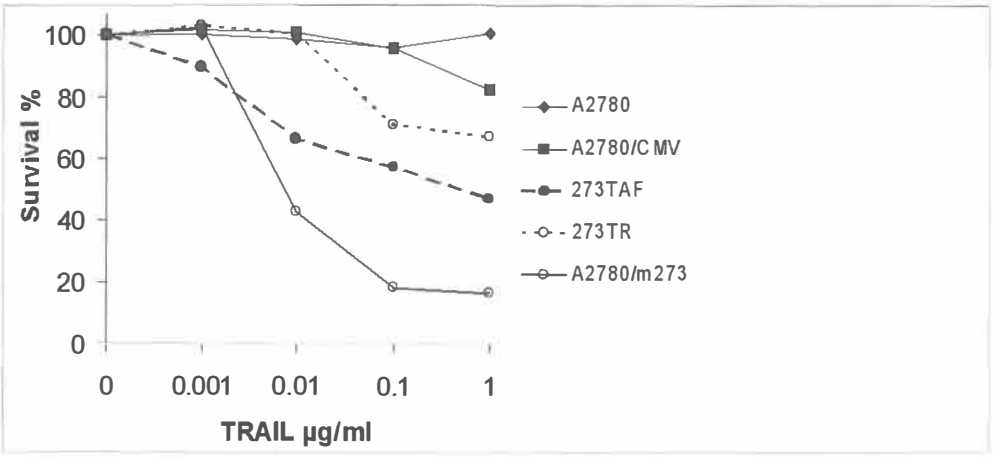

Figure 1. Sensitivity of ovarian carcinoma cells for rhTRAIL.
Cytotoxicity of ovarian carcinoma cell lines after continuous exposure to rhTRAIL for 4 days. Data represent the mean ± SD of three independent experiments.

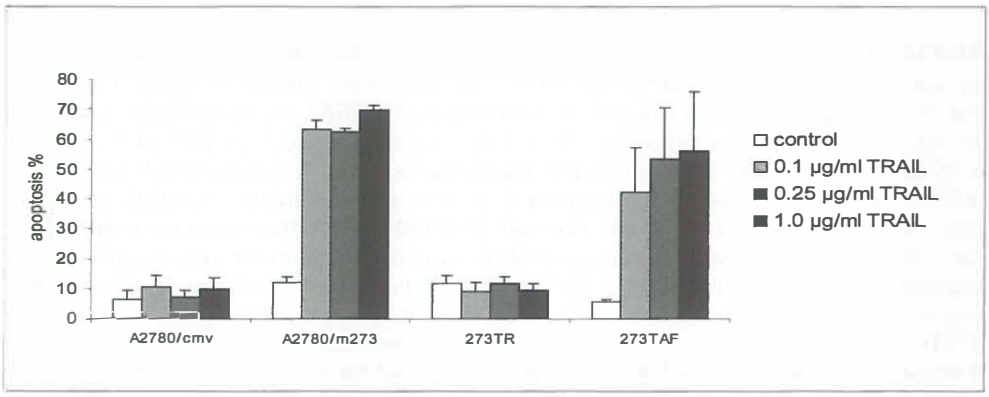

Figure 2. Apoptosis induction by rhTRAIL.
Induction of apoptosis by rhTRAIL incubation for 3 h. Data represent the mean ± SD of three independent experiments.

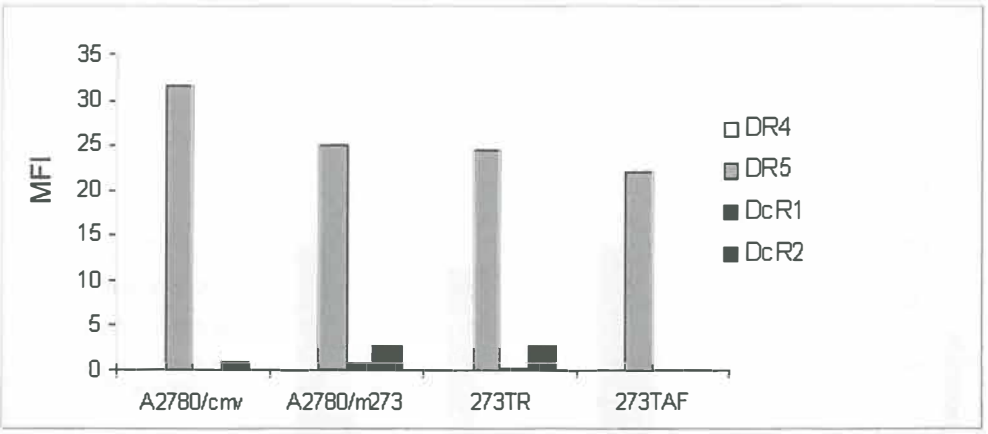

Figure 3. Cell membrane expression of death receptors.
Flow cytometry analysis of DR4, DR5 , DcR1 and DcR2 in A2780/cmv, A2780/m273, 273TR and 273TAF. A representative sample from multiple analyses is shown.

TRAIL receptor surface expression on the A2780 lines was investigated with flow cytometry (Fig.3). DR4 was not expressed on any of the cell lines, while DR5 surface expression was detected on all cell lines. However, no differences in DR5 expression were observed between the intrinsic TRAIL resistant cell lines A2780 and A2780/cmv, the acquired resistant cell line 273TR, or the revertant 273TAF cells compared to A2780/m273. In addition, no differences in DcR1 or DcR2 surface expression were found.

To determine whether resistance of 273TR was due to non-functional DR5, cells were treated with CHX (Fig.4). RhTRAIL sensitivity was restored in 273TR, suggesting the overexpression of an intracellular inhibitor of apoptosis. Similarly, sensitivity of 273TAF to rhTRAIL was enhanced in the presence of CHX. In contrast, A2780/cmv still maintained resistance to rhTRAIL.

Reduced caspase 8 and caspase 9 activation in 273TR

To map the point of the resistance mechanism, we determined caspase 8, 9 and 3 activation and Bid cleavage by Western blot analysis following short rhTRAIL exposure (Fig.5). Caspase 8 activation was detected by either the intermediate caspase 8 product (p43/41) or the caspase 8 (p18) subunit and Bid activation by the disappearance of Bid. Exposure of A2780/m273 to rhTRAIL resulted in cleavage of procaspase 8 as well as Bid activation, whereas in A2780/cmv and 273TR only caspase 8 activation was observed but not disappearance or cleavage of Bid. Remarkably, caspase 8 expression levels in A2780/cmv are much reduced compared to expression levels in A2780/m273, 273TR or 273TAF. In contrast to caspase 8, processing of caspase 9 was observed in A2780/m273, but to a much lesser extent in A2780/cmv and not in 273TR. However, caspase 9 processing was observed in revertant 273TAF cells (Fig. 5). Caspase 3 activation was detected in A2780/m273 and 273TAF but not in 273TR and A2780/cmv cells, which is related to the results observed in the apoptosis assay. Cells were treated with the specific caspase 8 inhibitor zIETD-fmk (20 μM) and the caspase 9 inhibitor zLEHD-fmk (20 μM) to investigate whether caspase activation was involved in rhTRAIL-induced apoptosis. Following pretreatment with zIETD-fmk or zLEHD-fmk, rhTRAIL-induced apoptosis was reduced with 87.3 % ± 4.9 and 74 % ± 13.3, respectively, in A2780/m273 and with 95.4 % ± 5.5 and 76.8 % ± 3.9, respectively, in 273TAF. These results demonstrate the involvement of caspase activation in rhTRAIL-induced apoptosis, which may depend on the mitochondrial apoptotic pathway.

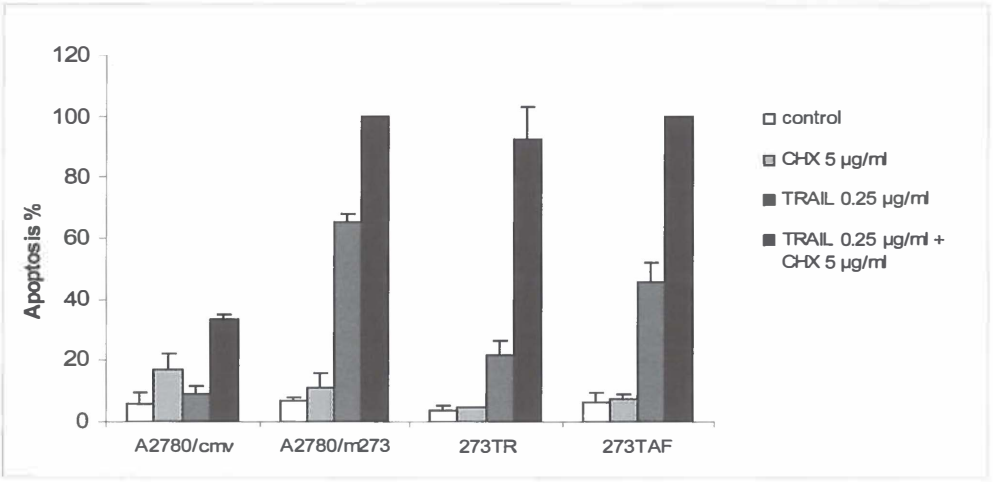

Figure 4. Cycloheximide increases sensitivity to rhTRAIL.
Cells were treated with cycloheximide 1 h prior to incubation with rhTRAIL for 3 h. Data represent the mean ± SD of three independent experiments.

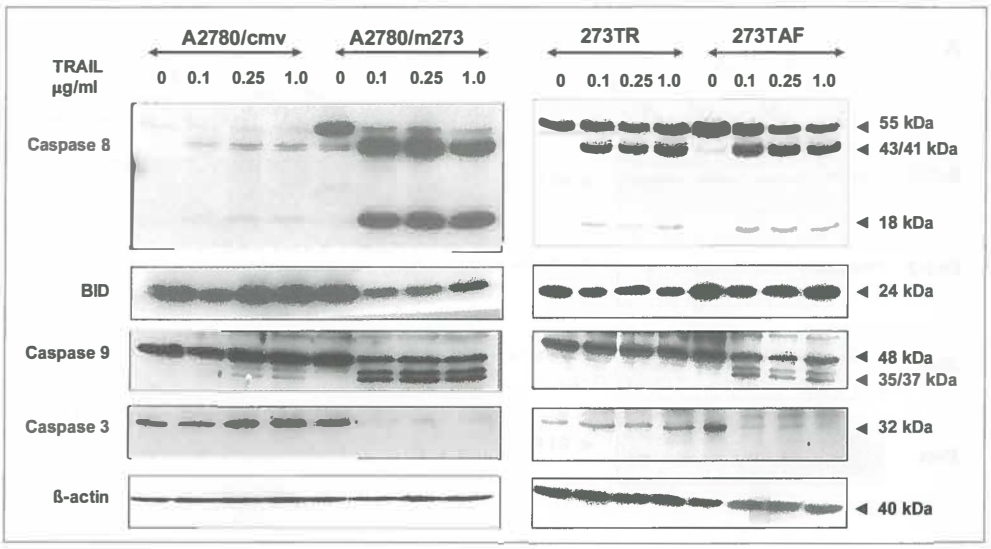

Figure 5. Lack of TRAIL-induced caspase 9 activation in 273TR.
Cells were treated with 0.1 µg/ml rhTRAIL for 3 h. A representative Western blot of at least three independent experiments is shown.

Loss of Bax expression in 273TR

Evaluation of the expression of several pro-apoptotic proteins and apoptosis inhibitory proteins revealed identical levels of caspase 8, caspase 10 and FLIP in A2780/m273 and 273TR. No distinct change was observed in XIAP, cIAP-1, or cIAP-2 expression (results not shown). When Bcl-2 family members were analyzed, no differences in Bid, Bak, Bcl-2 and Bcl-X_L were found between A2780/m273 and 273TR. Bcl-2 expression levels in A2780/m273 and 273TR, however, were reduced compared to A2780/cmv. The major difference between A2780/m273 and 273TR was the complete absence of Bax expression in 273TR (Fig.6A). The revertant cell line 273TAF still showed no Bax expression, and remained Bax negative over time (Fig.6B).

To further investigate the cause of Bax loss, RT-PCR was performed. Bax mRNA was detected in both cell lines. Sequence analysis revealed that in A2780/m273 a wild-type and a mutant BAX sequence were present, whereas only a mutant BAX sequence was detected in 273TR (Fig.7). The mutation in the BAX gene was located within a homopolymeric tract of 8 deoxyguanosines (G)8, resulting in a frameshift. DNA sequence analysis of the BAX gene demonstrated the same finding as with RT-PCR analysis, i.e. a wild-type and a mutant BAX gene in A2780/m273 but only mutant BAX genes in 273TR.

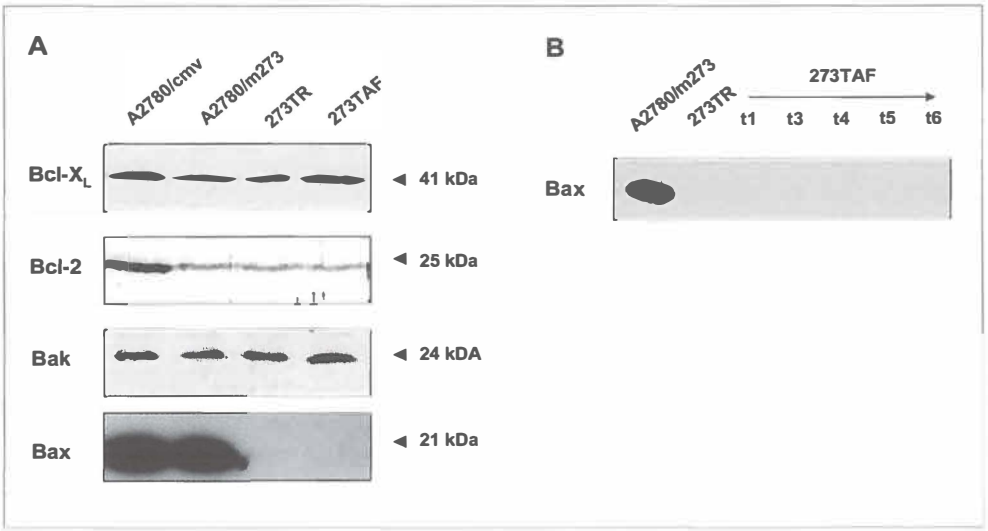

Figure 6. Bax is not expressed in the acquired TRAIL resistant 273TR cells.
(**A**) Expression of Bcl-2 family members was determined with Western blot analysis. A representative example of at least three independent experiments is shown.
(**B**) Bax expression is not detectable in 273TR in time (t1 = 2 weeks, t2 = 4 weeks,...., t6 = 12 weeks)

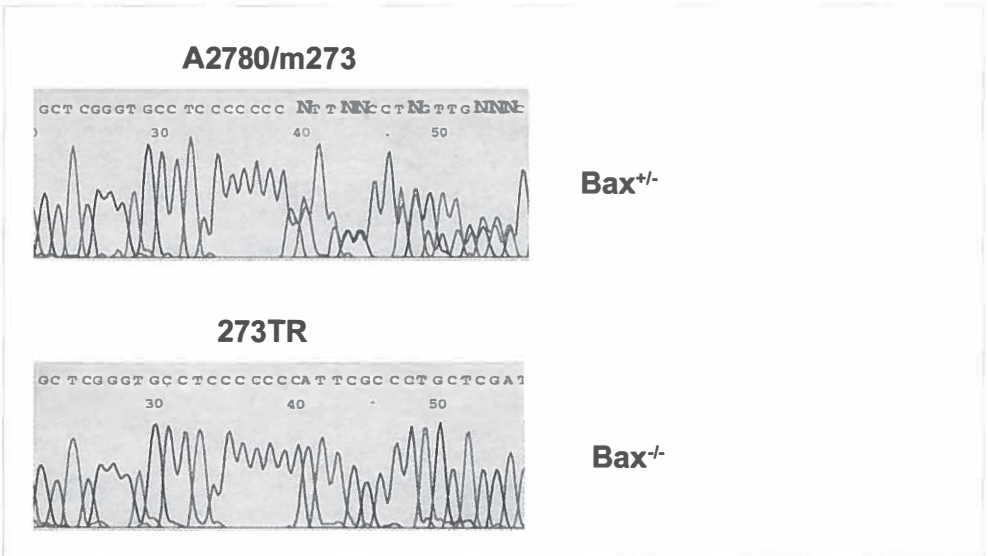

Figure 7. Deletion of a base in the homopolymeric G(8) repeat of Bax in 273TR.
DNA sequence analysis of the G(8) repeat of *BAX*, shown from the sequence of the antisense. DNA isolated from A2780/m273 shows two PCR products, one of the wild type allele and one of the mutated allele as a 1 base pair deletion in the C(8) repeat. DNA isolated from 273TR shows one PCR product with a 1 base pair deletion in the C(8) repeat.

Induction of TRAIL resistance in microsatellite instable A2780/m273 BAX $^{+/-}$ cells selects for BAX $^{-/-}$ cells

To determine whether the *BAX* $^{+/-}$ phenotype of A2780/m273 was due to a mixed population of *BAX* $^{+/+}$ and *BAX* $^{-/-}$ cells, we subcloned A2780, A2780/cmv and A2780/m273. DNA was isolated from a number of subclones and the *BAX* gene was sequenced following PCR. All subclones of A2780/m273 revealed a *BAX* $^{+/-}$ genotype at the genomic level and expressed Bax protein. We further investigated if A2780 or its sublines were mismatch repair (MMR) deficient by analyzing MSI in analogy with the resistance mechanism in MMR deficient colon cell lines (Le Blanc et al. 2002). All cell lines demonstrated MSI with A2780/m273 and 273TR having an identical pattern of MSI markers.

DISCUSSION

In the present study we investigated the effect of wild-type p53 and mutant p53 expression on rhTRAIL sensitivity in ovarian cancer cells. Expression of mutant p53His273 sensitized A2780/m273 cells to rhTRAIL as compared to mock transfected A2780/cmv, which is probably due to the enhanced caspase 8 expression in A2780/m273. To explore mechanisms of acquired resistance to rhTRAIL, we also established a subline of A2780/m273 with acquired TRAIL resistance. Following continuous selection with rhTRAIL, the TRAIL resistant 273TR cells no longer expressed Bax, whereas Bak was still present. Caspase 9 in contrast to caspase 8 was no longer activated by rhTRAIL, indicating a block in the mitochondrial apoptosis pathway. Genetic analysis demonstrated that the parental cell line A2780/m273 was *BAX* $^{+/-}$, while the TRAIL resistant cell line 273TR was *BAX*$^{-/-}$. A deletion of a G in a homopolymeric (G)8 repeat within the coding region of the *BAX* gene was responsible for the loss in Bax protein expression. The A2780/cmv, A2780/m273 and 273TR cell lines were characterized as being MSI high, suggesting a similar mechanism of resistance as observed previously in mismatch repair deficient colon carcinoma cell lines (19). However, the revertant cell line 273TAF, which was still *BAX*$^{-/-}$, regained partial sensitivity to rhTRAIL. Since rhTRAIL-induced caspase 9 activation was again observed in these revertant 273TAF cells, our results indicate that Bax is involved in TRAIL resistance but is not the sole determinant for rhTRAIL-induced apoptosis in these mutant p53 overexpressing ovarian cancer cells. This is in accordance with our findings that CHX treatment sensitized the 273TR to rhTRAIL despite its *BAX*$^{-/-}$ status suggesting the overexpression of an inhibitor of apoptosis in 273TR.

The cell line model we have established allows analysis of the effect of wild-type p53 and mutant p53 expression on determinants of TRAIL sensitivity. Cell surface expression of DR4 or DR5 is essential for rhTRAIL-induced apoptosis. However, whether the level of death receptor surface expression is important, is still not well established. DR4, DR5, DcR1 and DcR2 are p53-regulated genes (20,21,28,29). Our results indicated that overexpression of the p53His273 mutant, known as dominant-negative mutant p53 did not change basal DR5 surface expression suggesting that wild-type p53 is not an important determinant of DR5 surface expression, while DR4 was not detectable in both cell lines. Furthermore, in the acquired TRAIL resistant 273TR cells, no changes in DR5 expression were observed relative to the TRAIL-sensitive cells, suggesting that the observed difference in rhTRAIL sensitivity is not due to changes in DR5 surface expression, although we can't exclude DR5 redistribution at the cell surface affecting DISC formation (30).

At the intracellular level the most striking difference between A2780/cmv and A2780/m273 was the strongly elevated caspase 8 level in the latter. The relation between caspase 8 expression level and TRAIL sensitivity has been described in several reports (31,32). In accordance with caspase 8 expression levels being a limiting factor is the observation that even in the presence of CHX A2780/cmv was not sensitized to rhTRAIL. A previous report demonstrated that overexpression of exogenous wild-type p53 strongly enhanced caspase 8 gene promoter activity through an

unknown p53-inducible factor (33). The same study also described that basal caspase 8 promoter activity and caspase 8 mRNA expression levels were much higher in mutant p53-expressing hepatoma cells as compared to wild-type p53 hepatoma cells. A gain of function has been observed for several p53 mutants (for a review see (34)). A recent study also demonstrated that caspase 8 expression was elevated following transfection of p53His273 in p53 null human osteosarcoma cells but no relation with sensitivity to rhTRAIL was made (35). Thus, mutant p53 expression may be related to the strong expression of caspase 8 in A2780/m273. The present study shows that, although caspase 8 levels are increased, rhTRAIL to some extend depends upon the intrinsic pathway in A2780/m273 to induce cell death. In accordance with this observation is the cleavage of Bid and activation of caspase 9 in A2780/m273 following rhTRAIL exposure and the observation that LEHD-fmk inhibited rhTRAIL-induced apoptosis. Reduced expression of Bcl-2 but unchanged levels of Bax in A2780/m273, as compared to A2780/cmv, suggests a lower threshold for activation of the intrinsic pathway (14,36). Surprisingly, acquired resistance in 273TR was not induced via reduced caspase 8 expression as observed in colon cancer cells (37). The selection for Bax deficiency when cells were exposed long-term to rhTRAIL also indicates the involvement of the intrinsic pathway in A2780/m273 despite the elevated caspase 8 levels. Moreover, rhTRAIL treatment of these TRAIL resistant cells resulted in less caspase 8 activation and almost no cleavage of Bid and caspase 9. Similar to prior observations using an isogenic HCT116 colon carcinoma cell model with different Bax status, we found that Bax was not essential for caspase 8 activation (19). However, since TRAIL-induced caspase 8 activation is higher in A2780/m273 compared to 273TR, Bax may be involved in enhancing caspase 8 activation. Culturing 273TR cells in the absence of rhTRAIL partially restored TRAIL sensitivity, while Bax remained undetectable. This suggests similar to the results with CHX, which sensitized 273TR cells to rhTRAIL, the involvement of an overexpressed inhibitor of apoptosis. The most striking finding was the rhTRAIL-induced caspase 9 activation in the revertant 273TAF cells. This suggests that the intrinsic pathway was reactivated independently of Bax. In murine embryonic fibroblasts, tBid-mediated apoptosis is Bax and Bak-dependent (14). However, in human *BAX*$^{-/-}$ cancer cells overexpression of Bak or downregulation of Bak had no effect on TRAIL sensitivity (38,39). Bak overexpression enhanced TRAIL sensitivity only in combination with chemotherapeutic drugs (38). Both the TRAIL resistant 273TR as well as the intermediate TRAIL sensitive 273TAF cells are Bak positive. It remains to be established whether Bak is involved in the observed resensitization to rhTRAIL in our model. We also can not exclude differences in Bid phosphorylation between 273TR and 273TAF, which may be important in determining rhTRAIL sensitivity since phoshorylated Bid is less sensitive to caspase 8 cleavage (40).

Our study shows that in ovarian cancer cells with MSI, a mutation in one of the *BAX* alleles was present. We also found that rhTRAIL treatment selected for Bax$^{-/-}$ cells. These results are similar to previous studies in colon cancer models. A study with a *BAX* knock-out model of HCT116 provided evidence that Bax deficiency may confer resistance to rhTRAIL-mediated apoptosis (11,19). In addition, exposure of MMR-deficient HCT116 colon cancer cells to TRAIL selected for refractory subclones with homozygous *BAX* mutations in cell lines and xenografts (19). In hereditary non-polyposis colorectal cancer (HNPCC), mutations in one of several MMR genes such as hMSH2, hMLH1, hPMS1, hPMS2, hMSH3 or hMSH6, can lead to insertion/deletion mutations in microsatellites, while MSI was observed in 10 to 15% of sporadic colon cancers (41). MSI of colorectal cancers was associated with a high frequency of *BAX* mutations in the G8 stretch (42). Selection for Bax inactivation may even give tumor clonal growth advantages as demonstrated in colon cancer xenografts (43). In several subtypes of ovarian cancer such as serous and clear cell ovarian cancer, the MSI-high phenotype was present in 7 to 15 % of the cases (44,45). In endometrioid and clear cell ovarian carcinoma, in which the MSI-high phenotype was observed in almost 30 % of the cancers, Bax mutations were occasionally found (46,47). There is some indication that cisplatin refractory ovarian tumors become MSI compared to their untreated primary counterpart (48,49). These observations may have implications for a

subgroup of patients with MSI ovarian cancer when treated with apoptosis inducing agents such as rhTRAIL or TRAIL receptor agonistic antibodies.

Several studies have demonstrated that non-steroidal anti-inflammatory drugs and chemotherapeutic agents such as etoposide and camptothecin can overcome TRAIL resistance in HCT116 *BAX*$^{-/-}$ cells (19,38,39,50). Other mechanisms behind restoration of sensitivity to TRAIL in HCT116 *BAX*$^{-/-}$ cells involved upregulation of DR5 and enhanced activity of the extrinsic pathway (39,50). In several ovarian cancer cell lines, (sub)toxic concentrations of chemotherapeutic drugs, such as topotecan, paclitaxel, cisplatin and doxorubicin, and of proteasome inhibitors, such as PS-341 and MG132 sensitized cells to rhTRAIL by upregulation of DR4 and/or DR5 protein levels, either cellularly or at the cell surface independent from p53 status (51-56). Recently, we demonstrated expression of DR4 and DR5 in most primary ovarian cancers and a more frequent and enhanced expression of DR5 in residual tumor following chemotherapy (57). Combining in-vitro and in-vivo data suggests that chemotherapy combined with rhTRAIL could potentially increase susceptibility of ovarian tumors to rhTRAIL-induced apoptosis and overcome TRAIL resistance. Based on preclinical toxicity and activity profiling, native untagged rhTRAIL and TRAIL receptor agonistic antibodies are considered to be of clinical interest (58). TRAIL has entered phase I trials and the agonistic antibodies have entered phase I/II trials, also in combination with chemotherapeutic agents. It remains to be established, whether MSI, Bax mutations or mutant p53 will limit their therapeutic ability in ovarian cancer.

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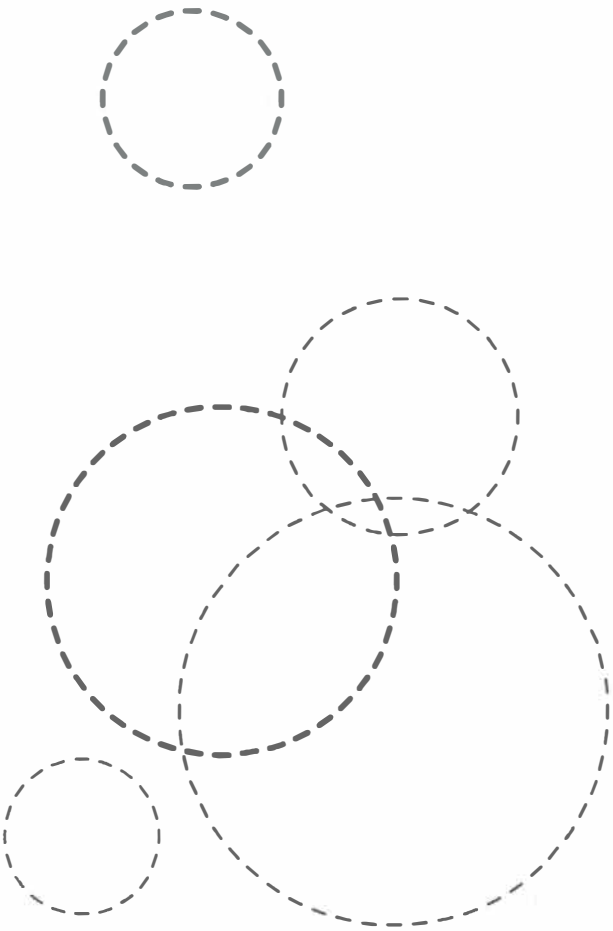

CHAPTER 5

DRUG-INDUCED CASPASE 8 UPREGULATION SENSITIZES CISPLATIN RESISTANT OVARIAN CARCINOMA CELLS TO RHTRAIL-INDUCED APOPTOSIS

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ABSTRACT

Drug resistance is a major reason for treatment failure in ovarian cancer. Triggering apoptosis directly through death ligands such as tumor necrosis factor related apoptosis inducing ligand (TRAIL) might overcome chemo-resistance. We established molecular determinants of recombinant human (rh) TRAIL sensitivity in an isogenic ovarian cancer cell line model consisting of A2780 and its cisplatin-resistant subline CP70. CP70 expressed lower caspase 8 protein with equal caspase 8 mRNA expression and was cross-resistant to rhTRAIL. Pre-exposure of CP70 to cisplatin or the proteasome inhibitor MG132 resulted in strongly increased caspase 8 protein levels, while caspase 8 mRNA levels slightly increased, leading to an enhanced apoptotic effect in combination with rhTRAIL. Caspase 8 mRNA turnover and levels before and after cisplatin exposure, as well as caspase 8 protein stability did not differ between both cell lines. Therefore, low caspase 8 protein levels in CP70 are likely caused by decreased caspase 8 protein translation. Downregulation of the caspase 8 inhibitor c-FLIP with siRNA even lowered apoptosis levels in CP70 in response to cisplatin and rhTRAIL, hereby endorsing that the cisplatin induced caspase 8 expression caused rhTRAIL sensitization and not an altered caspase 8/c-FLIP ratio. Additionally, p53 siRNA demonstrated that a p53-dependent increase in membrane expression of DR5 following cisplatin was not involved in sensitization to rhTRAIL. In conclusion, cisplatin enhances apoptosis induction by rhTRAIL in cisplatin resistant ovarian cancer cells without requirement of functional p53 or DR5 upregulation. An induction of caspase 8 protein expression is the key driver of sensitization to rhTRAIL.

INTRODUCTION

In ovarian cancer, the majority of tumors acquire drug resistance. Response rates to first-line platinum-based therapy are more than 80%, but most patients with advanced disease will finally relapse and die because of the occurrence of drug resistance (1). Resistance to cisplatin and/or taxanes is attributed to numerous mechanisms, which can be broadly divided into decreased DNA damage upon treatment and increased cell survival, mainly through defects in apoptosis programs (2). The tumor suppressor protein p53 is an important mediator of cell death through the mitochondrial or intrinsic apoptotic pathway after cisplatin induced DNA damage. Inactivation of p53 might therefore contribute to drug resistance. A meta-analysis showed that aberrant p53 status results in a worse 5 year survival for ovarian cancer patients (3). Circumvention of the escape mechanisms developed by cancer cells might improve cancer therapy. Triggering apoptosis directly by targeting the extrinsic apoptotic pathway through death receptors of the tumor necrosis factor (TNF) superfamily has attracted intense interest as a way to modulate programmed cell death. Especially the recombinant human (rh) form of the death ligand TNF-related apoptosis inducing ligand (TRAIL) is considered to be interesting for clinical use because of its ability to induce apoptosis in several types of cancer cell lines and xenografts (4-6). Preliminary data from a phase I trial with rhTRAIL showed no major toxicity (7). Monoclonal antibodies targeting death receptor 4 (DR4) or death receptor 5 (DR5) displayed minimal toxicity in phase I and II clinical trials (8) and recently showed to be safe in a phase Ib trial in combination with chemotherapeutics (9). TRAIL can induce apoptosis by binding to death receptor 4 (DR4) and death receptor 5 (DR5) (10-12). Binding to these death receptors causes trimerization of the receptors and recruitment of the adaptor protein Fas associated death domain (FADD). This in turn recruits caspase 8, resulting in the formation of the death inducing signaling complex (DISC) (13). Binding of caspase 8 to the DISC causes autocleavage and activation of caspase 8 (14,15), with subsequent activation of effector caspases such as caspase 3, 6 and 7, which will execute apoptosis. The cellular FLICE-inhibitory protein (c-FLIP), which is vastly homologous to caspase 8 but lacks enzymatic activity, can also associate with the DISC. It blocks activation of caspase 8 through competition for binding sites. However, it has also been stated that c-FLIP may act as an activator of caspase 8 under specific circumstances (16-18). In addition, the mitochondrial or intrinsic apoptotic pathway can be activated by caspase 8 through cleavage of the BH3-only protein Bid that triggers perturbation of the mitochondria through activation of Bax and Bak (19). This results in cytochrome c release and the formation of the apoptosome, which triggers the activation of caspase 9 and further activation of the effector caspases. Drug resistance in cancer cells, including ovarian cancer cells and in animal models could be prevented or overcome with combinations of rhTRAIL with cytotoxic drugs (20-25). Insight in the mechanisms that lead to synergy between drugs targeting different levels in the apoptotic pathway can assist in clinical trial design in ovarian cancer in the future. The described mechanisms involved in modulation in ovarian cancer cells were established by comparison of cell lines with different background and sensitivity-patterns, which impede establishment of causal relationships. Therefore, we investigated in an isogenic model of cisplatin resistance the molecular determinants for rhTRAIL sensitivity, the mechanism of synergy between cisplatin and rhTRAIL and the role of functional p53 in this synergy.

MATERIALS AND METHODS

Cell lines

The ovarian cancer cell lines A2780 and its 5-fold cisplatin resistant subline CP70 were a kind gift from Dr Hamilton (Fox Chase Cancer Center, Philadelphia) (26). Both cell lines carry wild type and functional p53. Cisplatin resistance in CP70 can partly be explained by an increased DNA repair mechanism and a higher intracellular glutathione (GSH) content (27). The cell lines grew as monolayers in RPMI 1640 (Life Technologies Breda, The Netherlands), supplemented with 10% heat inactivated fetal calf serum (FCS) (Bodinco BV, Alkmaar, The Netherlands) and 0.1 M L-glutamine. All cell lines were cultured in a humidified atmosphere with 5% CO_2.

Cytotoxicity assay

The microculture tetrazolium assay was used to measure cytotoxicity. The cell lines were cultured in HAM/F12 and DMEM medium, supplemented with 20% FCS and 0.1 M L-glutamine. Treatment consisted of continuous incubation with cisplatin (Pharmacochemie BV, Haarlem, The Netherlands) at 0 - 25 μM for A2780 and 0 - 100 μM for CP70. After a 4-day culture period, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT)-solution at a concentration of 5 mg/ml (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was added and formazan production was measured as described previously (28). Controls consisted of media without cells (background extinction) and cells incubated with medium instead of cisplatin. Cell survival was defined as the growth of treated cells compared to untreated cells. The mean IC50 (inhibitory concentration of 50% of the cells) +/- standard deviation was determined in three experiments each performed in quadruplicate.

Determination of apoptosis

In 96-well tissue-culture plates cells were incubated with either rhTRAIL (produced as described earlier (4)), cisplatin or both. The cells were exposed to cisplatin for 4 h, after which the cells were washed with phosphate buffered saline (PBS: 6.4 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 0.14 mM NaCl; 2.7 mM KCl; pH=7.2) twice and incubated in regular culture medium. 20 h after administration of cisplatin, rhTRAIL was added for 4 h. Exposure to rhTRAIL alone consisted of 4 h incubation. After incubation with rhTRAIL acridine orange was added to each well to distinguish apoptotic cells from vital cells. Staining intensity was determined by fluorescence microscopy and apoptosis was defined by the appearance of apoptotic bodies and/or chromatin condensation. Apoptosis was expressed as the percentage of apoptotic cells in a well.

SDS-polyacrylamide gel electrophoresis and Western Blotting

Exposure to rhTRAIL, cisplatin or cisplatin plus rhTRAIL was performed according to the same conditions as described for the apoptosis assay. Single exposure to 0.5 μM of the proteasome inhibitor MG132 (Calbiochem, The Netherlands) lasted 24 h, combined exposure with rhTRAIL consisted of adding rhTRAIL for the last 4 h. The caspase inhibitor I (zVAD) (Calbiochem, The Netherlands) was added 2 h before exposure to MG132 in the time course. Cycloheximide (CHX) exposure (Sigma-Aldrich, Zwijndrecht, The Netherlands) was performed at 5 μg/ml or 20 μg/ml as indicated. After treatment, cells were harvested at indicated time points by treatment with trypsin for 5 min at 37 °C and washed twice with cold PBS. Cells were lysed with standard Western blot sample buffer (50 mM TrisHCl, pH 6.8, 4% SDS, 10% glycerol, 10% 2-ß-mercaptoethanol, 0.002% bromphenol blue) and boiled for 10 min. Protein concentration was determined according to Bradford (29). Total cell lysates were size fractionated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto activated polyvinylidene difluoride membranes (Millipore BV, Etten-Leur, The Netherlands). Equal protein loading was confirmed by Ponceau red (Sigma-Aldrich) staining of membranes and by immunostaining with beta-actin antibody (ICN Pharmaceuticals, Zoetermeer, The Netherlands). After blocking for 1 h in Tris-buffered saline (100 mM Tris-HCl, 137 mM $NaCl_2$) supplemented with 5% milk powder (Fluka, Darmstadt, Germany)

and 0.05% Tween-20 (Sigma-Aldrich Chemie BV), immunodetection of caspase 8, FADD, cFLIP, caspase 9, caspase 3, Bax, Bak, Bcl-2, Bcl-X_L, XIAP, p53, p21, XIAP and beta-actin was performed according to the manufacturer's protocol. Mouse anti-FADD- and mouse anti-XIAP antibodies were obtained from Transduction Laboratories (Alphen a/d Rijn, The Netherlands), the rabbit anti-Bax, goat anti-Bak, mouse anti-Bcl-2, rabbit anti-Bcl-$X_{S/L}$ and mouse anti-p53-DO-1 antibodies were purchased from Santa Cruz (Heerhugowaard, The Netherlands). The rabbit anti-caspase 9 and mouse anti-Cip1/Waf1 (p21) antibody were obtained from BD Biosciences (Alphen a/d Rijn, The Netherlands) and the mouse anti-caspase 8 and rabbit anti-cleaved caspase 3 antibodies from Cell Signaling (Leusden, The Netherlands). The Bid-antibody was kindly provided by Dr J Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands), the FLIP NF6-antibody by Dr M E Peter (The Ben May Institute for Cancer Research, Chicago, IL). Binding of these antibodies was determined using horseradish peroxidase (HRP)-conjugated secondary rabbit anti-mouse, swine anti-rabbit and rabbit anti-goat antibodies (all from DAKO, Glostrup, Denmark). Visualization was performed with BM Chemiluminescence Blotting Substrate (POD) or LumiLight Plus Western Blotting Substrate from Roche Diagnostics (Almere, The Netherlands).

Flow cytometry

Analysis of TRAIL-receptor membrane expression was performed using a flow cytometer (Epics Elite, Coulter-Electronics, Hialeah, FL). Adherent cells were harvested by treatment with trypsin for 5 min at 37 °C and washed once with cold PBS at 4 °C. Cells were subsequently washed twice with cold PBS containing 2% FCS and 0.1% sodium azide and incubated with phycoerythrin (PE)-conjugated mouse monoclonal antibodies against DR4, DR5, decoy receptor 1 (DcR1) and decoy receptor 2 (DcR2). Mouse PE-labeled IgG1 and IgG2B were used as isotype controls. All PE-labeled antibodies were purchased from R&D systems (Oxon, UK). Membrane receptor expression was analyzed with Winlist and Winlist 32 software (Verity Software House, Inc., Topsham, ME) and is shown as mean fluorescent intensity (MFI) of all analyzed cells.

RNA interference

Small interfering RNAs (siRNAs) specific for human c-FLIP were designed according to the sequence AA(N19)TT, where AA and TT are present in the c-FLIP open reading frame at a spacing of 19 oligonucleotides. Double-stranded c-FLIP siRNA molecules 5'GAGGUAAGCUGUCUGUCGG-dTdT3' (sense) and 5'CCGACAGACAGCUUACCUC-dTdT3' (antisense) and siRNAs specific for human p53 according to Martinez et al. (30) were designed and synthesized by Eurogentec (Seraing, Belgium). The double stranded p53 siRNA sequence was 5'GCAUGAACCGGAGGCCCAU-dTdT3'(sense) and 5'AUGGGCCUCCGGUUCAUGC-dTdT3' (anti-sense). Oligonucleotides specific for luciferase mRNA served as a negative control (31). Cells were harvested with trypsin and plated in 6-wells plates (3.0×10^5 cells for A2780 and 2.5×10^5 cells for CP70). After attachment, the subconfluent cells were incubated in unsupplemented Optimem® medium and transfected with 200 nM oligonucleotide using Oligofectamine® reagent according to the manufacturer's protocol (Invitrogen). The day after transfection, the cells were exposed to cisplatin for 4 h at concentrations as indicated. Twenty h later, the cells were exposed to 0.25 μg/ml TRAIL for 4 h, harvested and used for protein isolation, FACS experiments, and plated in a 96-wells plate for an apoptosis assay.

Real Time RT-PCR

Total RNA was isolated by guanidine isothiocyanate-phenol-chloroform extraction with TRIzol® (Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol. Total RNA was purified using the RNeasy mini Kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's instructions. To remove trace amounts of DNA contamination, an on-column DNase I digestion was applied following the manufacturer's recommendations.

Yield and quality of the total purified RNA was assessed by measuring A260/280 nm and 260/230 nm ratios on a Nanodrop ND-1000 Spectrophotometer (Nanodrop, Isogen Life

Science B.V, IJsselstein, The Netherlands) and by RNA gel electrophoresis visualization of 18S and 28S rRNA bands. cDNA was synthesized from 800 ng purified RNA as described by the manufacturer's protocol (Life Technologies, Breda, The Netherlands) using oligo (dT)11 primers and MMLV transcriptase. Prior to the real time RT-PCR purity and integrity of the synthesized cDNA was examined by performing a qualitative RT-PCR for the housekeeping reference gene coding for the enzyme glycerylaldehyde-3-phosphate dehydrogenase (GAPDH). Real Time RT-PCR was performed in 96-wells plates with the SYBR Green method on a MyiQ® real time detection system (all from BioRad) with GAPDH as a control. A gradient RT-PCR was performed to assess primer specificity and to optimize the annealing temperature (T_{ann}) for each set of gene specific primers. The sequences for the primers used in real time PCR of caspase 8 were GGAGCTGCTCTTCCGAATTA (forward) and GCAGGTTCATGTCATCATCC (reverse) and those of GAPDH were CACCACCATGGAGAAGGCTGG (forward) and CCAAAGTTGTCATGGATGACC (reverse). Amplification of the samples in triplicate was carried out in a final reaction volume of 25 μL, containing 12.5 μl iQ™ SYBR Green Supermix (Bio-Rad), 1 μl of each gene specific primer (5 μM) and 5 μl cDNA (1:50). The thermocycling program used for each real-time RT-PCR consisted of an initial 3 min denaturation at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C, 20 s primer annealing at the primer specific T_{ann} and 30 s fragment elongation at 72 °C. Fluorescence data were acquired during the fragment elongation step at 72 °C. A melting curve was obtained at the end of each 40 cycles of amplification in order to determine the presence of a unique reaction product.

To determine RT-PCR efficiency and initial starting quantity of the samples, a standard curve was generated using samples from a 1:3 dilution series of total starting cDNA. Water controls were included to check for contaminating genomic DNA. Furthermore, the glycerylaldehyde-3-phosphate dehydrogenase (GAPDH) gene served as a housekeeping reference gene to correct for differences in the amount of starting cDNA between samples.

Statistical analysis

All experiments were performed at least three times on different occasions. Analysis included double-sided nonpaired Student's *t*-test. A p-value <0.05 was considered significant.

RESULTS

Combination of cisplatin and rhTRAIL causes enhanced induction of apoptosis

A2780 was moderately sensitive to cisplatin, with an IC50 of 2.6 μM as determined with an MTT assay. CP70 was 5 fold resistant to cisplatin, with an IC50 of 14.7 μM (Figure 1A). RhTRAIL alone induced moderate levels of apoptosis in A2780, ~ 30%, whereas CP70 was not sensitive to rhTRAIL. Combination of cisplatin with rhTRAIL enhanced apoptosis in both cell lines, with ~ 80% apoptosis in A2780 and ~ 40% apoptosis in CP70 (Figure 1B and 1C).

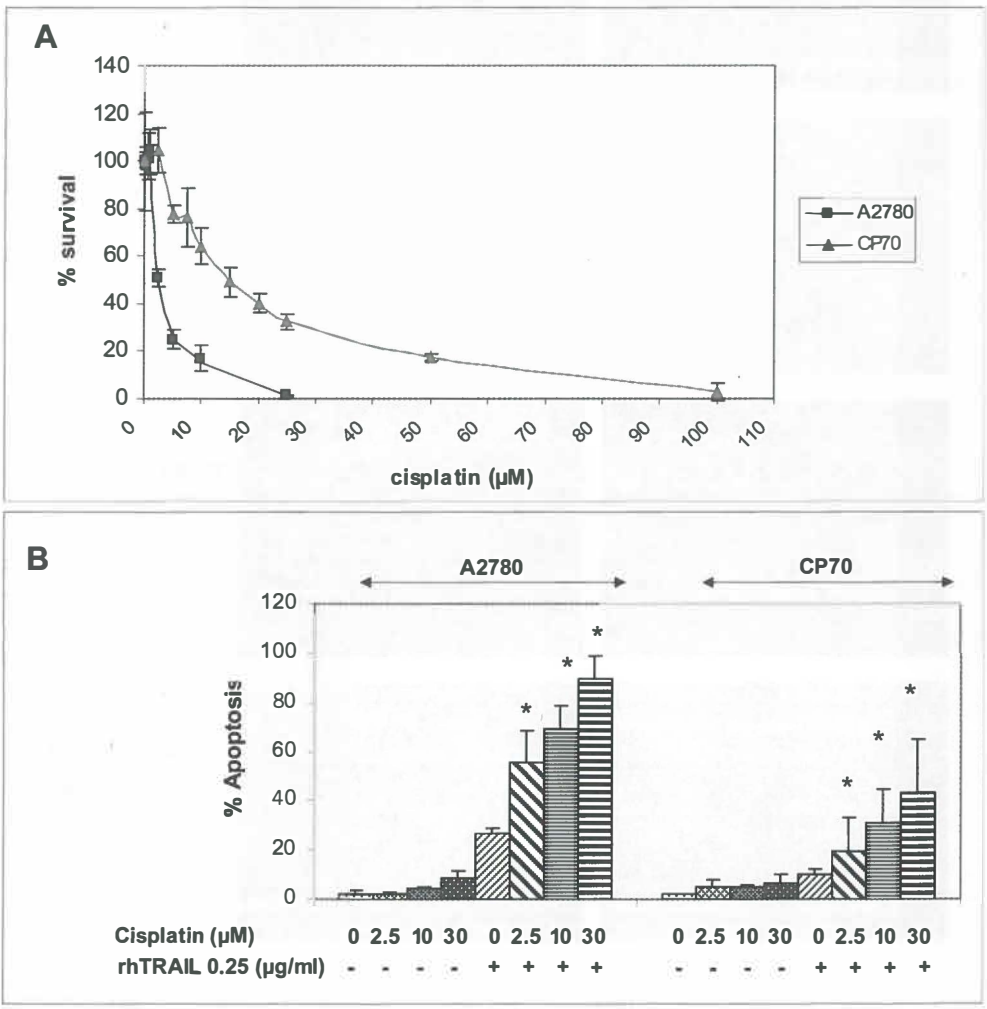

Figure 1 A, B Resistance to cisplatin causes cross-resistance to rhTRAIL. Combination therapy overcomes resistance. Survival (A) after 96 h exposure to 0 μM - 25 μM (A2780) or 0 μM - 100 μM (CP70) cisplatin as measured by cytotoxicity assays. To determine apoptosis induction, cells were treated for 4 h with cisplatin (2.5 μM; 10 μM and 30 μM) after which cisplatin was washed away. 20 h later the cells were treated for 4 h with 0.25 μg/ml rhTRAIL. Apoptosis was determined with acridine orange staining (B and C). Apoptosis in the combinations marked with * was significantly enhanced ($p < 0.05$) over apoptosis after single agent treatment. Data represent the mean ± SD of at least three independent experiments.

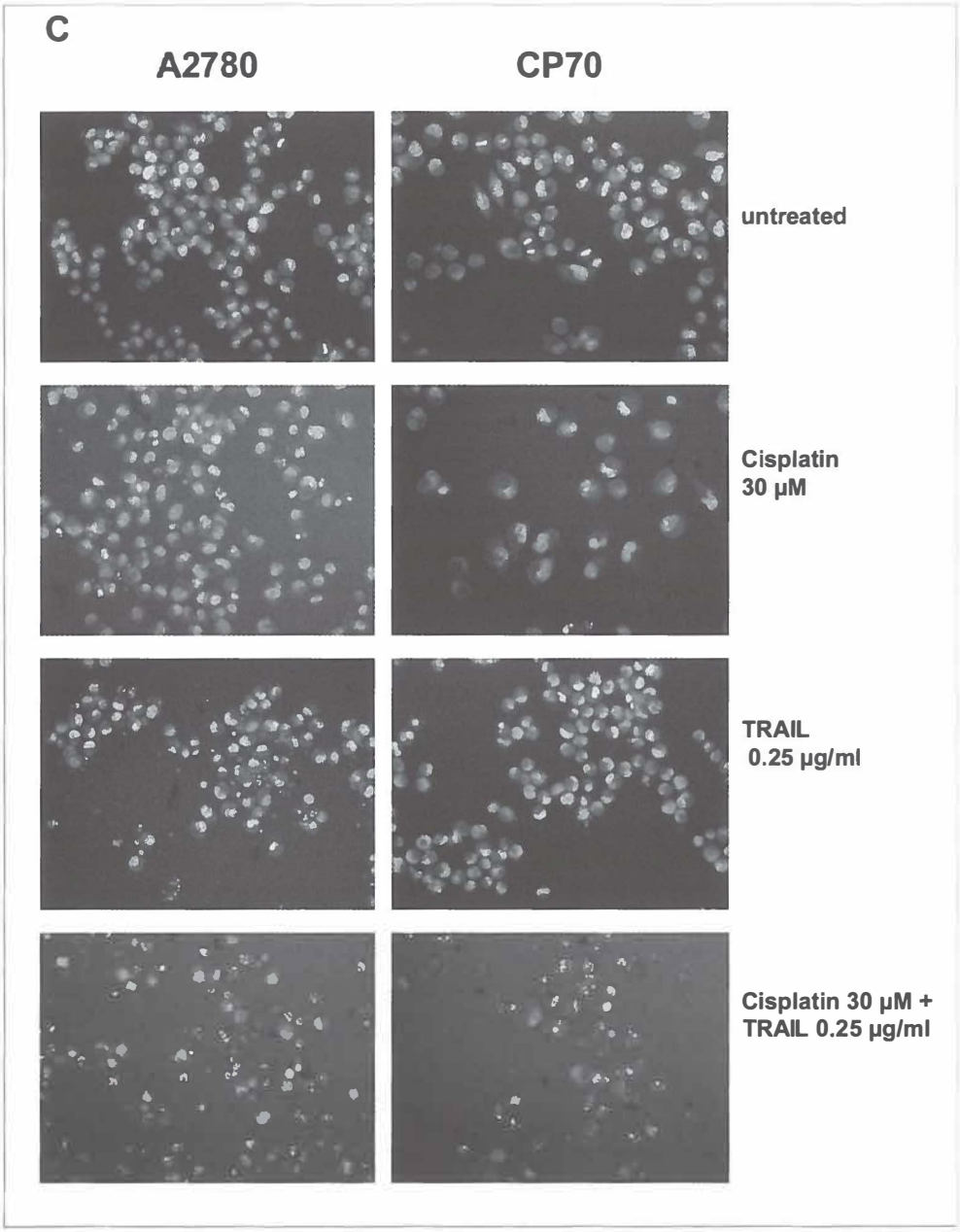

Figure 1 C Apoptosis induction in A2780 and CP70, as determined with acridine orange staining.

The resistant cell line CP70 has reduced caspase 8 protein levels

To determine which cellular characteristics could account for the different sensitivity patterns in response to rhTRAIL alone or combined with cisplatin, key components of the TRAIL signaling pathway were analyzed. DR4 and DcR1 were almost not detectable at the cell surface of both cell lines. DcR2 was expressed at similar levels, whereas DR5 was expressed at higher levels in CP70 (Figure 2A). At the level of the DISC, Western Blot analysis showed similar expression of DR4, DR5, FADD, c-FLIP$_L$ and c-FLIP$_s$ in A2780 and CP70. Procaspase 8 and levels were lower in CP70 than in A2780. Caspase 9 and 3 were equally expressed, as were the Bcl-2 family members Bid, Bax, Bak, Bcl-2 and Bcl-X$_L$. XIAP-levels did not differ between A2780 and CP70 (Figure 2B).

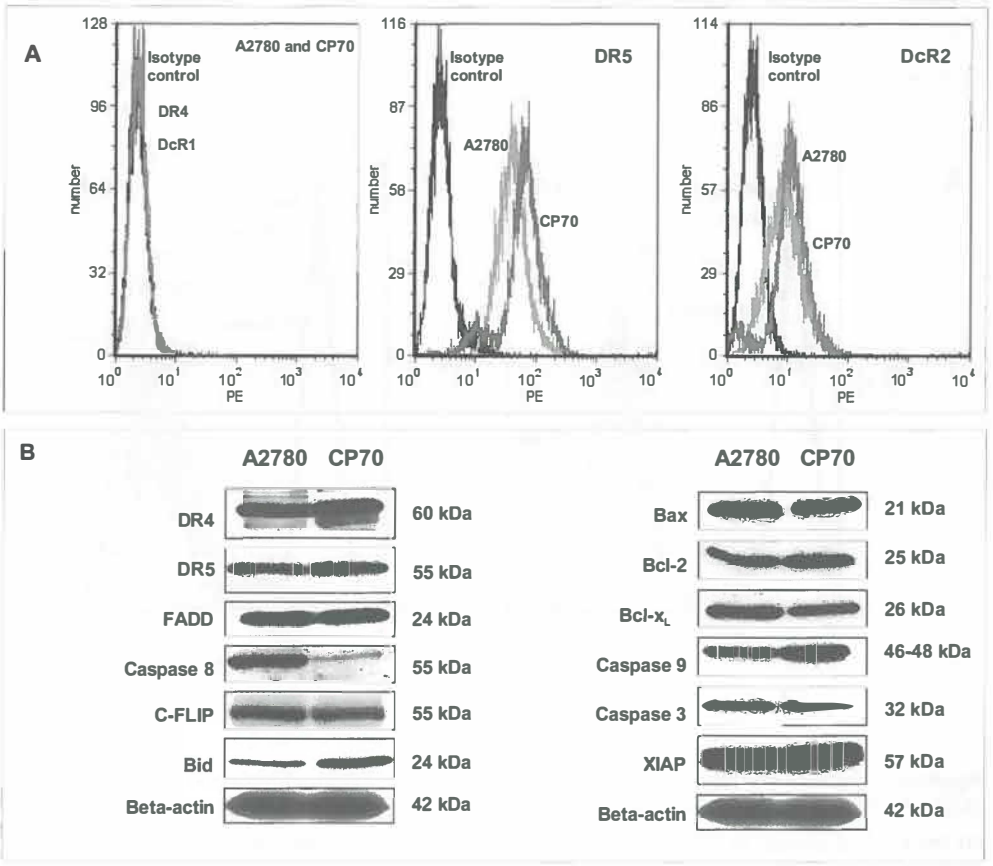

Figure 2 Cellular characteristics involved in sensitivity to rhTRAIL

(A) Basic levels of TRAIL receptor membrane expression in A2780 and CP70 as determined by FACS analysis. Receptor expression is expressed as mean fluorescence intensity (MFI).

(B) Western Blot analysis of basic protein expression levels of key determinants of the TRAIL pathway in A2780 and CP70. Beta-actin serves as a loading control. The blots are representative for at least three independent experiments.

Cisplatin induces upregulation of caspase 8 protein in the resistant CP70

Since pretreatment with cisplatin enhanced apoptosis synergistically in both cell lines, we evaluated the effect of cisplatin on rhTRAIL-receptor expression and subsequently the effect of different regimens on cleavage of caspase 8, 9 and 3. Cisplatin did not affect DR4 and DcR1 levels. DcR2 was moderately upregulated, but DR5 was strongly induced upon exposure to cisplatin in both A2780 and CP70 (Figure 3A). Moderate processing of caspase 8 to its active product was induced in A2780 in response to cisplatin, while caspase 9 and caspase 3 were not processed. rhTRAIL alone induced mild activation of caspase 8, 9 and 3, whereas all three caspases were strongly activated upon combination of cisplatin and rhTRAIL. In CP70, cisplatin induced upregulation of procaspase 8 levels, whereas caspase 9 and 3 were not activated. RhTRAIL induced a slight activation of caspase 8, 3 and 9, while the combination of cisplatin and rhTRAIL resulted in strong activation of the three caspases (figure 3B).

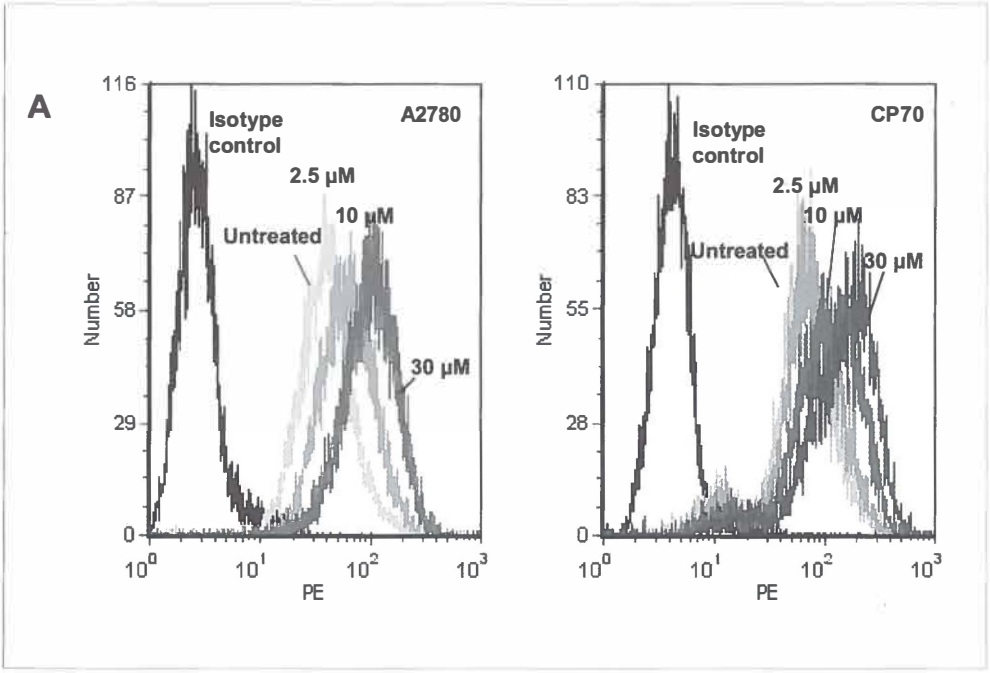

Figure 3 Co-treatment of cisplatin and rhTRAIL greatly sensitizes both cell lines to apoptosis. Cisplatin can induce caspase 8 protein in the low caspase 8 expressing CP70

(A) The effect of cisplatin on DR5 expression in both cell lines. Flow cytometry was performed 24 h after 4 h incubation with cisplatin.

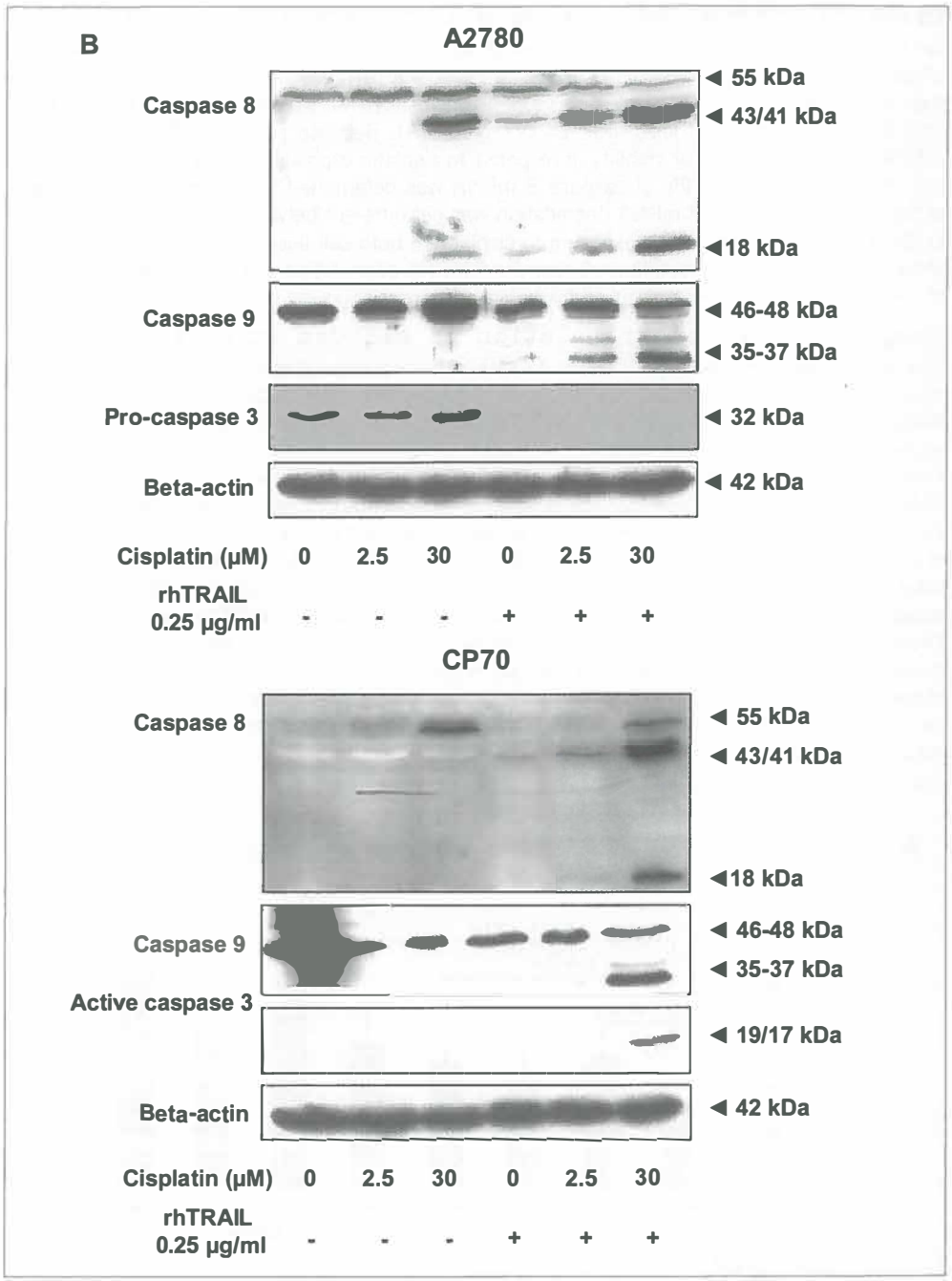

Figure 3 (B) Western Blot analysis of caspase 8, 9 and 3 after exposure to cisplatin and rhTRAIL. A2780 and CP70 were incubated with 2.5 μM or 30 μM cisplatin during 4 h, after which the cells were washed. 20 h later unexposed or cisplatin exposed cells were treated during 4 h with 0.25 μg/ml rhTRAIL. Subsequently caspase 8, 9 and 3 cleavage or pro-caspase 3 expression was determined with Western Blotting. Beta-actin serves as a loading control. The blots are representative for at least three independent experiments.

Cisplatin increases caspase 8 mRNA levels

We further investigated the mechanism causing reduced caspase 8 protein levels and upregulation of caspase 8 after cisplatin in CP70. Basal caspase 8 mRNA levels were slightly higher in CP70 than in A2780 (Figure 4A). Exposure to cisplatin resulted in 1.5 fold induction of caspase 8 mRNA levels in both cell lines after 24 h (Figure 4A). Because possible differences in basal mRNA stability over time or stability in response to cisplatin exposure are not demonstrated with this experiment, the half life of caspase 8 mRNA was determined by a chase experiment with actinomycin D. Caspase 8 mRNA degradation was not different between A2780 and CP70 ($p = 0.35$) and was not affected by exposure to cisplatin in both cell lines (Figure 4B). These results show that cisplatin does not influence caspase 8 mRNA degradation, indicating that the induction of mRNA after 24 h might be caused by increased transcription.

Caspase 8 protein translation is reduced in CP70

The basal levels of caspase 8 mRNA in A2780 and CP70 show that the difference in caspase 8 protein expression is not caused by a decreased transcription of the caspase 8 gene. Consequently, differences in protein translation or degradation might be involved in the reduced caspase 8 expression in CP70. To test whether increased proteasomal degradation causes the reduced caspase 8 protein levels in CP70, the cells were exposed to the proteasome inhibitor MG132. This induced upregulation of caspase 8, leading to concomitant synergy with rhTRAIL (Figure 5A). If enhanced proteasomal degradation is causing reduced caspase 8 levels in CP70, caspase 8 protein levels should increase in time after inhibition with MG132, or should decrease in time after blocking protein synthesis with CHX. Because exposure to MG132 induces apoptosis and substantial caspase 8 processing, the cells were incubated with MG132 for up to 24 h in the presence of zVAD, which did not lead to a change in caspase 8 protein levels (Figure 5B). CHX exposure for up to 24h did not affect caspase 8 protein levels either (Figure 5C). These results show that as decreased stability of caspase 8 protein is not responsible for the low caspase 8 expression in CP70, decreased translation of caspase 8 mRNA might be involved. The finding that MG132 exposure without zVAD induced caspase 8 protein, indicates that a caspase dependent mechanism releases a block at caspase 8 translation in CP70.

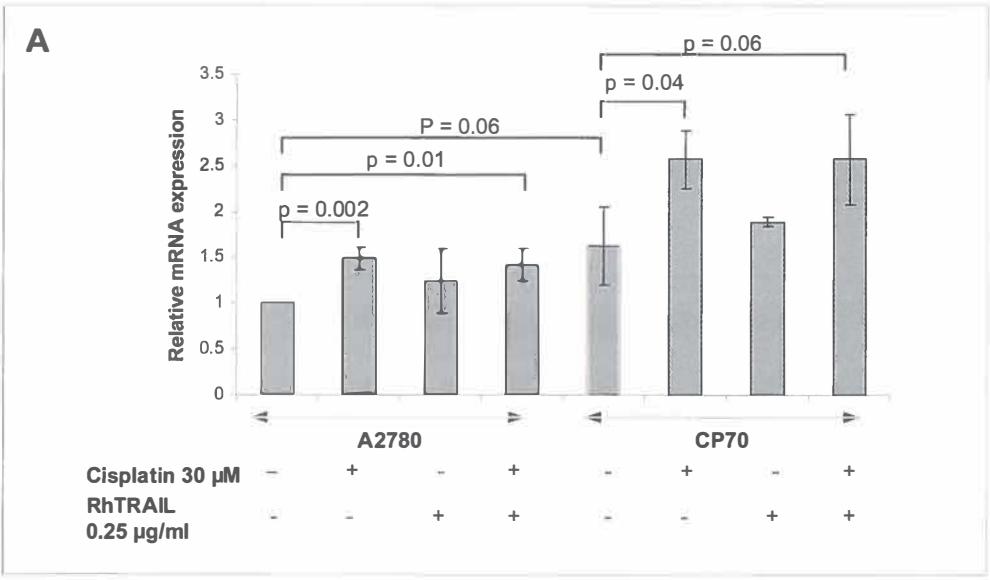

Figure 4A Regulation of caspase 8 mRNA in A2780 and CP70

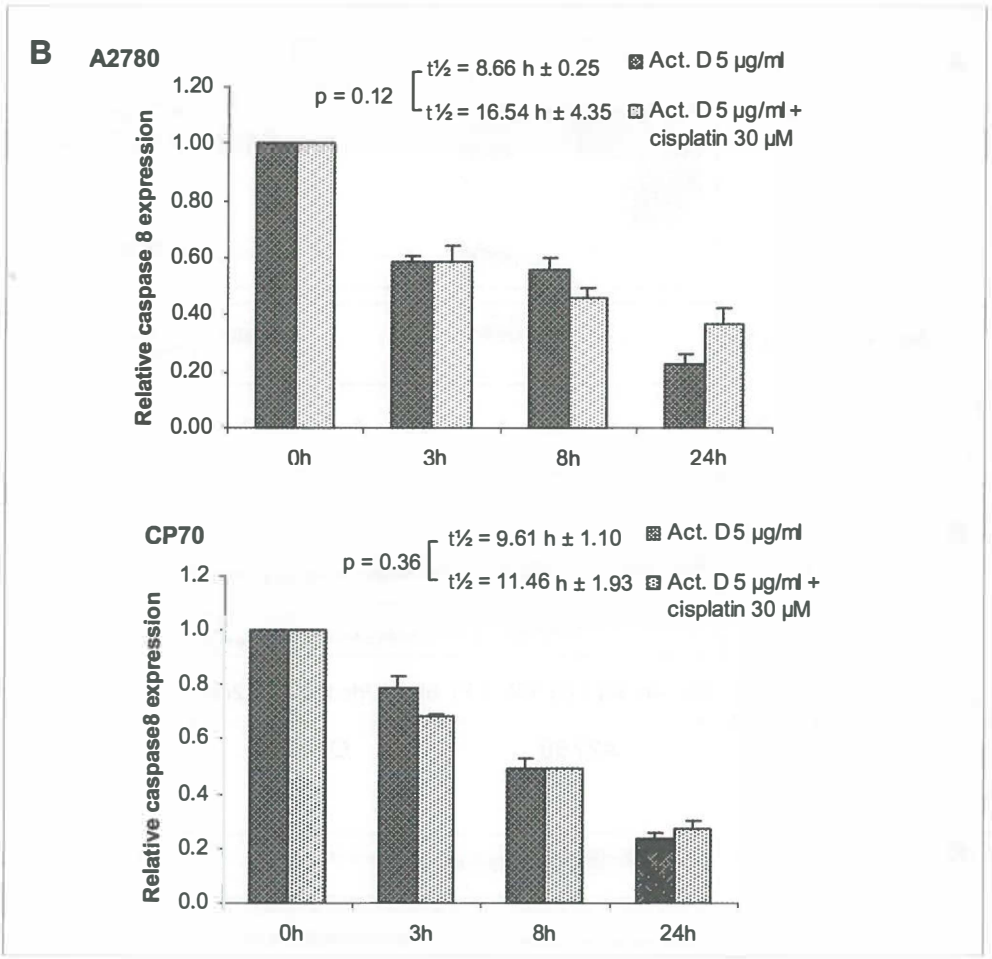

Figure 4B Regulation of caspase 8 mRNA in A2780 and CP70

(A) mRNA expression of caspase 8 was determined with quantitative RT-PCR after exposure to 30 μM cisplatin, 0.25 μg/ml rhTRAIL or the combination in both cell lines. Cells were incubated for 4 h with cisplatin, after which the cells were washed and total RNA was isolated with TRIzol® 20 h after cisplatin exposure. Total RNA was isolated 4 h after exposure to rhTRAIL. Quantitative RT-PCR was carried out on c-DNA with the SYBR Green method. Quantification was performed with the standard curve method with GAPDH as reference. Basic mRNA levels between A2780 and CP70 were not different. Cisplatin induced caspase 8 mRNA 1.5 fold. (B) To assess caspase 8 mRNA stability, cells were exposed to 30 μM cisplatin for 4 h or left untreated. Hereafter, all conditions were washed with PBS and received new medium, followed by addition of 5 μg/ml actinomycin D (Act D). Total RNA was extracted at the time of Act D addition (t = 0 h) and at the indicated time points. Quantitative RT-PCR was performed as in (A). Data represent the mean ± SD of at least three independent experiments.

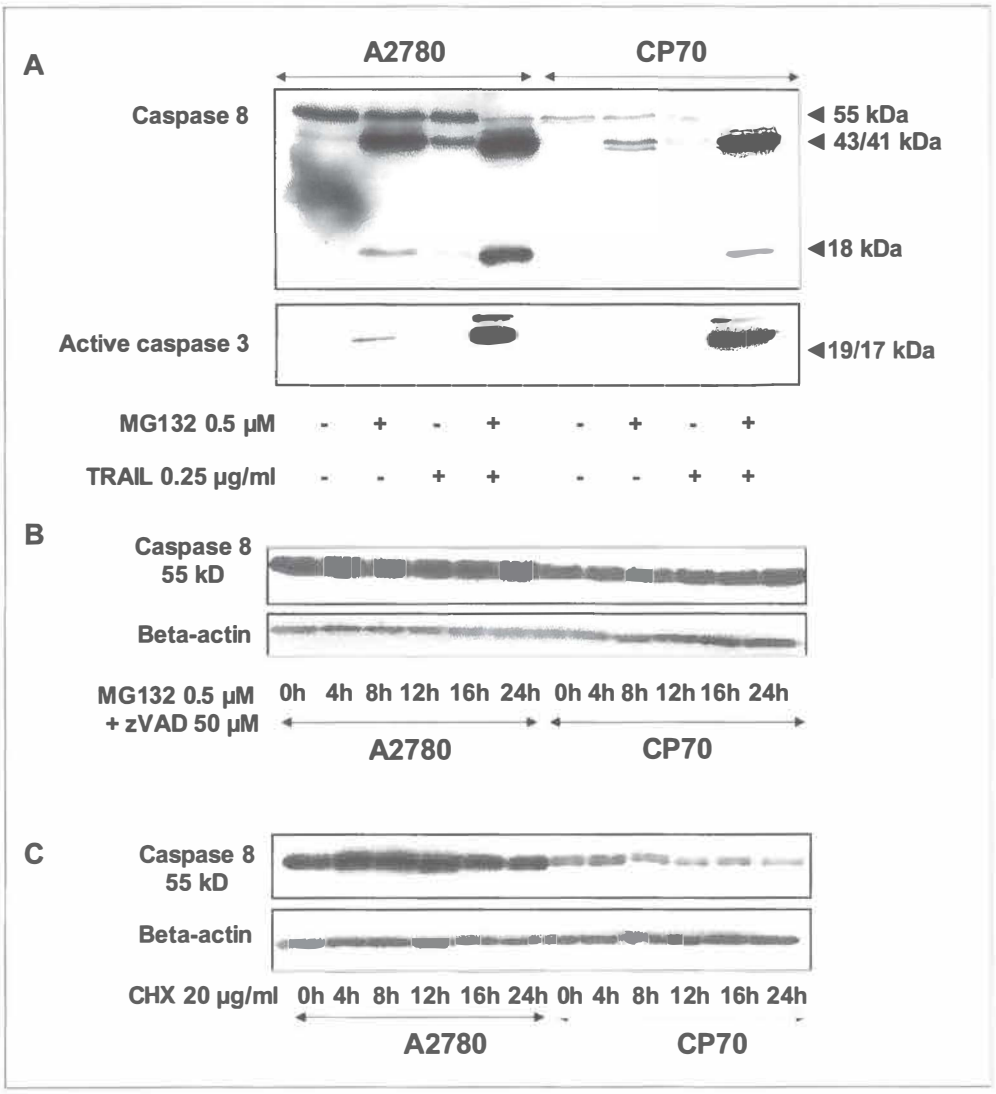

Figure 5 Differences in caspase 8 protein levels in CP70 are due to changes in protein translation

(A) A2780 and CP70 were exposed to 0.5 μM of the proteasome inhibitor MG132 for 24 h. The following day either rhTRAIL was added the last 4 h of incubation, or the cells were left untreated. Then cells were lysed and subjected to immunoblot analysis with caspase 8 and 3 antibodies. (B) A2780 and CP70 were treated with 0.5 μM MG132 for 0, 4, 8, 12, 16 and 24 h in the presence of 50 μM of z-VAD. Treated cells were lysed for SDS/PAGE and immunoblotted with caspase 8 antibodies. (C) Cells were treated with 20 μg/ml CHX for 0, 4, 8, 12, 16 and 24 h and lysed. Caspase 8 expression was determined with Western Blotting. Beta-actin serves as a loading control. All immunoblots are representative for at least three independent experiments.

An increase of the caspase 8/c-FLIP ratio is not involved in the response to cisplatin and rhTRAIL in the resistant cell line

Since a different caspase 8/c-FLIP ratio exists between A2780 and CP70 that might contribute to resistance in CP70, we downregulated c-FLIP with siRNA. Efficient downregulation of both c-FLIP$_L$ and c-FLIP$_s$ did not lead to an increase in apoptosis after either treatment regimen, with caspase 8 cleavage not being affected after downregulation of c-FLIP (Figure 6A). Quantitatively, downregulation even led to a significant decrease in apoptosis in CP70 and a small non-significant decrease in A2780 (Figure 6B). These results indicate that solely the elevated caspase 8 levels and not the change in caspase 8/c-FLIP ratio are involved in the onset of apoptosis after combination treatment in CP70. Moreover, c-FLIP even promotes caspase 8 activation in these cell lines.

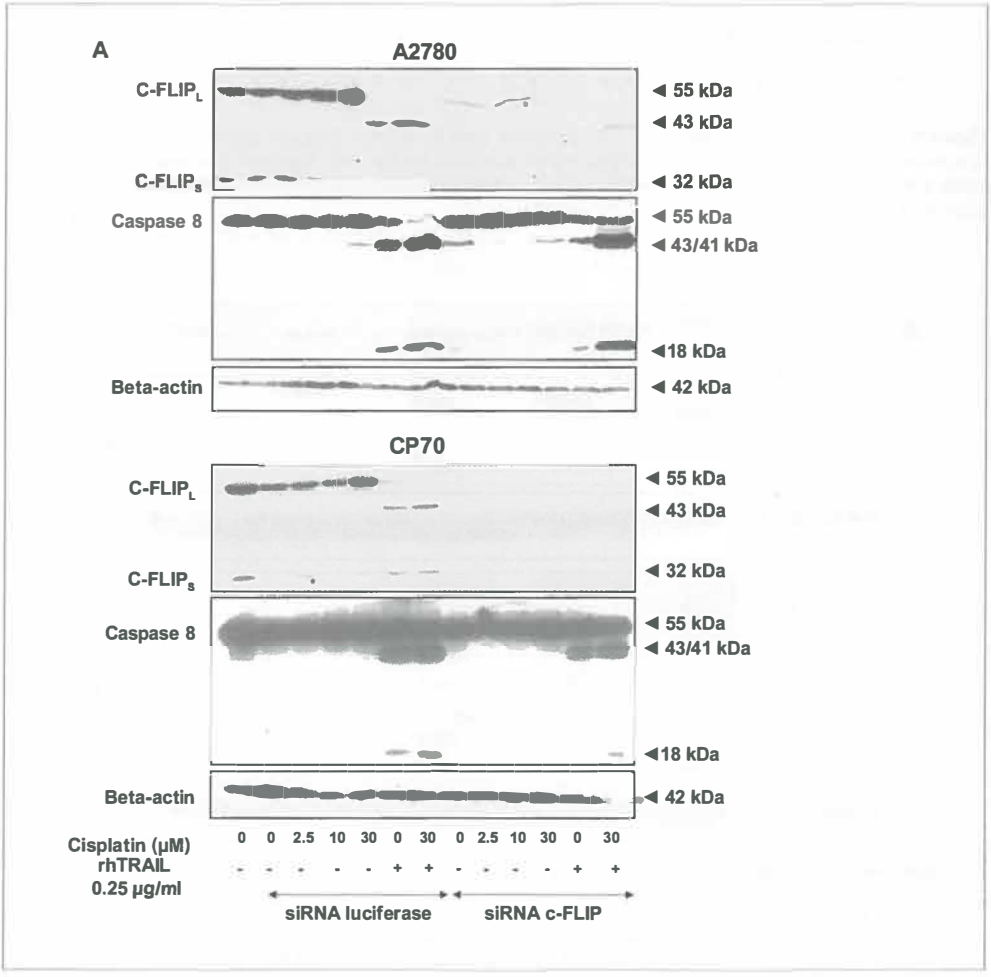

Figure 6. The caspase 8/c-FLIP ratio is not involved in resistance to rhTRAIL
Cells were transfected with siRNA against c-FLIP or luciferase. The next day, the cells were exposed to cisplatin for 4 h, after which all cells including those conditions left unexposed were washed. The following day after 4 h incubation with or without 0.25 μg/ml rhTRAIL cell lysates were made
(A) C-FLIP and caspase 8 cleavage was determined with Western Blot analysis. The blots are representative for at least three independent experiments.

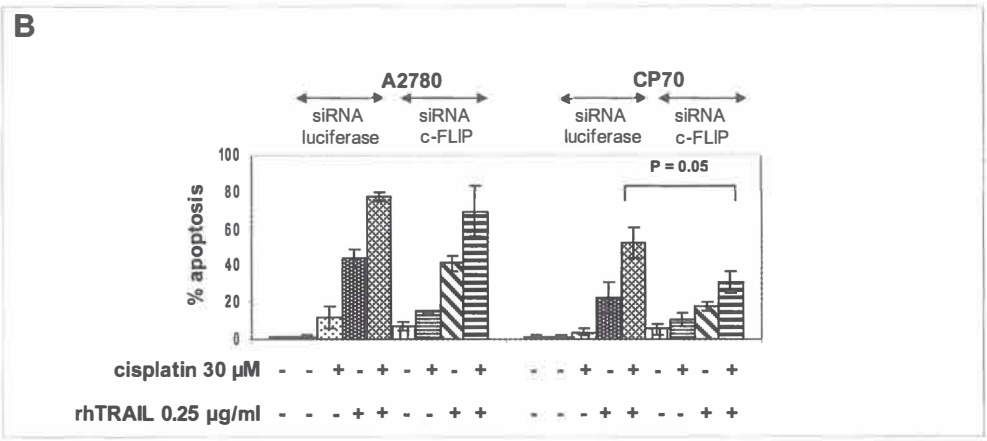

Figure 6 (B) A small fraction of the same cell suspension used for Western Blot was plated in a 96-wells plate and apoptosis levels were determined with acridine orange apoptosis assays. Data represent the mean ± SD of at least three independent experiments. After siRNA against c-FLIP a significant decrease in apoptosis occurred in CP70 after treatment with cisplatin and rhTRAIL ($p < 0.05$).

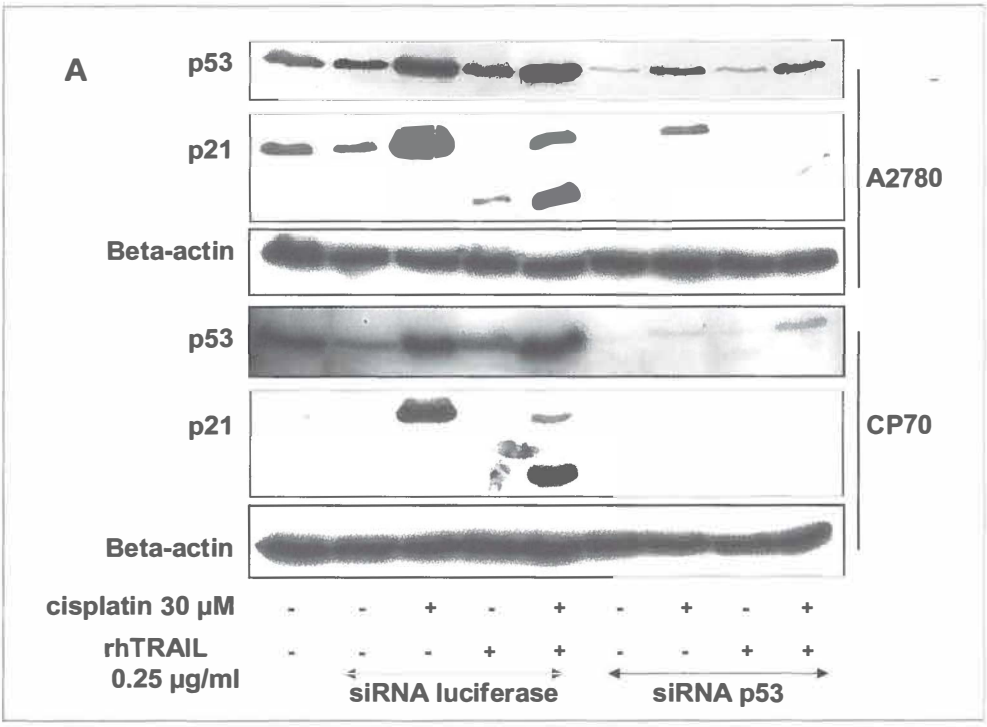

Figure 7 Transcriptionally active p53 is not required for sensitization of A2780 and CP70

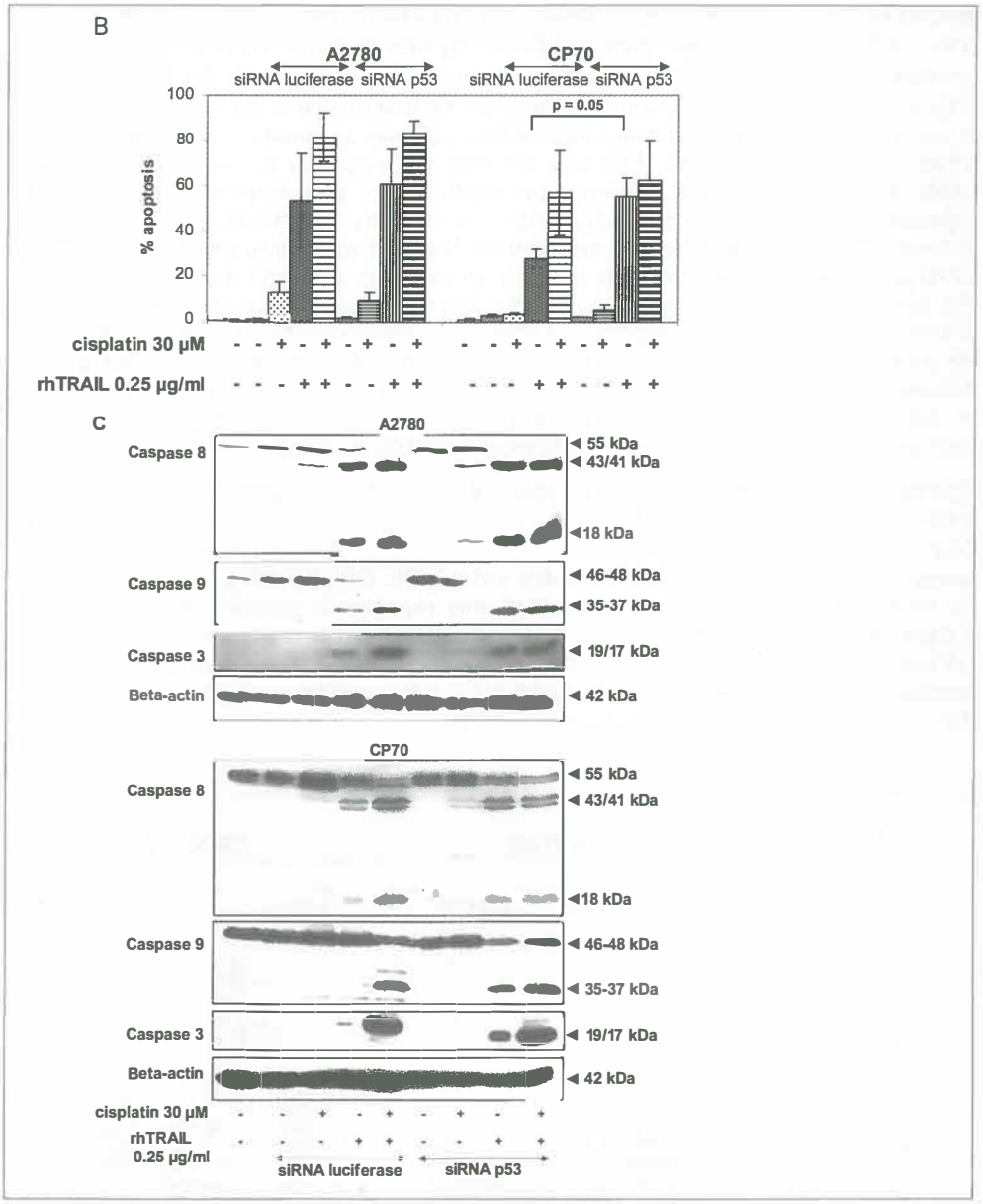

Figure 7 Transcriptionally active p53 is not required for sensitization of A2780 and CP70

A2780 and CP70 were transfected with siRNA against p53 or luciferase and were 24 h later exposed to 30 μM of cisplatin during 4 h, after which all cells including those not exposed to cisplatin, were washed. The next day the cells were treated with 0.25 μg/ml TRAIL for 4 h. (A) Subsequently, Western Blotting of p53 and p21 levels was performed on the lysates. Beta-actin serves as a loading control. (B) A small fraction of the same cell suspension used for Western Blot was plated in a 96-wells plate and apoptosis levels were determined with acridine orange apoptosis assays. After downregulation of p53, a significant increase in apoptosis occurred in CP70 after treatment with rhTRAIL (C) Cleavage patterns of caspase 8, caspase 9 and caspase 3 was determined on lysates after transfection with siRNA against luciferase and p53 as indicated above. Beta-actin serves as a loading control. All blots are representative for at least three independent experiments.

Modulation is not dependent on transcriptionally active p53: p53 siRNA does not affect synergistic effect

Combined activation of the intrinsic pathway through p53 together with the TRAIL mediated extrinsic pathway might lead to a shift in the apoptotic balance due to the initiation of apoptosis at two different levels. We therefore asked whether p53 was a requisite in the synergistic effect between cisplatin and rhTRAIL. P53 was efficiently downregulated in A2780 and CP70 with siRNA. In response to cisplatin, p53 levels rose slightly, but the levels remained below those in the untreated luciferase siRNA transfected cell lines. The effect on p21 (Waf1/Cip1) expression, which is transcriptionally activated by p53, demonstrates that p53 was functionally downregulated. In CP70 p21 expression was completely absent in all conditions after p53 downregulation (Figure 7A). Apoptosis induction was not affected by p53 siRNA. In response to rhTRAIL even higher levels of apoptosis were seen, suggesting that loss of p53 or a downstream target of p53 might increase the response to rhTRAIL (Figure 7B). These results were confirmed with Western Blot analysis. Activation of caspase 8 in response to cisplatin and rhTRAIL was unaltered after downregulation of p53, as was activation of caspase 9 and 3 (Figure 7C). Collectively, these results show that functional p53 is not involved in the sensitization to rhTRAIL by cisplatin.

Upregulation of DR5 by cisplatin is p53-dependent and not relevant in modulation

Drug-induced p53-dependent upregulation of DR5 is frequently described to be instrumental in the synergistic effect between chemotherapeutics and rhTRAIL (20). Following p53 downregulation, the membranous DR5 levels hardly increased after exposure to cisplatin. Cisplatin did cause a slight increase of the DR5 levels, but those remained at or below the levels in the untreated cell lines transfected with luciferase siRNA (Figure 8). Since p53 downregulation did not affect apoptosis levels, these results show that p53-dependent upregulation of DR5 is not involved in the synergistic effect between cisplatin and rhTRAIL.

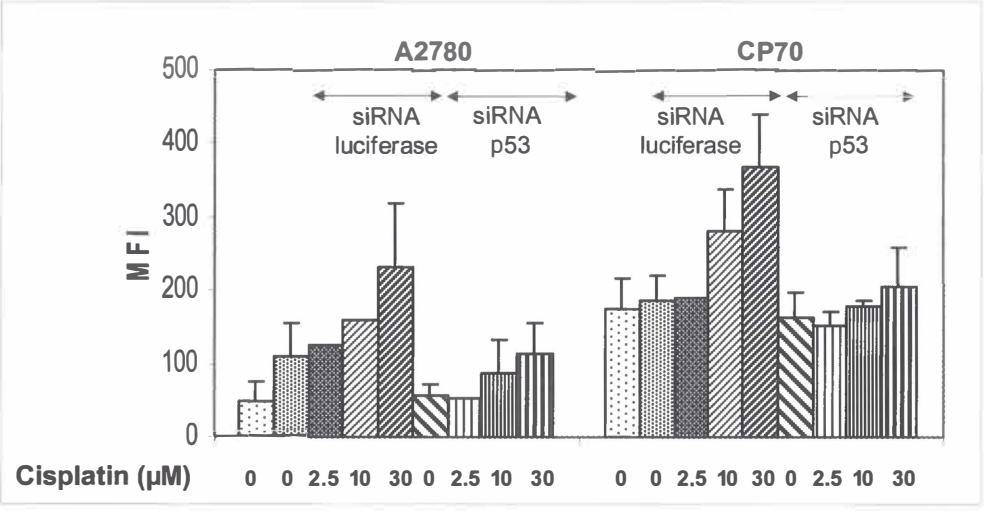

Figure 8 siRNA against p53 abrogates DR5 upregulation in response to cisplatin, without affecting apoptosis induction A2780 and CP70 were transfected with siRNA against p53 or luciferase and 24 h later the cells were exposed for 4h to 2.5 μM, 10 μM and 30 μM cisplatin, after which all cells including those unexposed to cisplatin, were washed. The next day the cells were harvested and analyzed for DR5 expression by flow cytometry. DR5 expression is represented as mean fluorescence intensity (MFI). Data represent the mean ± SD of at least three independent experiments.

DISCUSSION

In the present study we investigated in an isogenic ovarian cancer cell line model the effect of acquired resistance to cisplatin on sensitivity to rhTRAIL-monotherapy and -combination therapy with cisplatin. We show that combination of cisplatin and rhTRAIL overcomes resistance and induces apoptosis effectively, with the cisplatin-induced caspase 8 protein expression as the key driver of sensitization to rhTRAIL in CP70. The low caspase 8 protein levels in CP70 are likely to be caused by a decreased translation of caspase 8 protein rather than decreased transcription through methylation or genetic alterations that occur in neuroblastoma (32,33) and in other solid tumors (34,35), since caspase 8 mRNA levels do not differ significantly between A2780 and CP70. An increase in protein degradation in CP70 was ruled out, as caspase 8 levels remained stable after inhibition of protein synthesis and proteasomal degradation.

Translational and post-translational modifications are important drivers of the expression levels and activity of key proteins in the regulation of cell survival and apoptosis such as p53 (36), p73 (37), XIAP and APAF-1 (38). Aberrant mRNA translation plays a role in carcinogenesis (39). Recently attention has been drawn to microRNAs (miRNAs) as important regulators of translation and mRNA stability (40), with accumulating evidence showing that deregulation of miRNAs occurs in cancer. Several studies have shown that miRNAs influence apoptosis as well (41). An inducible post-translational modification of mRNA contributed to TRAIL resistance in melanoma cells, where cytosolic proteins could suppress DR5 protein expression by binding to the 3'-untranslated region of DR5-mRNA (42).These studies support our hypothesis that a post-transcriptional mechanism is involved in the low expression of caspase 8 in CP70. The fact that in CP70 translation could be enhanced by cisplatin or MG132 exposure argues in favor of a reversible block of translation. Low caspase 8 levels were previously shown to contribute to resistance to rhTRAIL in several cell line models and tumors (43-45). Concerning the role of caspase 8 and the death receptor pathway in chemotherapy induced cell death conflicting reports have been published. Death receptor dependent and independent activation of caspase 8 was involved in the response to different chemotherapeutic drugs (46-50). Although recruitment of the extrinsic pathway by chemotherapeutics does not seem to be a uniform mechanism of apoptosis induction, resistance to these drugs through defects in this pathway support the involvement of the extrinsic pathway in chemotherapy induced cell death (49,51,52). Considering these findings, the downregulation of caspase 8 protein levels in CP70 might contribute to cisplatin resistance, with subsequent cross resistance to rhTRAIL. Besides the differential expression of caspase 8 we found no other proteins that could account for the difference in sensitivity between A2780 and CP70. However, although levels of the anti-apoptotic protein c-FLIP did not vary, the caspase 8/c-FLIP ratio was lowered because of the decreased caspase 8 protein expression in CP70. A decreased ratio of caspase 8 to c-FLIP can result in resistance to death receptor signaling (53-56), whereas an increase in the ratio of caspase 8 to c-FLIP in the DISC can cause sensitization to rhTRAIL by chemotherapeutics (57,58). In our cell lines, downregulation of both c-FLIP$_L$ and c-FLIP$_s$ did not increase apoptosis induction. In the resistant CP70, downregulation of c-FLIP even resulted in a significant drop in apoptosis levels after exposure to cisplatin and rhTRAIL. This suggests that c-FLIP acts as a pro-apoptotic protein in CP70 and that the caspase 8 levels itself are the most important determinant in the response to rhTRAIL. Although extensive literature exists on the anti-apoptotic function of c-FLIP, a role of c-FLIP in activation of caspase 8 was also reported (16-18). It is postulated that the c-FLIP$_L$ concentration at the DISC determines whether activation or inhibitions occurs (18,59). Because c-FLIP$_L$ does not function as an anti-apoptotic protein in A2780 and CP70, it can be reasoned that the caspase 8-activating function of FLIP applies to these cell lines, especially when low caspase 8 levels are present and heterodimerization can occur more easily than homodimerization.

TRAIL receptor membrane expression in A2780 and CP70 did not correlate with sensitivity to rhTRAIL. The resistant CP70 even expressed more DR5 at its cell surface than A2780. DR4 was not expressed at the cell surface of both cell lines. This was not caused by hypermethylation of the DR4 promotor which was described to occur in ovarian cancers (60), as DR4 protein expression was not different between A2780 and CP70. A deficient translocation of DR4 to the cell membrane may be involved, as was described for colon carcinoma cells with acquired rhTRAIL resistance (61). Upregulation of DR4 or DR5, which can occur in a p53-dependent or –independent manner in response to chemotherapeutics (62-65), is often described as a key event in the synergistic effect between chemotherapeutics and rhTRAIL (20). We show that the increase of death receptors after chemotherapy treatment might just be an epiphenomenon, instead of a key event in modulation of rhTRAIL-induced cell death by chemotherapeutics, since downregulation of p53 did not affect apoptosis induction in either cell line, whereas the substantial increase of DR5 after treatment with cisplatin was almost completely abrogated.

Conclusively, these results show that cisplatin resistance may result in low caspase 8 protein levels with subsequent cross resistance to rhTRAIL therapy. The mechanism responsible for caspase 8 downregulation appears to be a decrease in caspase 8 protein translation. The fact that neither the level of DR5 expression, nor the functionality of p53 or the ratio between c-FLIP and caspase 8 contribute to rhTRAIL resistance further support the low caspase 8 levels being the key determinant of rhTRAIL resistance. This resistance can be overcome by treatment with cisplatin or MG132, which induces re-expression and activation of caspase 8.

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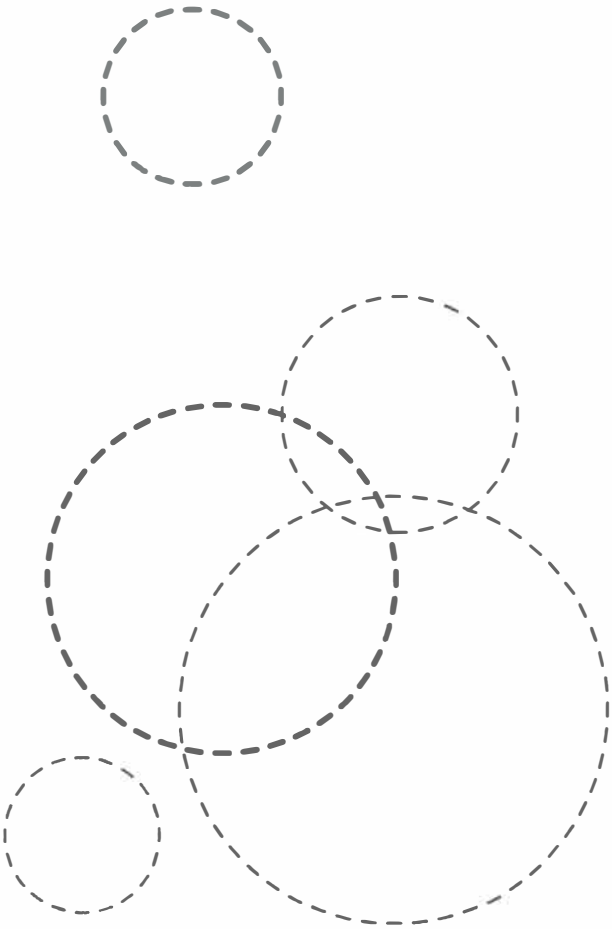

CHAPTER 6

THE EXTRINSIC APOPTOSIS PATHWAY AND ITS PROGNOSTIC IMPACT IN OVARIAN CANCER

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ABSTRACT

Purpose. The death ligand FasL, its agonistic receptor Fas, Tumor necrosis factor related apoptosis inducing ligand (TRAIL) and its agonistic receptors DR4 and DR5 are implied in carcinogenesis, tumor immune surveillance and response to chemotherapy. Fas and TRAIL receptor agonists are clinically evaluated as novel anticancer agents. This study aimed to relate expression of death ligands/receptors and downstream initiator caspase 8 and its anti-apoptotic homologue FLICE like inhibitory protein (c-FLIP) in a large group of epithelial ovarian cancers to response to chemotherapy and survival and to identify expression profiles with putative susceptibility to death receptor targeted therapy.

Methods. Immunohistochemical staining of Fas, FasL, TRAIL, DR4, DR5, caspase 8 and c-FLIP was determined on a tissue microarray (TMA) containing 382 ovarian cancers. Protein expression profiles were correlated with clinicopathologic variables, response to chemotherapy and survival.

Results. The majority of tumors expressed DR4, DR5, caspase 8 and c-FLIP. High c-FLIP expression was associated with expression of caspase 8 and both TRAIL receptors. TRAIL and Fas were associated with low tumor grade and better progression free (HR 0.63, $p = 0.018$ and HR 0.54, $p = 0.012$) and disease-specific survival (HR 0.49, $p = 0.009$) respectively in univariate analysis.

Conclusion. Loss of Fas and TRAIL is associated with dedifferentiation and a worse prognosis in ovarian cancers. Expression of pro-apoptotic DR4, DR5, caspase 8 and the anti-apoptotic c-FLIP by most cancers does not correlate with survival, but high c-FLIP expression should be taken into account for future death receptor targeted therapies in ovarian cancer.

INTRODUCTION

Ovarian cancer is the fifth most common cause of cancer deaths in women (1). Late stage disease at diagnosis and acquired resistance to chemotherapy are characteristic for the course of most ovarian cancers. These characteristics exemplify the complexity of ovarian carcinogenesis, of which a defined sequence of progression has not yet been established (2). The resulting heterogeneity among ovarian cancers and consequently in factors underlying clinical response, complicates the definition of prognostic and predictive factors for individualized treatment.

Distinctive for all cancers is deregulation of the apoptotic machinery (3). Apoptosis can be induced through two pathways. In the intrinsic pathway, diverse cellular stressors cause sensors within the cell to promote cytochrome c release from the mitochondria. This results in the formation of the apoptosome and subsequent activation of caspase 3, which sets the final execution phase of apoptosis in motion. The extrinsic apoptotic pathway is activated upon binding of death ligands from the tumor necrosis factor family (TNF) to their cognate receptors at the cell surface. The death ligands TNF related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL/CD95L) are well characterized members of the TNF family (4). TRAIL can bind five receptors of which death receptor 4 (DR4) and death receptor 5 (DR5) transmit an apoptotic signal (5). FasL binds to one agonistic receptor, Fas (CD95) and one soluble antagonistic receptor DcR3. Trimerization of the receptors upon ligand binding causes formation of a death inducing signaling complex (DISC) in which the initiator caspase 8 is activated. Active caspase 8 cleaves various designated cellular proteins including procaspase 3, resulting in apoptosis (4). An important regulator of caspase-8 activation is its anti-apoptotic homologue c-FLIP, which is upregulated in many tumor types and involved in resistance to chemotherapy and death receptor induced apoptosis (6).

Sensitivity of cancer cells to death ligand induced apoptosis has resulted in development of death receptor targeted drugs as anti-cancer agents. Because systemic administration of Fas targeted drugs resulted in severe hepatotoxicity in mice (7,8), only therapies directed at local administration are now investigated (9). The recombinant human (rh) form of TRAIL and agonistic antibodies targeting DR4 and DR5 show efficacy in a wide variety of tumor cell lines including ovarian cancer cell lines and in various xenograft tumor models in mice, without side effects (10). These results have led to clinical studies, which showed that these agents are well tolerated (11-13).

Considering the development of targeted therapies for TRAIL receptor and Fas activation, assessing expression of key proteins of the extrinsic pathway in ovarian cancer is of interest. Alterations in the expression of FasL, TRAIL, its receptors (14,15), caspase 8 (14,16) and c-FLIP (6) have been implied in carcinogenesis and may hamper future therapies directed at death receptors. Furthermore, because response to chemotherapeutic drugs can be mediated through death ligand dependent and independent activation of caspase 8, these alterations may cause resistance to chemotherapy (17-19). Robust co-expression data of proteins involved in the extrinsic pathway may define occurrence of these alterations in tumors and their impact on prognosis. Furthermore, they may assist in patient selection for future therapies targeting the extrinsic pathway.

Therefore, the aim of this study was to evaluate protein expression of Fas, FasL, TRAIL, DR4, DR5, caspase 8 and c-FLIP on a tissue microarray (TMA) containing tumor tissue of 382 ovarian cancer patients and to correlate these expression profiles with clinicopathological characteristics and disease-outcome.

MATERIALS AND METHODS

Patients

From ovarian cancer patients treated since 1985 at the University Medical Center Groningen or affiliated hospitals all clinical, pathological and follow-up data have prospectively been stored in a database. Tumor samples from 382 patients were collected on a TMA. Patients were included when sufficient paraffin embedded tissue and complete follow up data were present and excluded when they had borderline or non-epithelial tumors. Tumor stage was surgically defined according to International Federation of Gynaecology and Obstetrics (FIGO) criteria. Histology was assessed by a gynecological pathologist in agreement with World Health Organization (WHO) criteria. Standard surgical treatment consisted of abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, multiple peritoneal biopsies and peritoneal washings with cytology. Optimal or suboptimal debulking was defined according to a largest residual tumor lesion diameter of < 2 cm or ≥ 2 cm respectively. Chemotherapy consisted of platin-based regimens in 90% of the patients eligible for systemic treatment. In case of residual tumor, response to chemotherapy was determined after three or six cycles based on WHO criteria. When indicated, intervention surgery was performed after three cycles of chemotherapy and second look surgery after six cycles. Follow-up lasted up to 10 years with gradually increasing intervals. The last evaluation of follow-up for this data set was December 2006.

All relevant data were filed in a separate anonymous database in which patients were given unique codes to protect patient identity. Database management was restricted to two people with access to the larger database containing all patients' characteristics. Due to these procedures no additional patient or IRB approval was required according to Dutch Law.

TMA construction

TMAs were constructed as described previously (20). Representative tumor tissue samples were selected from hematoxylin and eosin stained slides. Four 0.6 mm cores were punched from each donor block containing ovarian tumor tissue and put into 12 recipient paraffin TMA blocks. Each array contained 240 tissue cores, representing 55 tumor samples in quadruplicate and 10 internal controls in duplicate (comprised of five tumor, one benign and four non-tumor samples). From each block 4 μm sections were cut and mounted on aminopropyltriethoxysilane-treated slides.

Antibodies

The TMAs were stained for TRAIL, DR4, DR5, Fas, FasL, caspase 8 and c-FLIP using the antibodies: polyclonal goat anti-TRAIL (1:100, clone K-18, Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal goat anti-DR4 (1:100, clone C20, Santa Cruz Biotechnology), polyclonal rabbit anti-DR5 (1:100, clone PC392, Calbiochem, San Diego, CA), monoclonal mouse anti-Fas (1:50, clone CH-11, Upstate Biotechnology, Temecula, CA), polyclonal rabbit anti-FasL (1:100, clone N-20, Santa Cruz Biotechnology), monoclonal mouse anti-caspase 8 (1:100, clone 1C12, Cell Signaling Technology, Danvers, MA) and monoclonal mouse anti-c-FLIP, detecting both $FLIP_L$ and $FLIP_S$ (1:10, clone NF6, Alexis, Lausanne, Switzerland).

Immunohistochemistry

Staining procedures for all antibodies were performed as described previously (21-23). Sections were dewaxed in xylene and rehydrated through graded concentrations of ethanol. Antigen retrieval methods used included incubation in citrate buffer at pH 6.0 for 15 min (min) at 96 °C by means of microwave treatment for DR5 and FasL, or autoclave treatment at 3 cycles of 5 min each at 115 °C in blocking reagent (2% block + 0.2% SDS in maleic acid, pH 6.0 Boehringer Mannheim, Mannhein, Germany) for c-FLIP and caspase 8. No antigen retrieval was performed for Fas, TRAIL and DR4 staining. After three washes with phosphate buffered saline (PBS: 6.4 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 0.14 mM NaCl; 2.7 mM KCl; pH=7.2), slides for Fas detection were blocked in 1% human AB serum for 30 min; all other slides were incubated in 0.3 % hydrogen

peroxide for 30 min to block endogenous peroxidase activity. For DR4, DR5, TRAIL and FasL endogenous avidin and biotin activity was blocked using an avidin/biotin blocking kit (Vector Laborotories, Burlingame, UK). Before primary antibody incubation, slides were pre-incubated with 1% human AB serum (DR4, DR5, FasL) or normal rabbit serum (TRAIL). Primary antibody incubation for c-FLIP was overnight; all other antibodies were applied for 1 h. C-FLIP staining was detected by incubation with EnVison (DAKO, Glostrup, Denmark), caspase 8 staining with rabbit anti-mouse peroxidase antibody (DAKO), followed by addition of goat anti-rabbit peroxidase antibody (DAKO) and for all other stainings with appropriate biotinylated secondary antibodies and peroxidase-labeled streptavidin (DAKO). For all slides, peroxidase activity was visualized with diaminobenzidine. Slides were counterstained with hematoxylin.

Normal tissue (kidney for FasL, liver for Fas) and tumor sections found positive on previous occasions served as positive control for Fas, FasL, DR4, DR5 and TRAIL staining. Paraffin embedded tumor cell lines GLC4 and GLC4-CDDP with respectively high endogenous and low expression levels of caspase 8 as defined with PCR and Western blotting served as positive control for caspase 8 staining, the Ramos B-cell line transfected with a c-FLIP$_L$ construct or with a mock vector served as a positive control for c-FLIP staining (24). Negative controls were obtained by omission of the primary antibody, and by incubation with normal mouse IgG1 for caspase 8, c-FLIP and FasL, with mouse IgGM for Fas, with goat IgG for DR4 and TRAIL and with rabbit IgG for DR5.

Evaluation of immunohistochemical staining

All sections were simultaneously reviewed by two observers (ED and WBvE), without knowledge of the clinical data. Independent scoring was performed prior to simultaneous evaluation with agreement of > 90% for all stainings. Discordant cases and final scoring were reviewed with a gynecological pathologist (HH) and assigned on consensus of opinion. Cores containing less than 10% tumor tissue were considered negative and excluded. From the final analysis all cases with < 2 cores were excluded. The intensity of staining was estimated and scored semi-quantitatively in four classes for DR4, DR5 and caspase 8 as negative (0), moderate (1), positive (2) and strong positive (3). Staining for Fas, FasL, TRAIL and c-FLIP was scored in three classes as negative (0), moderate (1) and positive (2). If heterogeneous staining intensity occurred between 4 cores of the same tumor, the highest staining intensity was chosen for final scoring if the core with highest staining contained > 50% tumor tissue. For statistical analysis all classes were initially studied separately and then dichotomized. For DR4, DR5 and caspase 8, positive and strong positive staining were considered positive and moderate and negative staining was considered negative. For TRAIL, FasL and Fas, positive staining was considered positive and moderate and negative staining as negative. For c-FLIP staining positive and moderate staining was considered positive and negative staining as negative (24).

Statistical analysis

Statistical analysis was performed with SPSS 14.0 for Windows (SPSS Inc., Chicago, IL). Comparisons between unpaired tumor samples obtained before and after chemotherapy were made using the Mann-Whitney U test. Comparisons between paired pre- and post- chemotherapy samples were made with the χ^2 test or Fisher exact test where appropriate, as were associations between proteins and between proteins and clinicopathologic characteristics. To exclude the possibility of a type I error in these comparisons, a p-value < 0.01 was considered statistically significant. Response to chemotherapy was analyzed using logistic regression analysis, and was restricted to patients with a residual tumor mass ≥ 2 cm, receiving platinum-based chemotherapy (n = 141). Differences in progression free survival and disease specific survival were analyzed with two-sided log-rank testing. Progression free and disease-specific survival were defined respectively as the time from primary surgery until the date of progression or relapse of the disease and as the time from primary surgery until death due to ovarian cancer. Cox proportional hazards analysis was applied for univariate and multivariate survival analysis. Only variables significant in univariate analysis were included in multivariate analysis. The following categorized covariates were used for

multivariate analysis: age at diagnosis (< 59 years (median), ≥ 59 years), FIGO stage (stage I/II, stage III, IV), tumor type (serous, non serous), tumor grade (grade I/II, grade III/undifferentiated) and residual tumor size after primary surgery (< 2 cm, ≥ 2 cm).

RESULTS

Patient characteristics

Clinicopathological data are summarized in Table 1. Median follow-up time was 29.3 months (range 0-213), one patient was lost to follow-up. In 75 (30.5%) FIGO stage III/IV (late stage) patients debulking surgery resulted in a residual tumor mass of ≤ 2 cm. 323 (84.6%) patients received first line chemotherapy, of which 173 (49.0%) received it as adjuvant therapy. In 55 patients no chemotherapy was administered because of stage Ia disease (34 patients – 9%), ineligibility or patient refusal. At the time of data analysis, 78 (72.2%) FIGO stage I/II (early stage) and 36 (13.3%) late stage patients were alive without evidence of disease, 3 (2.8%) early and 38 (14.1%) late stage patients were alive with disease and 22 (20.4%) early and 184 (68.1%) late stage patients had died of ovarian cancer. Median progression free survival (PFS) was 49.6 (range 0-207) months for early and 11.5 (range 0-149) months for late stage patients. Median disease-specific survival was 57.6 (range 0-207) months for early stage patients and 19.7 (range 0-213) months for late stage patients.

Table 1. Clinicopathological characteristics of the patients (n = 382).

	N	%		N	%
FIGO stage			Residual disease		
Stage I	73	19,1%	< 2 cm	183	47,9%
Stage II	36	9,4%	≥ 2 cm	173	45,3%
Stage III	223	58,4%	Missing	26	6,8%
Stage IV	47	12,3%			
Missing	3	0,8%	Type of chemotherapy		
			No chemotherapy	55	14,4%
Tumor type			Platinum monotherapy	164	42,9%
Serous	226	59,2%	Platinum/Taxane containing	119	31,2%
Mucinous	46	12,0%	Other regimen	40	10,5%
Clear cell	48	12,6%	Missing	4	1,0%
Endometriod	20	5,2%			
Adenocarcinoma NOS	19	5,0%	Age at diagnosis (years)		
Other	23	6,0%	Median	58,4	
			Range (years)	21.8 - 89.8	
Tumor grade					
Grade I	68	17,8%	Follow up (months)		
Grade II	90	23,6%	Median	29,3	
Grade III	159	41,6%	Range (months)	0-213	
Undifferentiated	14	3,7%			
Missing	51	13,4%			

	DR4 (%)	p	DR5 (%)	p	Caspase 8 (%)	p	c-FLIP (%)	p	TRAIL (%)	p	FasL (%)	p	Fas (%)	p
Age														
<58 years	129/169 (76.33)	0,27	159/169 (94.1)	0,62	68/87 (78.2)	0,60	141/166 (84.9)	0,88	33/166 (19.9)	0,25	53/167 (31.7)	0,09	19/166 (11.5)	0,86
>58 years	116/164 (70.7)		161/168 (95.8)		91/112 (81.3)		146/174 (83.9)		26/174 (14.9)		71/174 (40.8)		18/169 (10.7)	
Stage														
Early stage	70/98 (71.4)	0,58	88/93 (94.6)	0,78	73/93 (78.5)	0,70	81/97 (83.5)	0,74	25/97 (25.8)	0,01	40/97 (41.2)	0,26	16/96 (16.7)	0,05
Late stage	174/232 (75)		230/241(95.4)		198/239 (82.8)		204/240 (85.0)		33/240 (13.8)		82/241 (34.0)		21/236 (8.9)	
Histology														
Serous	148/192 (77.1)	0,10	191/199 (96.0)	0,32	113/144 (78.5)	0,44	168/197 (85.3)	0,65	33/198 (16.7)	0,77	65/197 (33.0)	0,14	20/196 (10.2)	0,60
Non-serous	97/141 (68.8)		129/138 (93.5)		46/55 (83.7)		119/143 (83.2)		26/142 (18.3)		59/144 (41.0)		17/139 (12.2)	
Grade														
Grade I/II	97/137 (70.8)	0,36	131/137 (95.6)	0,61	42/55 (76.4)	0,55	113/139 (81.3)	0,049	34/140 (24.3)	**0,006**	60/140 (42.9)	0,03	26/139 (18.7)	**< 0.001**
Grade III/ undiff	119/157 (75.8)		150/160 (93.8)		99/122 (81.1)		143/160 (89.4)		19/158 (12.0)		48/149 (30.2)		5/158 (3.2)	
Residual tumour														
<2 cm	113/164 (68.9)	0,10	154/162 (95.1)	1,00	46/58 (79.3)	1,00	135/165 (81.8)	0,38	36/165 (21.8)	0,08	69/165 (41.8)	0,01	22/163 (13.5)	0,02
≥ 2 cm	114/147 (77.6)		145/153 (94.8)		103/130(79.2)		131/153 (85.6)		21/153 (13.7)		44/154 (28.6)		8/150 (5.3)	

Table 2. Staining results and clinicopathological characteristics. P values are derived from χ^2-analysis

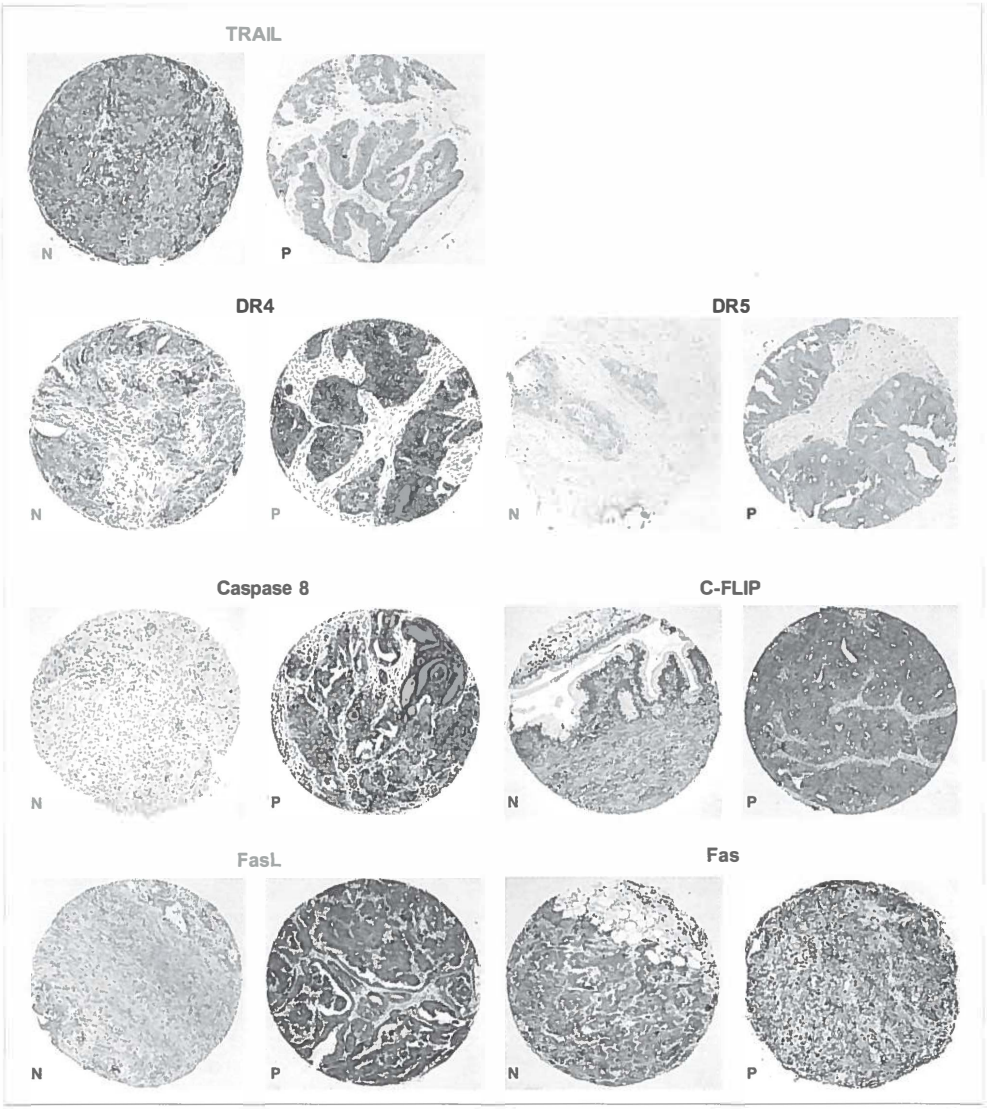

Figure 1 Results of immunostaining for TRAIL, DR4, DR5, caspase 8, c-FLIP, FasL and Fas. N stands for negative and P for positive staining. *See appendix, page 186 for color figure.*

Associations of proteins with clinicopathological characteristics

Immunohistochemistry on primary tumor samples from 359 patients provided staining results in 92.8% to 94.7% of the tumors (Table 2). Staining for all proteins was cytoplasmic, with no apparent membranous staining (Figure 1).

Most tumors expressed DR5, DR4, caspase 8 and c-FLIP (Table 2). Combining data of 322 tumors with expression results on both DRs showed that 228 (70.8%) tumors expressed both death receptors, 86 (26.7%) tumors expressed one receptor and only 8 (2.5%) tumors expressed neither DR4 nor DR5. Positive c-FLIP expression was associated with higher differentiation grade ($p = 0.049$). TRAIL, Fas and FasL were less frequently expressed. TRAIL expression was reduced in late stage tumors ($p = 0.01$). TRAIL was more frequently shown in low grade tumors ($p = 0.006$). Both FasL and Fas expression occurred more often in low grade tumors ($p = 0.03$ and $p < 0.001$ respectively) and were associated with a smaller residual tumor mass after primary surgery ($p = 0.01$ and $p = 0.02$ respectively). Separate analysis of early and late stage tumors did not show an association for TRAIL with grade, but late stage tumors of low grade expressed FasL and Fas more frequently ($p = 0.02$ and $p = 0.001$ respectively). After adjustment for multiple testing, only the associations of TRAIL and Fas with low grade tumors sustained.

Associations between proteins

Comparison of protein expression profiles of biological relevance revealed several associated profiles (Table 3A and 3B). In early stage tumors, DR5 was positively associated with caspase 8 staining ($p = 0.008$). In late stage tumors, DR4 staining correlated with positive FLIP staining ($p = 0.001$) and negative TRAIL staining ($p = 0.007$ respectively). Strikingly, 75.7% of the tumors expressing both DRs also expressed c-FLIP, while tumors with reduced expression of at least one DR were more often associated with negative c-FLIP expression (60.5%) ($p < 0.0001$) (Figure 2). These associations were also observed for early ($p = 0.004$) and late stage tumors ($p = 0.001$) separately. Caspase 8 staining was positively correlated with c-FLIP ($p = 0.008$) in late stage tumors. Finally, in late stage tumors negative Fas expression was associated with negative FasL staining ($p = 0.008$). For 188 patients data on p53 staining were available (20). No relationships were identified between the protein expression of the proteins under study and p53.

Protein expression in pre- and post-chemotherapy tumor samples

Staining patterns in paired tumor samples ($n = 43$) were compared and revealed no alterations in protein expression profiles after chemotherapy. When primary tumor samples were compared with all post-chemotherapy samples available, Fas expression was lower in the post chemotherapy samples ($p = 0.048$).

Table 3A. Associations between proteins of the TRAIL pathway. P values are derived from $\chi 2$-analysis or Fisher exact tests were appropriate. A p-value < 0.01 is considered significant.

	early					late				
	DR4	DR5	Caspase 8	c-FLIP	TRAIL	DR4	DR5	Caspase 8	c-FLIP	TRAIL
DR4	x	0.61	0.02	0.01	0.01	x	0.03	0.30	**0.001**	**0.007**
DR5		x	**0.008**	0.11	0.32		x	0.02	0.06	0.37
Caspase 8			x	0.15	1.0			x	**0.008**	0.31
FLIP				x	0.11				x	0.06
TRAIL					x					x

Table 3B. Associations between proteins of the Fas pathway. P values are derived from $\chi 2$-analysis or Fisher exact tests were appropriate. A p-value < 0.01 is considered significant.

	early				late			
	Caspase 8	c-FLIP	FasL	Fas	Caspase 8	c-FLIP	FasL	Fas
Fas	1.0	0.45	1.0	x	0.03	0.75	**0.008**	x
FasL	0.04	0.09	x		0.02	0.34	x	

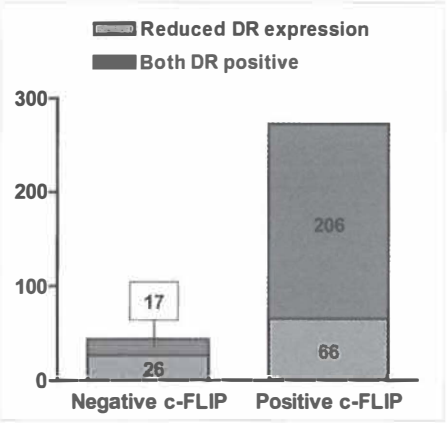

Figure 2 Death receptor expression in relation to c-FLIP expression in early and late stage ovarian tumors.

Response to chemotherapy and survival in relation to protein staining

To assess the presence of a correlation between expression of the apoptosis-related proteins under study and response to chemotherapy, univariate regression analysis was performed in 141 patients with a residual tumor mass ≥ 2 cm after initial surgery, who received platinum-based chemotherapy. Expression profiles were not correlated with response to chemotherapy.

Associations between progression free and disease specific survival and protein staining were evaluated using log-rank tests and univariate Cox proportional hazard analysis. Positive TRAIL expression was associated with a better progression free survival (HR 0.63, 95% CI 0.42 -0.92, p = 0.018) (Table 4). However, when the data were analyzed separately in early and late stages, this association was not found (Figure 3). Positive Fas staining was associated with better progression free and disease specific survival (p = 0.012 and p = 0.008 respectively) (Figure 4A and 4B). This was also observed in Cox proportional hazard analyses (HR 0.54, 95% CI 0.33 – 0.88, p = 0.012 and HR 0.49, 95% CI 0.28 – 0.84 p = 0.009 respectively). Subgroup analysis did not show an association for Fas with survival in early and late stage tumors.

In multivariate analysis including clinicopathological variables of prognostic value, only advanced stage and a residual tumor ≥ 2 cm after primary surgery were independent predictors of poor progression free survival (HR 3.92, 95% CI 2.17 – 7.084, p < 0.0001 and HR 1.94, 95% CI 1.33 – 2.83, p = 0.001 respectively) and disease-specific survival (HR 3.3, 95% CI 1.73 – 6.29, p < 0.0001 and HR 2.11, 95% CI 1.41 – 3.16, p < 0.0001 respectively).

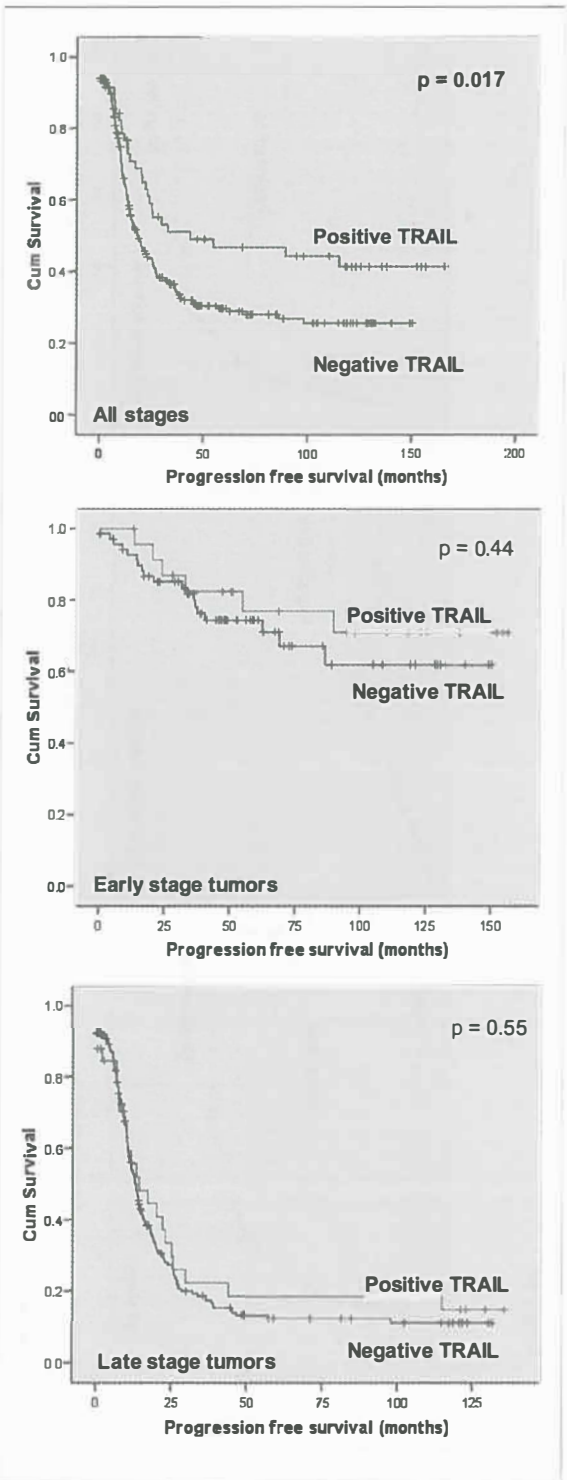

Figure 3 Progression free survival according to TRAIL expression in all patients and early and late stage tumors separately.

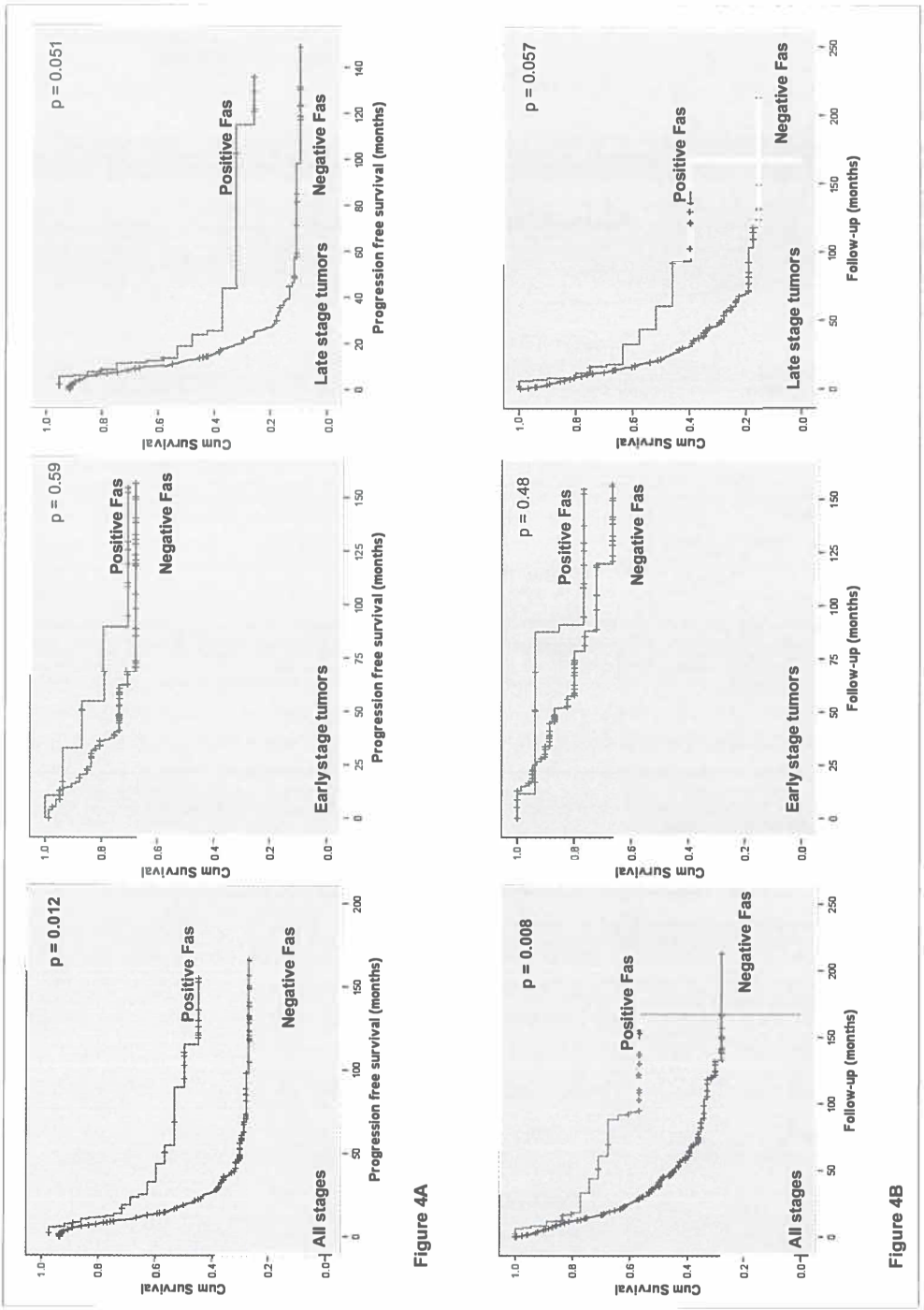

Figure 4 (A) Progression free survival according to Fas expression in all patients and early and late stage tumors separately.**(B)** Disease specific survival according to Fas expression in all patients and early and late stage tumors separately.

Table 4. Results of univariate survival analysis in early and advanced stage ovarian cancer patients.

	Univariate Cox regression analysis			
	Log rank P value	**Hazard ratio**	**95% confidence interval**	**P value**
Progression free survival				
DR4	0.86	1.03	0,75 - 1,42	0.86
DR5	0.59	1.2	0,62 - 2,34	0.59
Caspase 8	0.44	1.16	0,80 - 1,69	0.44
C-FLIP	0.65	0.92	0,63 - 1,34	0.66
TRAIL	**0.017**	**0.63**	**0,42 -0,92**	**0.018**
Fas	**0.012**	**0.54**	**0,33 - 0,88**	**0.013**
FasL	0.34	0.87	0,66 - 1,16	0.35
Disease specific survival				
DR4	0.58	0.902	0,6 - 1,28	0.58
DR5	0.97	1.2	0,51 - 1,93	0.97
Caspase 8	0.93	1.02	0,69 - 1,51	0.93
C-FLIP	0.49	1.17	0,75 - 1,81	0.49
TRAIL	0.76	0.7	0,47 - 1,04	0.78
Fas	**0.008**	**0.49**	**0,28 - 0,84**	**0.009**
FasL	0.55	0.91	0,67 - 1,24	0.55

DISCUSSION

In the largest study to date analyzing the protein expression of the death ligands TRAIL, FasL, their cognate agonistic receptors, caspase 8 and c-FLIP in ovarian cancers, we showed that the majority of cancers expressed at least one death receptor, as well as the initiator caspase 8 and the anti-apoptotic protein c-FLIP. FasL expression was expressed by approximately one third of the tumors. Expression of TRAIL and Fas was less abundant and more frequently observed in early than late stage tumors. Fas expression was lower in post-chemotherapy samples, but was not associated with a response to chemotherapy, nor were other proteins. Fas and TRAIL expression were associated with a higher tumor grade and with better progression free and disease-specific survival.

These results show that derangement of the Fas/FasL system which is implied in malignant transformation of the ovaries (25) is indeed the case in human ovarian cancer. In addition, these data show that loss of Fas expression in ovarian cancers is implied in dedifferentiation and acquisition of a higher malignant potential. In vivo studies in mice have shown that reduced or a complete loss of Fas expression in tumor cells leads to enhanced tumor development, while restoration of Fas expression results in suppression of tumor growth and restoration of chemosensitivity (26-28). Our results are the first to show that ovarian tumors that have retained Fas expression are better differentiated and have a better progression free and disease specific survival. Fas expression was reduced in tumor samples obtained after chemotherapy, suggesting that loss of Fas expression contributes to development of chemoresistance, although Fas expression was not associated with a response to chemotherapy. Previous studies examining protein expression of Fas, FasL or both in ovarian cancers showed substantial variation, which can be explained by differences in study design, use of antibodies and scoring systems (22,29-31). Most studies had a small sample size and consisted of tumors classified as benign, borderline and malignant, which made it difficult to draw strong conclusions on the relevance of protein expression in each subgroup. Moreover, we dichotomized staining classes according to their association with prognosis, in agreement with a study that investigated the prognostic relevance of Fas expression in colorectal cancers (32). This resulted in different cut-off values for definition of positive or negative staining.

FasL expression is commonly reported to increase with malignant progression and tumor grade, which was not observed in our study (28,31). A previous study in 63 ovarian carcinomas showed an association of FasL expression with reduced 5-year survival (29). Because of fewer CD8+ lymfocytes in FasL expressing tumors, a role for the tumor counterattack hypothesis (33) in ovarian cancer was suggested. Our results in a large group of tumors did not show an association of FasL expression with a worse prognosis and are therefore not supportive for the tumor counterattack hypothesis in ovarian cancers.

TRAIL expression was associated with lower tumor grade and better progression free survival when all tumors were analyzed. Previous studies in ovarian cancer patients also showed an association of TRAIL expression with low tumor grade (34) and early stage (21,34), but no correlation with prognosis (21,34,35). In contrast, TRAIL mRNA transcripts were increased in advanced stage patients and associated with a more favorable survival (36). Unfortunately, TRAIL protein expression was not determined in this study, precluding correlation of mRNA levels and protein expression. Among colon adenomas and carcinomas loss of TRAIL expression was shown in a subset of colon carcinomas (37) and in samples spanning oral cancer progression it was an early event in carcinogenesis (38). These results suggest that loss of TRAIL expression represents a survival advantage for tumor cells, possibly because they evade apoptosis induction by para- or autocrine released TRAIL. This is supported by a study which showed that in response to interferon-gamma Ewing tumor cells produce and secrete TRAIL which could induce apoptosis in unstimulated Ewing tumor cells (39).

The majority of tumors in our study expressed DR4, DR5, caspase 8 and c-FLIP. A striking finding was the association of c-FLIP expression with expression of both death receptors and with caspase 8, which suggests that apoptotic death receptor signaling is counteracted in ovarian cancers. These associations were however not correlated with prognosis, nor were the individual proteins. Upregulation of the anti-apoptotic caspase 8 homologue c-FLIP was reported in several tumor types (6) and associated with a poor clinical outcome in Burkitt lymfoma's (40) and bladder urothelial carcinomas (41). In vitro, c-FLIP induces resistance to Fas and TRAIL receptor targeted drugs in vitro (42-45) and is therefore a target for modulating death receptor induced apoptosis. In colon cancer patients, high DR4 expression was an independent prognostic factor for worse disease-free and overall survival (46). High DR5 expression was independently associated with decreased survival in breast and small lung cancers (47,48) and in ovarian cancers univariate analysis showed an association for high DR5 expression with decreased survival (34). These different results underline the complexity of death receptor signaling, which regulation is not only dependent on expression of its constituents, but also on external factors and the intracellular apoptotic machinery and might therefore be tissue specific. Moreover, it becomes increasingly evident that single prognostic factors e.g. HER2 and hormone receptors in breast and c-kit in GIST tumors are rather an exception than the rule. Considering the redundancy of signaling pathways, it is not surprising that in most tumors numerous factors are likely to influence prognosis (49). Furthermore, although alterations in the death receptor pathway are involved in chemoresistance (18,50,51), the main tumoricidal mechanism of most conventional drugs is not likely to act through the extrinisic pathway. Therefore, our results show that in ovarian cancers loss of TRAIL and Fas expression represent important aspects, namely dedifferentiation and escape from tumor immune surveillance. In addition, deregulation of the extrinsic pathway by c-FLIP expression occurs, but these changes are not of critical significance for disease outcome. They may however be of significance for future therapies targeting the extrinsic pathway. Clinical studies with rhTRAIL, agonistic antibodies directed at DR4 or DR5 and Fas are ongoing. Membranous DR expression on tumors is a necessary prerequisite for these drugs to be effective as anticancer agents, but functionality of the downstream signaling pathway is of equal importance. Therefore, it needs to be established whether these protein expression profiles correlate with functionality of the death receptor pathway in ovarian cancers, which can be achieved by relating clinical responses to TRAIL receptor agonists with tumor characteristics.

Resistance to death receptor targeted agents and to conventional chemotherapies can be overcome by combining these drugs. Many different mechanisms were described to be involved in this synergy, including downregulation of c-FLIP (52). Consequently, combinations of conventional chemotherapeutics and death receptor drugs warrant further development as novel strategies for cancer treatment.

In conclusion, loss of Fas and TRAIL is associated with dedifferentiation and a worse prognosis in ovarian cancers. Expression of anti-apoptotic C-FLIP is associated with caspase 8 and death receptor expression, which should be considered for future death receptor targeted therapies in ovarian cancer.

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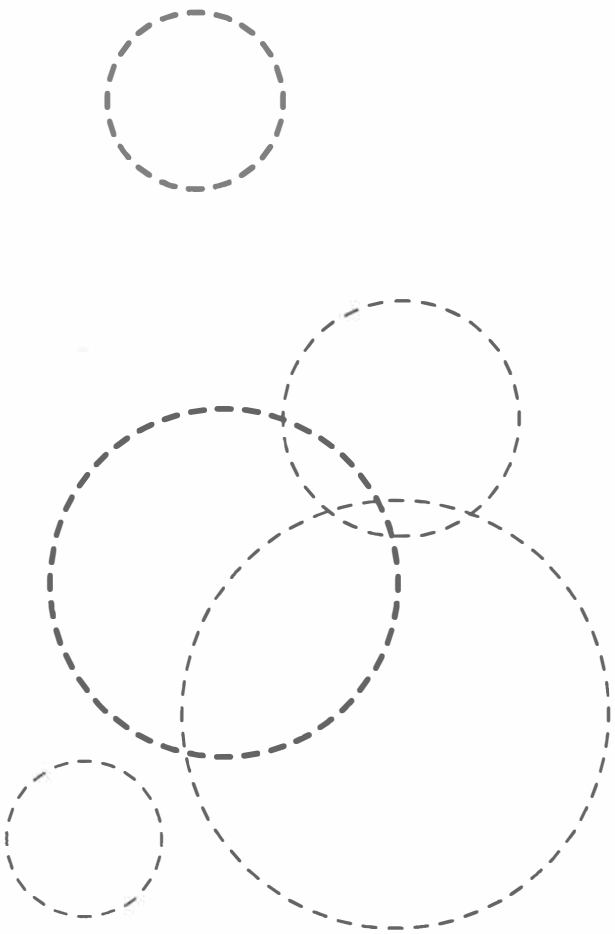

CHAPTER 7

DEVELOPMENT OF RADIOIODINATED RHTRAIL AND RADIOLABELLED AGONISTIC TRAIL RECEPTOR ANTIBODIES FOR CLINICAL IMAGING STUDIES

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ABSTRACT

Background and purpose. Tumour necrosis factor related apoptosis inducing ligand (TRAIL) induces apoptosis through activation of death receptors DR4 and DR5. Recombinant human (rh) TRAIL and monoclonal antibodies directed at DR4 or DR5 are currently clinically evaluated as anticancer agents. The objective of this study was to develop radiopharmaceuticals targeting DR4 and DR5 suitable for clinical use.

Experimental approach. RhTRAIL was radioiodinated and ITC-DTPA-conjugated antibodies were radiolabelled with ^{111}Indium. The radiopharmaceuticals were characterized, their in vitro stability and death receptor targeting capacities were determined and in vivo biodistribution was studied in nude mice bearing human xenografts with different expression of DR4 and DR5.

Key results. Labelling efficiencies, radiochemical purity, stability, and binding properties were optimal for the radioimmunoconjugates. In vivo biodistribution showed rapid renal clearance of ^{125}I-rhTRAIL, with highest kidney activity at 15 min and almost no detectable activity after 4 h. Activity rapidly decreased in almost all organs, except for the xenografts with maximal activity at 60 min. Tumour-to-blood ratios increased over time to a maximum at 240 min. Both antibodies displayed similar blood clearance, with a gradual decrease between 24 and 168 h. Tumour-to-blood ratios of HGS-TR2J in the DR4 and DR5 positive xenograft increased over time and remained low in the receptor negative xenograft.

Conclusions and Implications. RhTRAIL and antibodies can be efficiently radiolabelled. The new radiopharmaceuticals can be used clinically to study pharmacokinetics, biodistribution and tumour targeting, which could support evaluation of the native targeted agents in phase I/II trials.

INTRODUCTION

Tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) or Apo2L is a member of the TNF family that was originally identified based on its sequence homology to Fas and TNF (1). TRAIL is expressed as a transmembrane type II glycoprotein, whose extracellular domain can be proteolytically cleaved from the cell surface to form a soluble homotrimeric form. Both full-length surface bound TRAIL and the soluble form are biologically active. In contrast to the recombinant forms of FasL and TNF whose systemic administration is hampered by severe side effects (2,3), soluble recombinant human (rh) TRAIL can induce apoptosis in numerous preclinical models, without severe toxicity to normal tissues (4).

The engagement of surface expressed TRAIL receptors by TRAIL or agonistic antibodies leads to triggering of the intracellular apoptosis cascade. Currently, four membranous TRAIL receptors have been identified: DR4, DR5, DcR1, and DcR2. DR4 and DR5 contain a cytoplasmic death domain (DD), whereas DcR1 completely lacks and DcR2 possesses a truncated DD (5). The soluble receptor osteoprotegerin, which is not exclusively a TRAIL receptor, is described as a third decoy receptor that can bind TRAIL with low affinity at physiological temperatures (6). Most tumour types as well as a wide range of normal tissues express TRAIL receptors (7-11). While TRAIL can bind all TRAIL receptors, agonistic antibodies will bind either DR4 or DR5. Trimerization of DR4 or DR5 upon ligand or antibody binding leads to recruitment of the adaptor protein Fas-associated death domain (FADD) to the death domain. FADD can recruit and activate the initiator proteases caspase 8 and 10 that will in turn activate downstream proteins and effector caspases leading to apoptosis.

RhTRAIL and antibodies targeting DR4 (HGS-ETR1) or DR5 (HGS-ETR2 and HGS-TR2J) show in vitro efficacy in a wide variety of tumour cell lines. This anti-tumour efficacy could be confirmed in various xenograft tumour models in mice (12-14). A phase I study in patients with solid cancers and non-Hodgkin lymphomas (NHL) showed that rhTRAIL/Apo2L administration is safe and well tolerated (15). Phase 1-2 studies proved safety and good tolerability for HGS-ETR1 (16). Currently HGS-ETR1 is evaluated in combination studies with chemotherapy and bortezomib (17) (www.hgsi.com). An ongoing phase I study in patients with solid malignancies showed that HGS-ETR2 can be administered safely and with good tolerance (18). Two other monoclonal antibodies directed at DR5 were well tolerated and safe (19,20) and are further evaluated. The eventual clinical efficacy of the recombinant ligand and the antibodies may vary based on differences in receptor specificity, pharmacokinetics and biodistribution. Availability of radiolabelled rhTRAIL and antibodies would offer the possibility of molecular imaging in humans to study biodistribution and whole body pharmacokinetics. Furthermore, imaging will enable to illustrate whether the drug reaches the target. In this paper we describe the development of radioiodinated rhTRAIL and ^{111}In-ITC-DTPA conjugated HGS-ETR1 and -TR2J, their in-vitro characterization and receptor binding and the in-vivo death receptor targeting and biodistribution in a human tumour bearing mice model.

MATERIALS AND METHODS

RhTRAIL and monoclonal antibodies

RhTRAIL was produced non-commercially in cooperation with IQ-corporation based on a protocol described earlier (21). Briefly, a rhTRAIL expression vector (pET15b-TRAIL114-281) that harbours the gene encoding soluble human TRAIL corresponding to the extracellular part of the TRAIL molecule (i.e. amino acids 114-281) was transformed in competent *E.coli* BL21-salt-inducible (SI) cells. Soluble rhTRAIL was recovered from host BL21-SI cell lysates by sonication and subsequently purified by SP-Sepharose Fast Flow cation exchange chromatography, nickel-nitrilotriacetic acid affinity chromatography and dialysis. The recombinant protein was formulated in a TRIS buffer containing zinc and glycerol.

The agonistic monoclonal antibodies against DR4 (HGS-ETR1 - mapatumumab) and DR5 (HGS-TR2J) are fully human monoclonal antibodies of the IgG_1 isotype which were provided by Human Genome Sciences (Rockville, MD, USA).

Radioiodination of rhTRAIL

A rhTRAIL solution of 1 mg ml^{-1} in a pH 7.4 TRIS buffer, containing 100 μM zinc sulphate and 10% glycerol was used. ^{123}I-NaI and ^{125}I-NaI in 0.05 M NaOH, pH 9.0 were obtained from GE Healthcare (Eindhoven, The Netherlands). Several available oxidizing agents were tested for iodination of rhTRAIL, including Iodogen (Pierce Biotechnology, Rockford, IL), Chloramine T (Merck, Amsterdam, The Netherlands) and N-bromosuccinimide (Merck Schuchardt OHG, Hohenbrunn, Germany), of which chloramine T proved most efficient with lowest amount of impurities. 45 μg rhTRAIL and 50 μg chloramine T were allowed to react with 70 MBq ^{123}I-NaI during 3 min at pH 8. The labelling reaction was stopped with sodium metabisulphite (Acros Organics, Geel, Belgium). Unbound $^{125/123}$I was removed by gel filtration chromatography. The PD-10 column (Sephadex™ G-25M, Amersham Biosciences AB, Uppsala, Sweden) was eluted with TRIS buffer, containing 100 μM zinc sulphate, 10% glycerol and 0.5% human serum albumin (HSA, Cealb 20%, Sanquin, Amsterdam, The Netherlands).

Conjugation and radiolabelling of the antibodies with ^{111}In

The antibodies were conjugated with the chelator 2-(4-Isothiocyanatobenzyl)-diethylenetriami nepentaacetic acid (ITC-DTPA, Marcrocyclics, Dallas, TX, USA) according to Ruegg (22) with 1-2 chelate groups per antibody molecule. Briefly, to 1 mg of antibody 50 molar excess ITC-DTPA was added. After 1 h incubation at room temperature, the reaction mixture was purified by ultracentrifugation (vivaspin-2, vivascience) with ammonium acetate (50 mmol l^{-1}; UMC Groningen), pH 5.5 to eliminate the excess unconjugated ITC-DTPA. Subsequently, 1 mg purified ITC-DTPA-ETR1/TR2J was allowed to react with 50 MBq $^{111}InCl_3$ (370 MBq ml^{-1}, >1.85 GBq μg^{-1} ^{111}In, 99.9% radionuclide purity, Tyco Health Care, Petten, The Netherlands) for 1 h at room temperature. The product was diluted in normal saline and sterilized by filtration through a 0.2 μm Millex GV filter (Millipore). The biodistribution study was conducted with the cDTPA-conjugated antibodies, according to the method described for trastuzumab (23).

Glassware, materials and solutions used for the conjugation and labelling procedures were sterilized, pyrogen-free and metal-free.

Quality control

Radiochemical purity was determined by size exclusion high performance liquid chromatography (SEC-HPLC), instant thin-layer chromatography (ITLC) and trichloroacetic acid (TCA) precipitation, to differentiate the labelled product from aggregates and unlabelled ^{125}I or ^{111}In. The radiochemical purity of the final product was preferably over 95%. The HPLC system used, consisted of a Waters 1500 series manual injector with 20 μl injection loop (RheodyneTM 7725i Injector, Milford, MA, USA), a Waters 1525 Binary HPLC pump, a Waters 2487 dual-wavelength absorbance detector and an in-line radioactivity detector made of a sodium iodide crystal coupled to a multichannel

analyzer (Ortec, Nieuwegein, The Netherlands). Chromatograms were analyzed using the Breeze software (Waters, Etten-Leur, The Netherlands). The size exclusion column used was either a TSK SW3000 XL 300 x 7.8 mm column (Tosoh Bioscience GmbH, Stuttgart, Germany) or a Bio Silect SEC 250-5, 300 x 7.8 mm column (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). The mobile phase consisted of phosphate-buffered saline (PBS; NaCl 500 mmol l^{-1}, Na_2HPO_4 9.0 mmol l^{-1} and NaH_2PO_4 1.3 mmol l^{-1}; pH 7.4) to which 1% methanol was added. The flow was 1.0 ml min^{-1} and the UV detector wavelengths were set at 220 and 280 nm. The column performance was tested using a reference Bio-Rad Gel Filtration standard. The retention time of radioiodinated rhTRAIL was 9.8 min, unbound radioiodine eluted at 12 min. The retention time of the radiolabelled antibodies was 8 min, ^{111}In-DTPA eluted at 11 min. If (radiolabelled) aggregates were present they eluted early at 5-6 min (TSK-column). Recovery from the HPLC column was assessed by collecting fractions and counting them for radioactivity (well-type LKB-1282-Compugamma system, LKB Wallac, Turku, Finland). ITLC was performed on silica-impregnated glass fibre sheets (ITLC-SG 2.5 x 10 cm, Pall Gelman Sciences, Ann Arbor, MI, USA), using 0.15 mol l^{-1} citrate buffer (pH 6.0) as the mobile phase for the antibodies and 0.9% NaCl for ^{125}I-rhTRAIL. Radioactivity was determined by an ITLC scanner (VCS-101; Veenstra Instruments, The Netherlands).

For standard TCA precipitation analysis, 2 ml TRIS buffer or ammonium acetate containing 0.5% serum albumin and 2 ml 20% TCA were added to 10 μl of final solution. The mixture was vortexed and centrifuged for 10 min at 2,000 g. After centrifugation 2 ml of the supernatant and the remaining precipitate were counted for radioactivity. Radiochemical purity was the percentage of (precipitated) protein bound radioactivity.

In-vitro stability evaluation of the radiolabelled compounds

After optimization of the labelling procedure and assessment of (radio)chemical purity, the stability of the radiopharmaceuticals was determined in final buffer solution (TRIS with 0.5% HSA for ^{125}I-TRAIL, and ammonium acetate for the ^{111}In-antibodies) and in human serum. Solutions were stored at 4 °C and 37 °C in a humidified incubator maintained at 5% CO_2 and 95% air. Frequent HPLC and TCA analyses were performed. ^{125}I-TRAIL stability was assessed during the first 4 h and after 24 h. Stability of the ^{111}In-ITC-DTPA labelled antibodies was determined during 7 days.

Cell lines

Various human tumour cell lines were used including human colon (Colo320 and SW948), breast (SKBR3) and small cell lung cancer (GLC4) cell lines. All cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2. GLC4 and Colo320 were cultured in RPMI 1640 medium (Life Technologies, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS) (Bodinco BV, Alkmaar, The Netherlands). SKBR3 cells were cultured in Dulbecco's Modified Eagle's Medium High (Life Technologies) with 10% FCS. Cells were harvested by short treatment with trypsin. SW948 was cultured in Leibovitz L15-RPMI 1640 (1:1) enriched with 10% FCS, 0.05 M pyruvate, 0.1 M glutamine and 0.025% ß-mercaptoethanol. SW948 was harvested following treatment with protease.

TRAIL-receptor membrane expression (DR4, DR5, DcR1 and DcR2) was analyzed using a flow cytometer (Epics Elite, Coulter-Electronics, Hialeah, FL, USA) as described before (24). TRAIL receptor membrane expression, expressed in mean fluorescence intensity were DR4 - 0.24, DR5 - 13.77 for GLC4, DR4 - 7.93, DR5 - 10.68 for Colo320, DR4 - 28.85, DR5 - 10.61 for SW948 and DR4 - 2.13, DR5 - 4.45 for SKBR3.

Immunoreactivity

For ^{125}I-rhTRAIL the immunoreactive fraction was determined by cell-binding assays at infinite antigen excess, as described by Lindmo (25). For this assay, SW948, Colo320 and GLC4 were used. SKBR3 served as control to determine aspecific binding and is further referred to as 'negative' control. Serial dilutions in culture medium ranging from 1 to 32 were made of the cell suspension. A fixed amount of radiolabelled product was added to the cells and incubated at 21 °C for 1 h under rotation. Cell suspensions were subsequently centrifuged (10 min, 167 g) and washed with PBS 5% FCS. Specific binding was calculated as the ratio of cell-bound (pellet obtained after the last centrifugation step) to total radioactivity applied minus non specific binding, determined by the same procedure after adding a 200-fold excess of the unlabelled compound.

Antibody immunoreactivity was assessed with a surface plasmon resonance assay with the BIAcore 3000 analyzer (Biacore AB, Uppsala, Sweden). The receptor molecules were directly immobilized to the surface of separate flow cells of a CM5 chip by amine coupling to a signal of approximately 900 RU, with one lane treated by the amine coupling reagents as a control flow cell. A loop-method was created with multiple injections cycles of 120 μl of a concentration between 1-480 nM of antibody, followed by a dissociation phase and a regeneration of the chip surface with a 1:1 mixture of 10 mM glycine, pH 2.0, 1.5 M NaCl, and ethylene glycol for 90 s. The experiment was performed at a flow rate of 70 μl/min standard HBS-EP buffer at 25 °C.

In-vivo biodistribution in tumour-bearing mice

The in-vivo behaviour of the radiolabelled compounds was assessed using nude mice bearing SKBR3 or SW948 xenografts. The SW948 cell line is considered as DR positive cell line and the SKBR3 cell line is used as 'negative' control. Male nude mice (Hsd:Athymic Nude-*nu*) were obtained from Harlan Nederland (Horst, The Netherlands) at 4–6 weeks of age (30 g). They were housed in groups of five in individually ventilated solid bottom polycarbonate cages with wood-chips floor bedding (changed weekly). Temperature in the animal rooms was set for a range from 18 to 26°C, with a 12 h light-dark cycle. Mice were provided with deionised autoclaved water and standard rodent chow. After 10 days of acclimatizing, the mice were injected subcutaneously with either 1 x 10^6 SKBR3 cells or 1 x 10^6 SW948 cells mixed equally with 0.1 ml Matrigel™ (Becton Dickinson, Bedford, MA, USA). The study was conducted approximately 1-2 weeks after inoculation. The radiopharmaceutical was injected through the penile vein (0.15 ml; 150 kBq), which represented 0.5 μg rhTRAIL and 5 μg of HGS-ETR1 or HGS-TR2J. At four time points after ^{125}I-rhTRAIL injection (t = 15, 30, 60, 240 min) and three time points after injection of the ^{111}In labelled HGS-ETR1 and HGS-TR2J (t = 24 h, 72 h, 168 h), groups of mice (n = 5) bearing a SKBR3 or SW948 tumour were sacrificed and organs and tissues excised, rinsed for residual blood and weighed. An additional experiment was performed in four groups of mice (n = 5) bearing a SKBR3 or SW948 tumour. A 100-fold excess amount of unlabelled antibody was injected together with the labelled antibody. At 72 h after injection, the mice were sacrificed and tissues and organs were processed as described earlier. Samples were counted for radioactivity in a calibrated well-type LKB-1282-CompuGamma counter.

Tissue activity was expressed as percentage of the injected dose g^{-1} tissue (%ID g^{-1}). Tumour-to-blood and tumour-to-muscle ratios were also calculated. Differences between activity of both antibodies were determined according to the formula $\Delta = 100 - (\%ID\ g^{-1}\ ETR1/\ \%ID\ g^{-1}\ TR2J)*100$.

All data were corrected for physical decay and compared with a known standard sample. The animal studies were conducted in accordance with the Law on Animal Experimentation and local guidelines, and were approved by the local ethical committee.

Statistical analysis

Data are presented as mean ± s.e.m. Statistical analysis was performed using an unpaired two-tailed Student's *t*-test (SPSS version 14.0) or a non-parametric Mann-Whitney U-test in the case of unequal variances. A *P*-value of 0.05 was considered significant.

RESULTS

Conjugation, radiolabelling and quality control

Radioiodination of rhTRAIL under optimized conditions resulted in a mean labelling efficiency (± s.e.m.) of 70.3 ± 1.1% (n = 24). Radiochemical purity as determined by TCA-precipitation was always more than 98%. The maximal specific activity was 1.3 MBq μg^{-1}, mean specific activity was 0.75 ± 0.05 MBq μg^{-1} (n = 24).

Radioiodination was also assessed for the antibodies. Radiolabelling with iodine proved to destroy binding capacity of HGS-ETR1 to DR4 (data not shown) and therefore the radiolabelling process was proceeded with ^{111}In. Labelling of the antibodies with ^{111}In resulted in labelling efficiency of 92.0 ± 1.5% (n = 9). After purification radiochemical purity was 96.0%. Specific activity was 150 MBq mg^{-1}. No impurities were detected.

In vitro stability of the radiolabelled compounds

The stability of ^{125}I-rhTRAIL was determined in human serum and in a TRIS buffer containing 0.5% HSA. Stability in the TRIS buffer at 4 °C lasted at least 24 h. After 24 h denaturation of the radiolabelled preparation occurred, indicated by aggregation and dehalogenation over time. The percentage of radiolabelled aggregates was 8% after 24 h, increasing to 13% and 16% after 48 h and 72 h respectively. Free iodine could not be detected after 24 h, but 16% was dehalogenated after 48 h and 19% after 72 h.

Upon storage in human serum at 37 °C, already after 4 h high molecular weight impurities were seen (8%), but no free iodine could be detected. Given the short serum elimination half-life of less than 30 min this is acceptable (26,27).

The radiolabelled antibodies were very stable. Only a small decrease (< 6%) in protein-bound radioactivity was observed for the antibodies stored at 4 °C in ammonium acetate and at 37 °C in serum for 1 week. The stability of the radiopharmaceuticals ensures optimal measurement and imaging during 1 week.

Receptor cell binding assays

Radiolabelling might reduce receptor binding properties. Cell binding assays have been performed to study immunoreactivity and receptor binding. Three different DR4 and DR5 positive cell lines were used. The specific cell binding of ^{125}I-rhTRAIL versus the cell number is shown in figure 1. From the inverse plot the immunoreactive fraction is calculated. The immunoreactive fraction varied with the cell line used and was 0.80 ± 0.08 (n = 4) determined with Colo320, 0.76 (n = 2) with GLC4 and 0.55 (n = 2) with SW948.

For the radiolabelled antibodies the immunoreactive fraction was determined by BIAcore analysis. For HGS-ETR1 the equilibrium dissociation constant Kd was approximately 60% of the unmodified antibody. The Kd of HGS-TR2J was not significantly different for the labelled and unlabelled antibody.

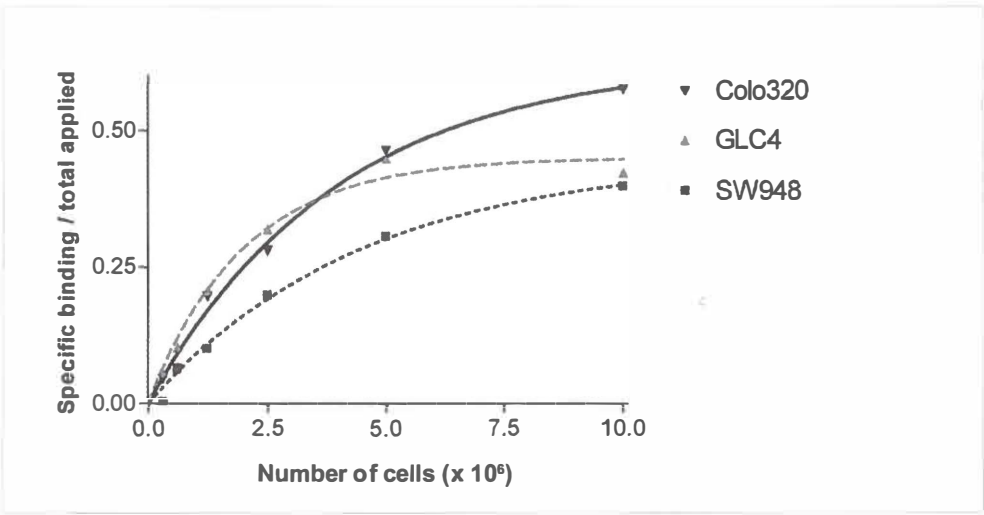

Figure 1. Typical plot of specific cell binding of 125I-rhTRAIL versus the number of cells (Colo320, GLC4 and SW948). From the inverse plot the immunoreactive fraction is calculated. The immunoreactive fraction varied per cell line used and was 0.80 ± 0.08 (n = 4) for Colo320, 0.76 (n = 2) for GLC4 and 0.55 (n = 2) for SW948.

In vivo biodistribution of ^{125}I-rhTRAIL in tumour-bearing mice

The in-vivo behaviour of ^{125}I-rhTRAIL was assessed using athymic mice bearing DR4 and DR5 positive SW948 and very low expressing DR4 and DR5 SKBR3 xenografts. ^{125}I-rhTRAIL biodistribution data of mice bearing SW948 tumors are presented in Table 1. Similar activity in normal tissues was measured in mice with SKBR3 xenografts. ^{125}I-rhTRAIL biodistribution is characterised by fast renal elimination. Already 15 min after intravenous (i.v.) injection considerable amounts of radioactivity were detected in the urine. SEC-HPLC revealed that no intact rhTRAIL trimer, but smaller fragments were present in the urine. Kidney uptake expressed as %ID g^{-1}, rapidly decreased from 45.6 ± 5.4 at 15 min after injection to 0.86 ± 0.2 after 4 h. The total blood activity (%ID g^{-1}) decreased from 7.3 ± 0.7 at 15 min to 0.93 ± 0.2 after 4 h. Tumour uptake (%ID g^{-1}) reached a maximum at 60 min (4.05 ± 0.47 vs. 2.21 ± 0.52, 3.26 ± 0.36 and 1.11 ± 0.22 resp. at 15, 30 and 240 min). The tissue-to-blood ratios in Figure 2 illustrate that the uptake in organs that are all well-circulated, for example lungs, liver and spleen, simultaneously decreased in 4 h with blood pool activity. In contrast, the tumour uptake increased over time as is illustrated by the tumour-to-blood-ratio versus time curve, although activity in %ID g^{-1} (1.11 ± 0.22 for SW948) and the tumour-to-blood ratio (1.3 ± 0.11) were low.

As is illustrated in Figure 2 no differences in normal tissue uptake were found in the human SKBR3 or SW948 xenograft groups. Interestingly ^{125}I-rhTRAIL tumour uptake increased, also in the SKBR3 tumours with very low levels of DR4 and DR5 membrane expression. Histological analysis showed necrosis in the SW948 tumours but not in the SKBR3 tumours, which were substantially smaller in size.

Stomach activity slightly increased over time. No activity could be detected in the brain.

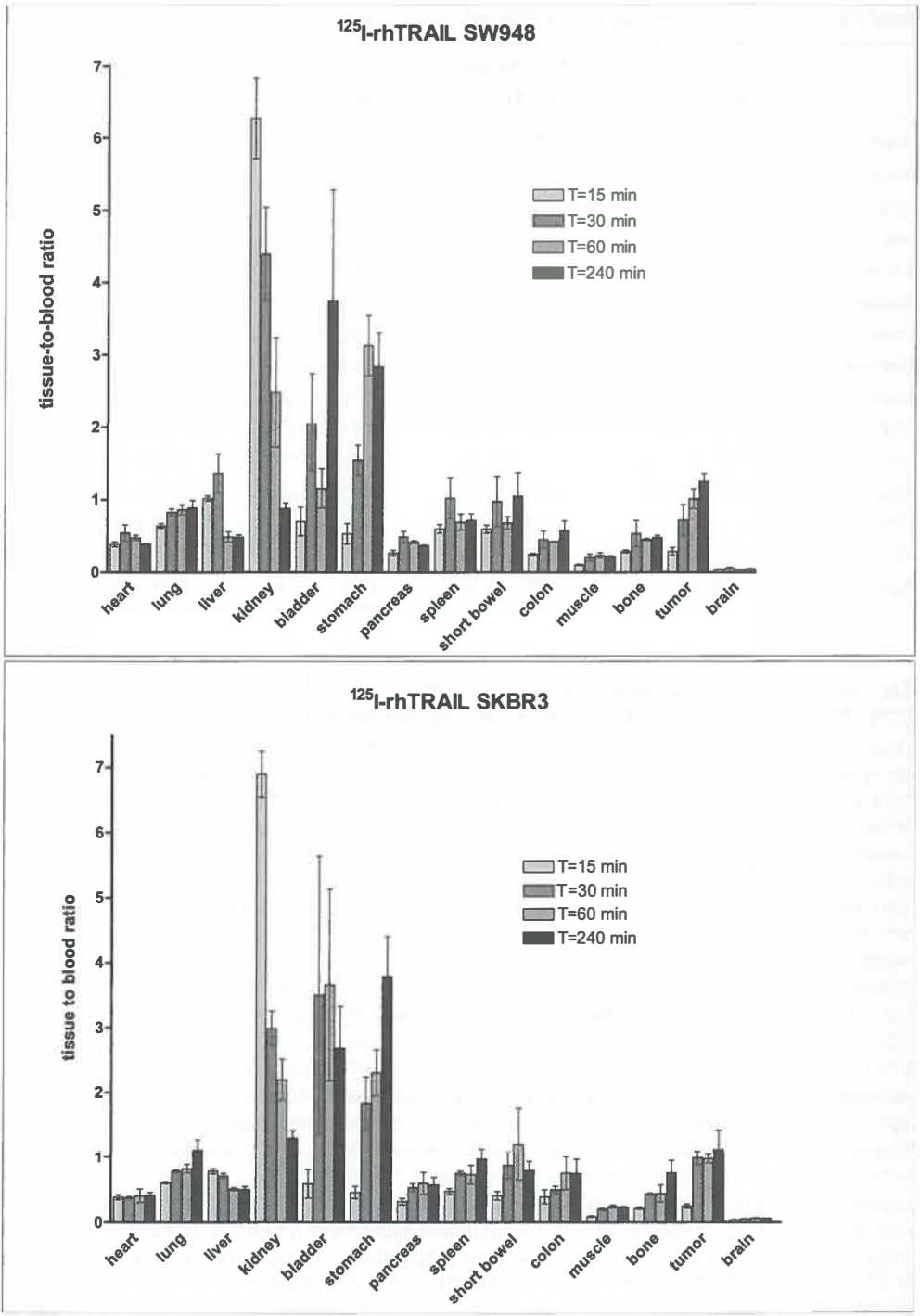

Figure 2. ^{125}I-rhTRAIL biodistribution in SW948 or SKBR3 tumour bearing athymic mice. Tissue-to-blood ratios.

Table 1. Biodistribution of ^{125}I-rhTRAIL in human SW948 tumour bearing mice displayed as %ID g^{-1} ± s.e.m.

	t = 15 min (n=4)	t = 30 min (n=4)	t = 60 min (n=4)	t = 240 min (n=4)
Heart	2.79 ± 0.23	2.56 ± 0.15	1.90 ± 0.13	0.36 ± 0.09
Blood	7.34 ± 0.73	5.10 ± 0.65	4.02 ± 0.13	0.93 ± 0.24
Lung	4.70 ± 0.53	4.13 ± 0.37	3.44 ± 0.20	0.76 ± 0.14
Liver	7.41 ± 0.63	6.44 ± 0.21	1.95 ± 0.23	0.43 ± 0.09
Kidney	45.63 ± 5.37	21.80 ± 3.56	9.82 ± 2.79	0.86 ± 0.20
Bladder	5.28 ± 1.70	9.26 ± 1.26	4.59 ± 0.97	2.50 ± 0.62
Stomach	4.10 ± 1.23	7.55 ± 0.72	12.54 ± 1.56	2.96 ± 1.20
Pancreas	2.00 ± 0.39	2.33 ± 0.05	1.68 ± 0.08	0.34 ± 0.09
Spleen	4.44 ± 0.72	4.67 ± 0.45	2.76 ± 0.37	0.61 ± 0.11
Small intestine	4.31 ± 0.43	4.32 ± 0.75	2.71 ± 0.29	0.79 ± 0.08
Colon	1.82 ± 0.26	2.07 ± 0.17	1.70 ± 0.07	0.49 ± 0.10
Muscle	0.78 ± 0.13	0.95 ± 0.05	0.93 ± 0.13	0.20 ± 0.05
Bone	2.13 ± 0.30	2.39 ± 0.36	1.83 ± 0.10	0.43 ± 0.09
Tumor	2.21 ± 0.52	3.26 ± 0.36	4.05 ± 0.47	1.11 ± 0.22
Brain	0.28 ± 0.02	0.27 ± 0.02	0.15 ± 0.01	0.04 ± 0.02

In vivo biodistribution of ^{111}In -labelled HGS-ETR1 and HGS-TR2J in tumour bearing mice

Table 2 shows the biodistribution of ^{111}In-ETR1 and ^{111}In-TR2J in mice bearing SKBR3- or SW948-xenografts. Since no significant differences occurred in tissue biodistribution of ETR1 or TR2J in mice with either SW948 or SKBR3 xenografts, as was illustrated for rhTRAIL in Figure 2, data of the individual antibodies were pooled except for xenograft data. At all time points ^{111}In-ETR1 showed in virtually all tissues assessed, a lower uptake compared with ^{111}In-TR2J. In most organs activity declined over time for both antibodies, whereas the tissue-to-blood ratios showed an increase over time with a maximum at t = 168 h (Table 3). For both antibodies liver and kidney activity reached a maximum at 72 h (Table 2), with absolute levels of activity in the kidneys being higher than in the liver. Although absolute levels of ETR1 were lower than those of TR2J, the differences between the antibodies in liver, kidney and spleen uptake remained rather stable over time. The difference in blood activity between ETR1 and TR2J was highest at 168 h (74.1% vs. 58.4% and 37.4% at 24 h and 72 h respectively). As a consequence the tissue-to-blood ratios of ETR1 were higher at t = 168 h. The rate of blood clearance however was not significantly different between ETR1 and TR2J. Tumour uptake of ETR1 was not significantly different ($p = 0.32$ at 168 h) in the receptor negative SKBR3 xenograft as to the receptor positive xenograft SW948 (Figure 3A). TR2J showed an increase of the tumour-to-blood ratio over time for the SW948 xenograft, whereas it remained at a constant low level for SKBR3 (Figure 3B). As only one SKBR3 tumour sample was available at 168 h after TR2J administration, statistical analysis including this time point could not be performed. In an additional experiment an excess amount of unlabelled antibody was injected together with the labelled preparations and biodistribution was determined at 72 h. In the SKBR3 tumour the excess amount of antibody resulted in an increased signal for ETR1 and TR2J ($p = 0.02$ and 0.04 resp), whereas no differences were detected in the SW948 xenografts (Figure 4A + B).

Table 2. Biodistribution of ^{111}In-ETR1 and ^{111}In-TR2J in mice bearing SW948 or SKBR3 xenografts. Except for the tumour values, data of SW948 and SKBR3-xenograft bearing mice are pooled. Data are expressed as %ID g^{-1} ± s.e.m.

	t = 24h				t = 72h				t = 168h			
	^{111}In-ETR1 ± s.e.m.		^{111}In-TR2J ± s.e.m.		^{111}In-ETR1 ± s.e.m.		^{111}In-TR2J ± s.e.m.		^{111}In-ETR1 ± s.e.m.		^{111}In-TR2J ± s.e.m.	
Heart	3.38	0.46	10.27	0.93	2.88	0.43	5.53	0.74	1.27	0.21	3.34	0.40
Blood	10.28	1.94	24.70	4.79	6.92	1.55	11.07	3.06	0.96	0.53	3.70	1.58
Lung	2.84	0.61	10.00	3.40	2.49	0.55	4.27	1.02	1.03	0.30	2.08	0.53
Liver	7.48	1.46	24.17	3.84	8.14	0.75	23.53	2.83	5.29	0.87	12.13	2.00
Kidney	11.53	1.67	29.30	2.90	16.30	1.75	31.81	2.72	9.29	0.91	21.17	2.52
Urine	4.48	1.13	10.11	1.76	4.67	0.65	9.42	1.26	1.76	0.16	3.71	0.79
Bladder	3.04	1.24	10.99	0.57	3.41	0.61	6.45	1.11	1.70	0.31	2.77	0.82
Stomach	1.38	0.32	4.54	1.84	1.30	0.38	2.17	0.56	0.96	0.19	1.71	0.44
Pancreas	1.00	0.22	2.17	0.54	2.67	0.50	2.88	0.55	1.12	0.31	1.27	0.39
Spleen	7.25	2.64	19.42	9.89	5.74	2.59	15.11	4.77	5.33	1.98	13.57	4.14
Small intestine	5.47	1.92	3.94	0.96	5.09	1.74	6.70	3.24	2.60	0.80	3.21	1.39
Colon	1.19	0.34	8.12	4.52	1.29	0.35	2.83	1.16	0.68	0.18	0.73	0.22
Muscle	0.41	0.10	2.23	0.51	0.46	0.15	1.09	0.65	0.56	0.17	0.32	0.10
Bone	3.00	0.78	13.55	3.68	5.68	1.31	13.41	3.16	4.03	1.29	10.50	2.07
Brain	0.12	0.03	0.49	0.09	0.10	0.03	0.23	0.06	0.09	0.02	0.13	0.03
SKBR3 xenograft	6.23	1.10	18.76	7.58	7.55	3.54	5.66	1.56	3.66	1.83	2.99	0.00
SW948 xenograft	3.42	0.90	14.26	4.47	6.21	2.03	11.51	8.06	5.06	2.06	3.61	1.16

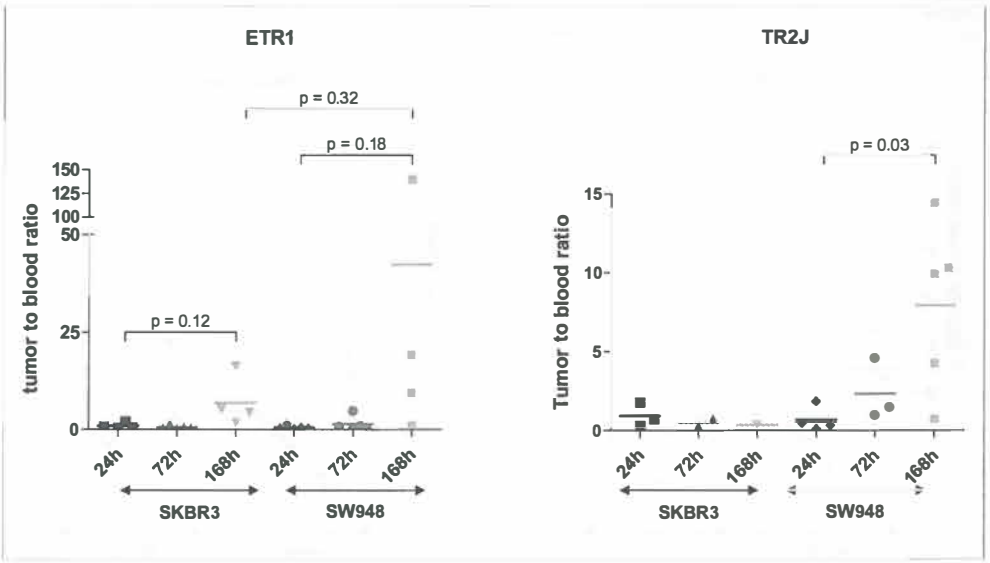

Figure 3. Tumour-to-blood ratio of (A) 111In-ETR1 and (B) 111InTR2J in SKBR3 vs. SW948 xenograft.

Table 3. Biodistribution of ^{111}In-ETR1 and ^{111}In-TR2J in mice bearing SW948 or SKBR3 xenografts. Except for the tumour values, data of SW948 and SKBR3-xenograft bearing mice are pooled. Data are expressed as tissue-to-blood ratios ± s.e.m.

	t = 24h				t = 72h				t = 168h			
	^{111}In-ETR1 ± s.e.m.		^{111}In-TR2J ± s.e.m.		^{111}In-ETR1 ± s.e.m.		^{111}In-TR2J ± s.e.m.		^{111}In-ETR1 ± s.e.m.		^{111}In-TR2J ± s.e.m.	
Heart	0.42	0.10	0.58	0.10	0.36	0.05	2.26	0.86	6.72	2.03	4.64	1.22
Blood	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
Lung	0.36	0.12	0.55	0.28	0.29	0.08	1.44	0.57	2.93	0.75	2.88	0.86
Liver	1.12	0.42	1.50	0.42	0.97	0.35	11.44	4.73	33.38	10.79	30.78	10.68
Kidney	1.43	0.35	1.55	0.31	1.94	0.83	13.82	5.32	50.04	12.27	35.30	11.37
Urine	0.72	0.37	0.65	0.32	0.40	0.08	6.07	3.84	10.35	2.53	6.41	3.62
Bladder	0.36	0.17	0.49	0.17	0.45	0.20	3.54	1.41	7.48	2.14	4.56	1.68
Stomach	0.19	0.08	0.37	0.24	0.12	0.06	0.96	0.51	4.51	1.75	2.19	0.87
Pancreas	0.10	0.02	0.10	0.03	0.22	0.06	0.68	0.28	4.25	1.11	1.79	0.78
Spleen	1.55	0.72	3.10	1.77	0.23	0.06	12.63	7.13	38.30	18.78	37.21	15.89
Small intestine	0.97	0.55	1.71	1.43	0.65	0.41	4.63	2.26	13.46	6.67	8.33	3.65
Colon	0.22	0.10	0.59	0.43	0.18	0.11	1.35	0.61	3.73	1.71	1.49	0.71
Muscle	0.05	0.02	0.13	0.04	0.06	0.02	0.81	0.72	3.36	1.96	0.61	0.24
Bone	0.43	0.20	1.10	0.45	0.47	0.20	9.96	4.17	24.64	8.80	25.05	9.48
Brain	0.01	0.00	0.02	0.00	0.01	0.00	0.06	0.02	0.31	0.10	0.13	0.04
SKBR3 xenograft	0.89	0.40	0.94	0.44	0.50	0.21	0.49	0.23	6.86	3.21	0.33	0.00
SW948 xenograft	0.50	0.21	0.68	0.39	1.33	0.89	2.33	1.14	42.31	32.64	7.92	2.42
SKBR3 tumor to muscle	23.03	8.69	12.02	5.34	26.90	16.91	12.13	8.76	18.08	4.95	3.86	0.00
SW948 tumor to muscle	23.65	12.66	12.00	4.01	18.88	5.96	90.19	84.94	46.52	37.96	36.41	21.95

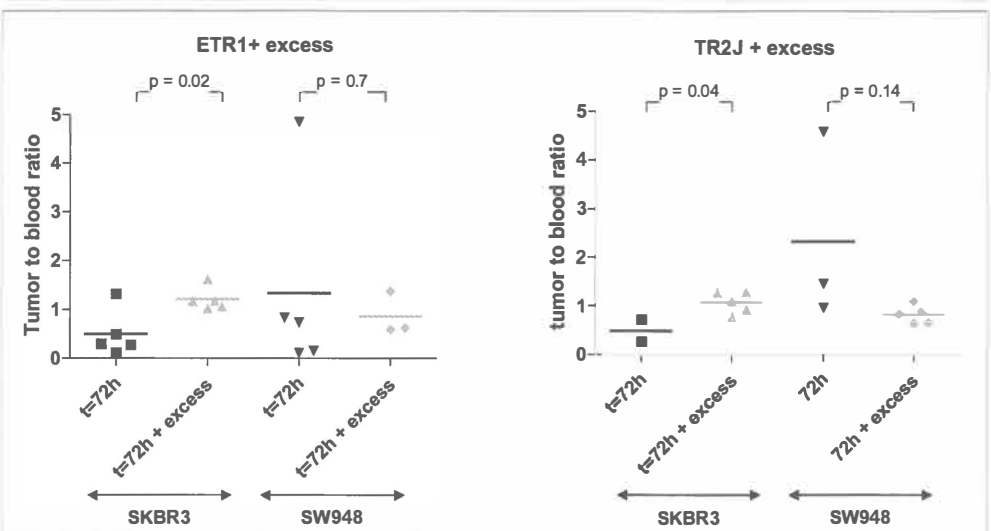

Figure 4 Tumour-to-blood ratio of **(A)** 111In-ETR1 and **(B)** 111In-TR2J at 72 h in SKBR3 vs. SW948 xenograft after administration of excess 111In-ETR1 or 111In-TR2J respectively.

DISCUSSION AND CONCLUSIONS

The present study shows the successful development of radioiodinated rhTRAIL and ^{111}In radiolabelled ETR1 and TR2J for clinical use. The new radiopharmaceuticals have suitable radiochemical purity, stability and receptor binding capacities. The biodistribution studies in nude mice bearing human xenografts showed tumour targeting for both the radiolabelled cytokine and the monoclonal antibodies. Based on the experiments described in this paper, it is therefore concluded that these radiopharmaceuticals are suitable for molecular imaging in humans.

Radioiodination of rhTRAIL was conducted as electrophilic substitution reaction, for which iodide must be oxidized. A difference with the Xiang study, who also analyzed radioiodinated rhTRAIL biodistribution, is that they choose to radioiodinate rhTRAIL using an oxidative enzyme (27). The use of lactoperoxidase offers a very mild method of oxidation, however routine clinical use of enzymes for radioiodination is undesirable as the enzymes themselves can be radioiodinated and radioiodinated proteins have to be separated from the enzyme. Because we aimed to develop a tracer suitable for imaging in patients, we have not tested lactoperoxidase but preferred chloramine T and Iodogen. Chloramine T showed the highest labelling efficiency and least amount of impurities, possibly related to its short reaction time compared to Iodogen. In oxidative environments however, free cysteines on rhTRAIL monomers may form intermolecular disulfide bridges, giving rise to free-standing dimers as well as disulfide-linked rhTRAIL dimer species within trimeric forms of rhTRAIL, which may lead to aggregation, precipitation and/or deactivation of rhTRAIL [patent WO01/00832-PCT/US00/17579, Ashkenazi]. Presence of divalent metal ions in the formulations may protect against such disulfide bond formation. We were able to radioiodinate zinc-stabilised rhTRAIL, resulting in a stable compound without signs of aggregation. Considering the rapid half life in humans (26), the small amount of aggregates present after storage of the radiolabelled compound at 37 °C is not worrisome. This was confirmed in the in-vivo biodistribution study. Minimal liver activity was detected at 15 min (7.4 %ID g^{-1}) which rapidly decreased (0.43 %ID g^{-1} at 4 h after injection). The liver-to-blood ratio declined over time, indicating that the liver uptake was non-specific and blood pool related.

Whereas we intend to use ^{123}I-rhTRAIL for imaging in humans, we used ^{125}I for most experiments to be more flexible and reduce costs. As specific activity may vary between ^{123}I and ^{125}I, differences in labelling efficiency could occur. This will however not affect stability, receptor binding properties or pharmacokinetic behaviour.

Radioiodination of rhTRAIL did not interfere with binding of rhTRAIL to its receptors. As the immunoreactive fraction is a measure for damage of binding capacity of the protein due to the radiolabelling process, no variation is expected when it is determined on different cell lines. The observed differences in immunoreactive fractions between the cell lines might be explained by varying ratios of DR4 and DR5 expressed on the cell lines and a different affinity of rhTRAIL for DR4 or DR5, with DR5 being the highest affinity receptor (6).

Since iodine is a non-residualising isotope, a disadvantage in in-vivo experiments could be the release into the circulation after receptor binding, internalisation and lysosomal catabolism (28,29). This together with some dehalogenation could have contributed to the amount of radioactivity found in the stomach (12.54 ± 1.6 %ID g^{-1} at 60 min) in the biodistribution experiment. However because of rapid rhTRAIL clearance this is considered not a major issue for imaging with radioiodinated rhTRAIL in humans.

Rapid internalisation of the death receptors (30) is the main reason for using ^{111}Indium for labelling the monoclonal antibodies. Moreover, iodination of the monoclonal antibodies resulted in complete loss of immunoreactivity. The indium labelled monoclonal antibodies retained receptor binding properties.

The biodistribution of ^{125}I-rhTRAIL and the ^{111}In-antibodies was determined in nude mice bearing human xenografts. We and others showed that ^{125}I-rhTRAIL biodistribution is characterised by fast renal clearance, with highest activity in the kidneys at 15 min (27,31). In most organs maximal activity was seen at 15 min after injection and the decrease of activity over time followed blood pool activity, suggesting that distribution to normal tissues was limited. The tumour uptake of ^{125}I-TRAIL in xenografts reached a maximum at 60 min after injection ($p = 0.027$ for 60 vs. 15 min) and was higher at 240 min than activity in for example kidney, liver, heart and intestine. Moreover, the tumour-to-blood ratio increased over time, showing that although activity of ^{125}I-rhTRAIL in the xenografts was rather low, specific tumour retention takes place.

Regarding the biodistribution of the antibodies, both radiolabelled antibodies showed high uptake in the liver, kidneys, spleen and bone, with highest activity in liver and kidneys at 72 h after injection. Whereas uptake in liver and spleen is in agreement with known antibody metabolism and retention of ^{111}In in the reticuloendothelial system (32), the high kidney activity is surprising. The fact that kidney activity did not follow blood pool activity suggests accumulation of ^{111}In in the kidney. Because the size of the antibodies excludes renal filtration, specific antigen-binding with possible subsequent metabolism might have occurred. In mice, one death domain containing receptor, also called mouse killer (MK), has been identified with high expression levels in heart, lung and kidney (33,34). MK possesses 76 and 79% amino acid homology with DR4 and DR5 respectively and can bind human TRAIL. These data together with data on the kidney being the main clearance site of rhTRAIL (27) might indicate that specific renal binding and possible metabolism might have taken place. Dissociation of ^{111}In-DTPA might also have caused high kidney activity, but if this occurred, this is expected to result in fast renal ^{111}In clearance which is in contrast to our findings.

For both antibodies tumour activity in %ID g^{-1} declined over time in the receptor positive and negative xenograft. This is indicative for low tumour binding since high specific tumour uptake would have caused trapping of ^{111}In and an increase in activity as for instance is seen for HER2 binding ^{111}In-labelled trastuzumab (23). Considering these differences, it should be taken into account that protein overexpression due to gene amplification in the tumour, as seen for HER2 is not described for rhTRAIL receptors. The increase of the tumour-to-blood ratios over time, with a substantial difference between the receptor positive and negative tumour for ^{111}In-HGS-TR2J, does show however that while retention was low, over time specific tumour uptake had occurred. One explanation for the lack in differences of ^{111}In-HGS-ETR1 uptake between the TRAIL-receptor negative and positive xenografts might be necrosis in the receptor positive tumours. The experiment with an excess amount of unlabeled monoclonal antibody nicely confirms the level of aspecific binding. For the SW948 xenograft a decrease in binding seems present, however the numbers are to small to reach significance.

In conclusion, our data show that radioiodinated rhTRAIL and radiolabelled antibodies can target human tumour tissue in vivo. Consequently, further evaluation of the radiopharmaceuticals in humans is justified.

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Statement of conflicts of interest

The authors state no conflict of interest.

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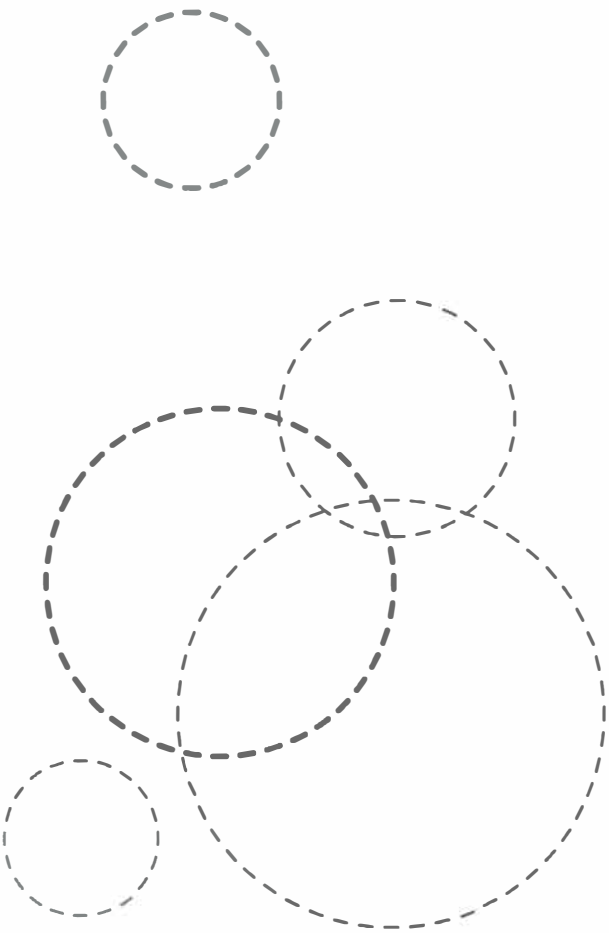

CHAPTER 8

ENHANCED ANTITUMOR EFFICACY OF A DR5-SPECIFIC TRAIL VARIANT OVER RHTRAIL IN A BIOLUMINESCENT OVARIAN CANCER XENOGRAFT MODEL

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Submitted for publication

ABSTRACT

Purpose: Recombinant human (rh) TRAIL is clinically evaluated as novel anticancer drug. A rhTRAIL variant that specifically binds to DR5 receptor (rhTRAIL-DR5) has recently been developed. This study aimed to compare antitumor efficacy of rhTRAIL-DR5 versus rhTRAIL alone and in combination with cisplatin in a bioluminescent ovarian cancer xenograft model.

Experimental design: In vitro efficacy of rhTRAIL and rhTRAIL-DR5 on the human A2780 ovarian cancer cell line was determined with cytotoxicity and apoptosis assays. Biodistribution analysis with ^{125}I-rhTRAIL was performed in mice to explore the best administration route: intravenous (IV) versus intraperitoneal (IP). Antitumor efficacy against IP A2780-Luc xenografts was determined with bioluminescence.

Results: In vitro, rhTRAIL-DR5 induced more apoptosis than rhTRAIL. Cisplatin enhanced induction of apoptosis by both ligands, with cisplatin and rhTRAIL-DR5 inducing most apoptosis. IP administration of ^{125}I-rhTRAIL resulted in a higher area under the curve and increased tumor exposure than IV administration. IP administration of rhTRAIL-DR5 or cisplatin delayed tumor progression, reflected in a mean light reduction of 68.3% ($p = 0.015$) and 72.3% ($p = 0.009$) as compared with vehicle treated animals, while rhTRAIL or rhTRAIL-DR5 plus cisplatin resulted in 85% ($p = 0.003$) and 97% ($p = 0.002$) reduction. Combination of rhTRAIL-DR5 with cisplatin was more effective than cisplatin alone ($p = 0.027$).

Conclusion: RhTRAIL-DR5 and rhTRAIL showed marked efficacy in vitro and in vivo against ovarian cancer, with maximal efficacy when combined with cisplatin. RhTRAIL-DR5 was superior over rhTRAIL. IP administration of these agents warrants further exploration in ovarian cancer because of a higher tumor drug exposure.

INTRODUCTION

In developed countries, ovarian cancer is the fifth leading cause of deaths related to cancer in women (1). Although initial response rates to first-line treatment are up to 80% in advanced stage patients, the overall 5-year survival is low due to the occurrence of drug resistance (2). A reduced tendency of cancer cells to undergo apoptosis is due to defects in the intrinsic apoptosis pathway, which contributes to drug resistance (3,4). Therefore, an attractive strategy for targeting cancer cells involves shifting cellular balance in favor of cell death. Such a shift can be achieved by targeting the extrinsic apoptotic pathway. This pathway is activated after binding of death ligands of the tumor necrosis factor family (TNF) to their respective receptors at the cell membrane (5). The recombinant human (rh) form of the death ligand TNF related apoptosis inducing ligand (TRAIL) is regarded as the most promising death ligand due to its selective toxicity against tumor cells while sparing most normal tissues (6). RhTRAIL (Apo2L) is currently evaluated in clinical trials. A recent phase I study showed that rhTRAIL can be administered safely and is well tolerated (7). This ligand can bind four membrane-bound receptors, of which death receptor 4 (DR4) and death receptor 5 (DR5) act as agonistic receptors and decoy receptor 1 (DcR1) and 2 (DcR2) act as antagonist or decoy receptors (8).

Besides rhTRAIL, alternative strategies for targeting death receptors such as activating/agonistic antibodies (9) and TRAIL variants that selectively activate DR4 or DR5 (10,11) have been developed. By avoiding competition with other TRAIL receptors, targeting of a single receptor may increase efficacy. Moreover, receptor specific rhTRAIL variants possess increased binding capacities for the designated receptor (10,11), which may further enhance efficacy and improve the therapeutic window. RhTRAIL effectively induced apoptosis in various ovarian carcinoma cell lines in vitro. Use of a combination of classical chemotherapeutics with rhTRAIL mostly enhanced the tumoricidal effect and could overcome resistance to either drug (12-14). So far, studies examining the efficacy of combinations of classical chemotherapeutic drugs with single death receptor targeting drugs have not been performed in ovarian cancer models.

The eventual clinical efficacy of death receptor targeted therapies in ovarian cancer depends on their biological activity and pharmacological behavior. As the peritoneal cavity is the main site of disease in ovarian cancer, intraperitoneal (IP) drug administration may result in increased tumor penetration and drug exposure with reduced systemic toxicity (15). IP cisplatin administration increases survival as compared to intravenous (IV) adminstration in advanced stage ovarian cancer patients (16). As IV rhTRAIL administration in humans results in rapid renal clearance with a half life of approximately 30 minutes (17), IP administration may delay its clearance and lead to increased anti-tumor activity, especially in ovarian cancer.

The aim of the present study was to compare the in vitro and in vivo efficacy of a novel rhTRAIL variant directed at DR5 (rhTRAIL-DR5) with that of rhTRAIL, alone and in combination with cisplatin in a bioluminescent human A2780 IP ovarian cancer model. The rhTRAIL variant, obtained by computational design, contains two amino acid mutations, D269H and E195R that ensure high affinity binding specifically to DR5 (11). The optimal route of in vivo variant or rhTRAIL administration, i.e. IV or IP was evaluated by biodistribution analysis with radiolabeled rhTRAIL. In vivo efficacy was determined by bioluminescence.

MATERIALS AND METHODS

Cell lines and transfection procedure

The human ovarian cancer cell line A2780, a kind gift from Dr Hamilton (Fox Chase Cancer Center, Philadelphia, PA), forms IP xenografts mimicking peritonitis carcinomatosis in nude mice (18). The A2780-Luc cell line was generated with HindIII and XbaI restriction enzymes (Roche Applied Science, Almere, The Netherlands). The luciferase gene was excised from pGL3-basic (Promega, Madison, WI) and ligated into a pcDNA3 vector under the control of the cytomegalovirus

promotor. A2780 cells were cultured to 70% confluency and transfected by incubation with 2.5 μg plasmid DNA and 5 μl Fugene6 (Roche Applied Science, Almere, The Netherlands) in 250 μl OptiMem (Invitrogen, Breda, The Netherlands). Two days after transfection, transfectants were selected by adding geneticin (1 mg/ml) (Roche Applied Science, Almere, The Netherlands). Stable transfectants were obtained with a clonogenic assay followed by subcloning of positive clones by limiting dilution. The cell lines were cultured in RPMI 1640 (Life Technologies Breda, The Netherlands), supplemented with 10% heat inactivated fetal calf serum (FCS) (Bodinco BV, Alkmaar, The Netherlands) and 0.1 M L-glutamine in a humidified atmosphere with 5% CO2 at 37 °C. Geneticin was added once a month to the A2780-Luc culture. Luciferase expression was regularly tested with the luciferase assay (# E1500, Promega, Leiden, The Netherlands) and the BioRad ChemiDoc XRS system (BioRad, Veenendaal, The Netherlands).

Cytotoxicity assays and determination of apoptosis

The microculture tetrazolium assay, performed as described earlier (19), was used to measure cytotoxicity. The cells were cultured in HAM/F12 and DMEM medium, supplemented with 20% FCS and 0.1 M L-glutamine. Treatment consisted of continuous incubation with 0 - 100 ng/ml rhTRAIL-DR5 or rhTRAIL. RhTRAIL and rhTRAIL-DR5 were produced as described earlier (11). Binding capacity to DR4 and DcR1 is virtually absent for TRAIL-DR5, whereas affinity for DcR2 is reduced (11). In cell viability assays assessing combination treatment with cisplatin, the cells were pre-incubated for 4 hours (h) with 2.5 μM cisplatin (inhibitory concentration 20% - IC20), before addition of 0 – 25 ng/ml rhTRAIL or rhTRAIL-DR5.

Apoptosis was determined with a caspase 3/7 fluorometric assay (Zebra Biosciences, Groningen, The Netherlands) and with acridine orange staining. For the fluorometric detection of DEVD-ase activity, cells were plated in 6-wells plates and left to adhere overnight. The cells were exposed to 2.5 μM cisplatin for 4 h, after which cisplatin was washed away with phosphate buffered saline (PBS: 6.4 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 0.14 mM NaCl; 2.7 mM KCl; pH=7.2) and fresh medium was added to the cells. 20 h later 50 ng/ml rhTRAIL-DR5 or rhTRAIL were added for 4 h. Thereafter the cells were harvested with trypsin and washed twice with ice-cold PBS. Before performing the caspase 3 activity assay according to the manufacturer's protocol, protein content of the lysates was determined with Bradford analysis (21).

For the apoptosis assay 10,000 cells were incubated in 96-well tissue-culture plates. The cells were exposed to 2.5, 10 or 30 μM of cisplatin for 4 h, after which they were washed with PBS twice and incubated in regular culture medium. Twenty hours after administration of cisplatin, 100 or 250 ng/ml rhTRAIL-DR5 or rhTRAIL were added for 4 h. The same procedure was performed in the presence of 2.5 μg/ml mouse anti-DcR2 antibody (R&D Systems, Oxon, UK), with the exception that 1 h pre-incubation with the blocking antibody preceded rhTRAIL-DR5 and rhTRAIL-incubation. After drug incubation acridine orange was added to each well to distinguish apoptotic cells from viable cells. Staining intensity was determined by fluorescence microscopy and apoptosis was defined by the appearance of apoptotic bodies and/or chromatin condensation. Apoptosis was expressed as the percentage of apoptotic cells counted in three fields containing minimally 300 cells.

Flow cytometry

Analysis of TRAIL-receptor membrane expression was performed by FACS analysis as described previously (22). For death receptor expression after cisplatin exposure, cells were exposed for 4 h, washed with PBS and incubated for 20 h in regular culture medium, after which FACS analysis was performed. All PE-labeled antibodies were purchased from R&D Systems. Membrane receptor expression was analyzed with Winlist and Winlist 32 software (Verity Software House, Inc., Topsham, ME) and is shown as mean fluorescent intensity (MFI) of all analyzed cells.

Animals and bioluminescence imaging procedure

Female nude mice (Hsd:Athymic Nude-*nu*) were obtained from Harlan Nederland (Horst, The Netherlands) at 6 - 8 weeks of age (~ 21 g). Inoculation was performed after 10 days of acclimatization. All animal studies were conducted in accordance with the Law on Animal Experimentation and local guidelines, and were approved by the local ethical committee.

Imaging was conducted with the IVIS 100 series (Xenogen Corporation Alameda, CA), composed of a cooled charge-coupled device camera connected to a light tight black chamber. Before in vivo imaging animals were anesthetized with 4% isoflurane and injected IP with D-luciferin (150 mg/kg, Xenogen) reconstituted in sterile PBS. Mice were placed in prone position on a warmed stage (37 ℃) in the imaging chamber, and grayscale reference images were obtained under dim illumination. Pseudocolor images representing bioluminescent intensity were acquired with LivingImage software (version 2.50, Xenogen) 10 and 15 min after D-luciferin injection in complete darkness. These images were superimposed on the grayscale images for analysis with Igor Pro software (version 4.09, WaveMetrics, Lake Oswego, OR). All bioluminescence imaging (BLI) data are depicted in radiance units (photons/sec/cm^2/sr) enabling absolute comparisons between bioluminescent images and represent final data obtained after subtraction of the background signal.

Characterization of the IP bioluminescent model

The A2780-Luc peritonitis carcinomatosis model, characterized in 45 mice, showed exponential tumor growth from 5 days after IP inoculation with 2 x 10^6 A2780-Luc cells. Approximately 10 days later, the increment of the bioluminescence signal was delayed, characterized by a flattening of the BLI log-growth curve that evolved in an almost flat slope. This flattening preceded the development of macroscopic disease, bloody ascites formation and deterioration of general condition. Animal survival based on clinical condition was on average 4 weeks after inoculation. Flattening was most likely due to superposed tumor tissue that absorbed and scattered light emitted from tumor cells situated deeply in the peritoneal cavity. To employ flattening of the BLI-growth curve in the definition of a uniform end point, we used logistic regression analysis. The exponential curve was represented by: $y = a \cdot e^{bx}$. *Y* stands for the bioluminescent signal (in radiance), *a* for the intercept with the y-axis (BLI-signal at day 5), *e* for the uniform mathematical constant, *x* for time (in days) and *b* for the equation-specific constant, which is calculated by logistic regression. The equation can be used to predict BLI signal upon consecutive imaging days. Definition of flattening, which serves as a surrogate endpoint for survival, was a bioluminescent signal of less than 50% as the expected signal based on the equation. We utilized this endpoint in the efficacy studies with one refinement. Treatment induced alterations in the log growth which did not allow determining an equation representing the BLI curve for each mouse during treatment. The time from cessation of treatment to flattening was too short to reliably employ logistic regression analysis. Therefore, the mean signal at flattening in the vehicle-treated group, minus 1 x standard deviation (SD) was defined as absolute cut-off value (3.1 x 10^8 photons/sec/cm^2/sr), which was valid as a uniform survival-endpoint in 85% of the mice.

In vivo biodistribution with ^{125}I-rhTRAIL

Radioiodination of rhTRAIL was performed with a rhTRAIL solution of 1 mg/ml in a pH 7.4 TRIS buffer, containing 100 μM zinc sulphate and 10% glycerol. 45 μg rhTRAIL and 50 μg chloramine T (Merck, Amsterdam, The Netherlands) were allowed to react with 70 MBq ^{125}I-NaI in 0.05 M NaOH (pH 9.0, GE Healthcare, Eindhoven, The Netherlands) during 3 min at pH 8.0. The labeling reaction was terminated with sodium metabisulfite (Acros Organics, Geel, Belgium). Non-bound ^{125}I was removed by gel filtration chromatography. The PD-10 column (Sephadex™ G-25M, Amersham Biosciences AB, Uppsala, Sweden) was eluted with TRIS buffer, containing 100 μM zinc sulphate, 10% glycerol and 0.5% human serum albumin.

The in vivo biodistribution study was conducted in 50 mice after establishment of A2780-Luc IP xenografts. ^{125}I-rhTRAIL (0.15 ml; 150 kBq, 0.5 μg) was administered IV through retro-orbital injection in 25 mice, and IP in 25 mice. At five time points after injection (t = 15, 30, 60, 90 and 360 min) groups of 5 mice were sacrificed and organs and tissues were excised, rinsed for residual blood and weighed. Tumor tissue was additionally fixed in 10% buffered formalin for histological assessment. Samples were counted for radioactivity in a calibrated well-type LKB-1282-CompuGamma counter. Tissue activity was expressed as percentage of the injected dose/g tissue (% ID/g). Tumor-to-blood and tumor-to-muscle ratios were also calculated. All data were corrected for physical decay and compared with a known standard sample. Pharmacokinetic parameters were derived using the KINFIT module of the MW/PHARM computer program package (version 3.50, MediWare, Groningen, The Netherlands). Clearance rates of ^{125}I-rhTRAIL from the circulation were calculated using non-linear regression analysis.

In vivo imaging of antitumor activity

A2780-Luc cells (2 x 10^6) were injected IP into 60 nude mice. 5 days after inoculation the mice were randomized in groups of 10 mice per treatment arm. Treatment consisted of IP injections with vehicle (NaCl, 5 mice, days 5 and 12, and rhTRAIL-buffer, 5 mice, days 5-10 and 12-16), cisplatin (4 mg/kg at day 5 and 12), rhTRAIL or rhTRAIL-DR5 (5 mg/kg, days 5-10 and 12-16), or the combination of cisplatin with rhTRAIL or rhTRAIL-DR5. Cisplatin in the combination therapy was administered 4 h prior to rhTRAIL or rhTRAIL-DR5 injections. The MTD of cisplatin (4 mg/kg IP, weekly x 2) (23) was determined in a pilot study based on max. 15% weight loss in tumor bearing mice. This dose is equivalent to ~ 12 mg/ m^2 (24). Mice were monitored daily for general condition and weight. BLI was performed at 2 to 3 day intervals. When the signal reached a value $\geq$ 3.1 x 10^8 photons/sec/cm^2/sr as described in the characterization section, mice were sacrificed. Tumor and liver tissue samples were excised for histological assessment.

Immunohistochemistry

Tissues were fixed in 10% buffered formalin, embedded in paraffin and cut in 4 μm sections, which were mounted on APES coated glass slides and deparaffinized by a standard procedure. Tumor tissue was stained for cleaved caspase 3 (rabbit anti-caspase 3, clone 9661, 1:200, Cell Signaling Technology, Danvers, MA) following manufacturers instructions. Negative controls were obtained by omission of the primary antibody and by incubation with normal rabbit IgG1. Slides were counterstained with hematoxylin. Histological assessment of liver tissue was carried out on hematoxylin and eosin stained slides.

Statistical analysis

In vitro data and results from the biodistribution study were assessed for differences with unpaired two-tailed Student's *t*-test. Results from the in vivo efficacy study, with bioluminescence signals depicted in radiance (photons/sec/cm^2/sr), are represented as means ± SEM. Percent signal reduction compared to vehicle treated mice at the end of treatment (day 16) was calculated according to the formula: 100 - (signal intensity at day 16)/(mean signal intensity vehicle group at day 16) x 100. One-way ANOVA was performed to determine differences in signal intensity between groups and between differences in percent signal reduction; significant differences were subjected to post hoc analysis with Tamhane's T2 and Dunnett's T3 tests assuming unequal variances. Survival (days) was estimated by Kaplan Meier analysis and compared with log-rank tests. A p-value < 0.05 was considered significant. Statistical analyses were generated using GraphPad Prism software (version 4.0 GraphPad software, San Diego, CA) and SPSS 14.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

In vitro activity of rhTRAIL-DR5, rhTRAIL and cisplatin on A2780

A2780 cells express DR5 and low levels of DcR2 at the cell surface, whereas DR4 and DcR1 are undetectable (Figure 1A). Treatment with cisplatin resulted in a dose-dependent increase in DR5 and DcR2 expression (Figure 1A). Long term exposure (96 h) of A2780 cells to low concentrations of rhTRAIL and rhTRAIL-DR5 induced a dose dependent loss of viability (Figure 1B). Pre-incubation with a low dose cisplatin (2.5 μM) prior to continuous treatment with rhTRAIL or rhTRAIL-DR5 caused a further drop in cell survival (Figure 1C). RhTRAIL-DR5 decreased cell survival more effectively than rhTRAIL, both as a single agent ($p < 0.01$) and in combination with cisplatin ($p < 0.001$). Consistent with these results, short term exposure to rhTRAIL or rhTRAIL-DR5 induced apoptosis, as determined by caspase 3 activity. Caspase activity was enhanced in all conditions upon pre-incubation with 2.5 μM of cisplatin (Figure 2A). Combination of cisplatin with rhTRAIL-DR5 was more effective than combined exposure to cisplatin and rhTRAIL ($p < 0.001$). Similar results were obtained with an acridine orange apoptosis assay (Figure 2B). The apoptosis assays for rhTRAIL and rhTRAIL-DR5 were also performed with co-incubation of a DcR2 blocking antibody. Blocking of DcR2 did not enhance apoptosis induction by rhTRAIL or rhTRAIL-DR5 (Figure 2C).

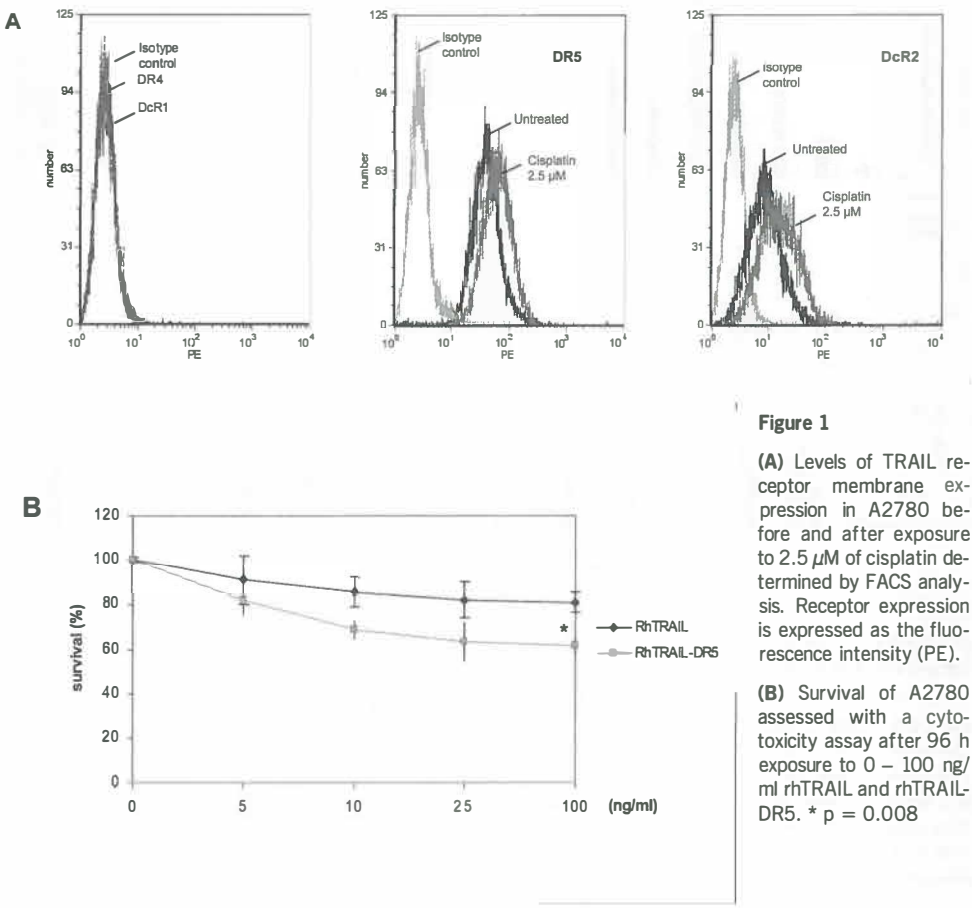

Figure 1

(A) Levels of TRAIL receptor membrane expression in A2780 before and after exposure to 2.5 μM of cisplatin determined by FACS analysis. Receptor expression is expressed as the fluorescence intensity (PE).

(B) Survival of A2780 assessed with a cytotoxicity assay after 96 h exposure to 0 – 100 ng/ml rhTRAIL and rhTRAIL-DR5. * $p = 0.008$

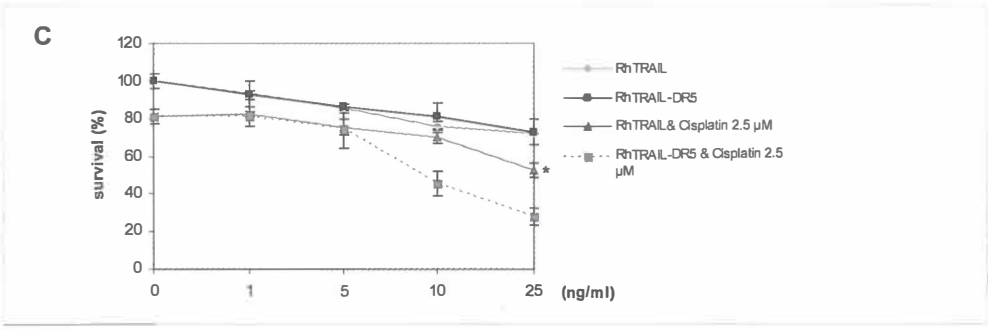

Figure 1

(C) Survival of A2780 determined with a cytotoxicity assay. Cells were pre-incubated for 4 h with 2.5 μM of cisplatin, after which the cells were washed and exposed to 0 - 25 ng/ml rhTRAIL or rhTRAIL-DR5 for 92 h. * rhTRAIL vs cisplatin and rhTRAIL $p < 0.01$, rhTRAIL-DR5 vs. cisplatin and rhTRAIL-DR5 $p < 0.01$, cisplatin and rhTRAIL vs cisplatin and rhTRAIL-DR5 $p < 0.001$.

Data represent the mean ± SD of at least three independent experiments.

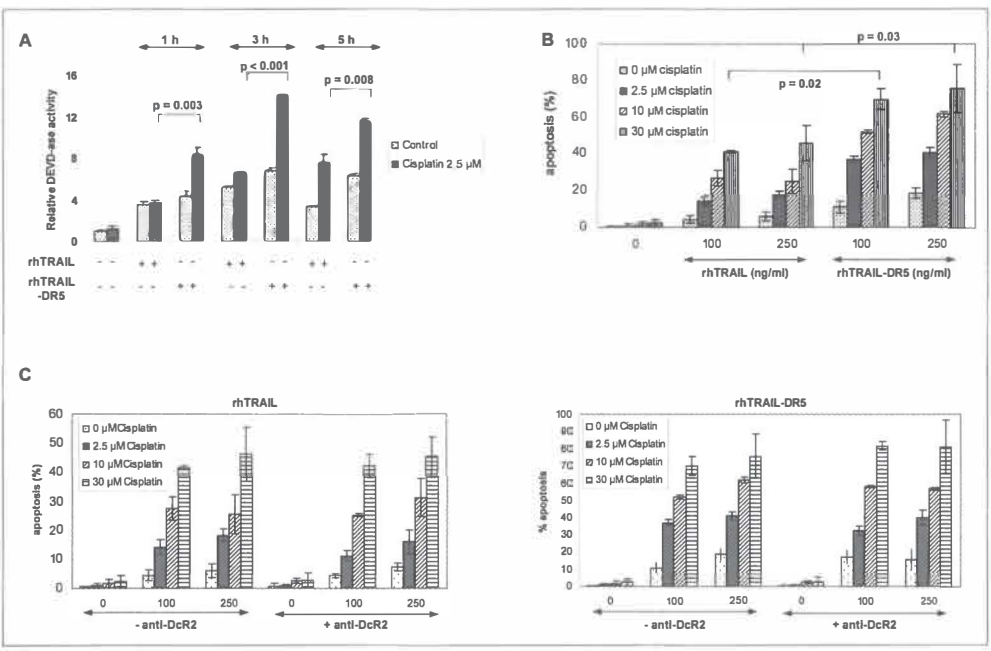

Figure 2

(A) Induction of apoptosis after exposure to 50 ng/ml rhTRAIL or rhTRAIL-DR5 alone or after pre-incubation with 2.5 μM cisplatin for 4 h, 20h prior to rhTRAIL or rhTRAIL-DR5 administration. Caspase activation was determined with a caspase 3 activity assay after 1, 3 and 5 h of treatment.

(B). Cells were pre-incubated with medium, 2.5, 10, 30 μM of cisplatin for 4 h and then washed. After 20 h, the cells were exposed for 4 h to 100 or 250 ng/ml rhTRAIL or rhTRAIL-DR5, and apoptosis was assessed by means of acridine orange staining.

(C). Acridine orange apoptosis assay. The cells were pre-incubated with 2.5, 10 or 30 μM of cisplatin for 4 h and then washed. After 20 h, the cells were exposed for 1 h to 2.5 μg/ml anti-DcR2 antibody or medium, after which the cells were exposed to 100 or 250 ng/ml rhTRAIL for 4 h. Anti-DcR2 antibody co-incubation did not cause significant changes in apoptosis induction. Data represent the mean ± SD of at least three independent experiments.

^{125}I-rhTRAIL biodistribution in tumor-bearing mice

Tissue biodistribution and tumor uptake of IV (Table 1A) and IP (Table 1B) administered ^{125}I-rhTRAIL were compared in nude mice with IP A2780-Luc xenografts. The administration route influenced the disposition of ^{125}I-rhTRAIL. Blood activity (% ID/g) was higher at 15 (43.29 ± 11.04 vs. 25.30 ± 5.04) and 30 min (30.51 ± 12.40 vs. 15.33 ± 3.78) after IV vs. IP injection, whereas it was lower at 90 (7.52 ± 1.22 vs. 23.74 ± 6.85) and 360 min (2.63 ± 0.56 vs. 8.26 ± 1.74). The blood kinetics of ^{125}I-rhTRAIL in blood could be described by a two-compartment model. The resulting blood activity vs. time profiles (Figure 3A) showed a higher area under the time curve (AUC) after IP administration (89.8) than after IV administration (53.7). After IP injection the peak blood activity is lower than after IV injection, but remains higher for a longer period in time. Kidney uptake (% ID/g) showed the same pattern as blood pool activity, with higher activity after IV administration vs. IP administration at 15 (199.2 ± 40.69 vs. 19.83 ± 1.38) and 30 min (126.6 ± 49.68 vs. 21.45 ± 1.90) and lower activity at 90 (12.73 ± 2.47 vs. 17.87 ± 1.28) and 360 min (2.06 ± 0.56 vs. 4.45 ± 0.31). Activity in well-perfused organs such as the lungs, liver and spleen displayed similar kinetics as the blood pool activity in both administration routes. Stomach activity increased over time, which can be attributed to in vivo dehalogenation. IP administration resulted in high tumor activity at 15 min (11.31 ± 1.51) and 60 min (12.91 ± 3.29) with a gradual decrease to 360 min, whereas after IV administration initial low tumor activity increased to a maximum at 60 min (6.85 ± 1.29 IV). The tumor to blood ratios were higher after IP administration at 15 (0.13 ± 0.02 vs 0.48 ± 0.03) and 60 min (0.38 ± 0.04 vs. 0.55 ± 0.06). Tumor to blood ratios remained constant over time after IP injection and increased over time after IV administration (Figure 3B).

Table 1 A. Biodistribution of IV administered 125I-rhTRAIL

	t = 15 min (n = 3)		t = 30 min (n = 5)		t = 60 min (n = 5)		t = 90 min (n = 4)		t = 360 min (n = 5)	
organ	% ID/g ± SEM		% ID/g ± SEM		% ID/g ± SEM		% ID/g ± SEM		% ID/g ± SEM	
heart	14.68	3.37	7.86	1.87	7.35	0.79	2.69	0.41	1.01	0.21
blood	43.29	11.04	30.55	12.40	19.03	1.94	7.52	1.22	2.63	0.56
lung	32.83	5.23	58.91	41.51	28.67	7.21	8.46	2.66	5.62	1.92
liver	60.67	15.20	25.55	6.48	14.92	2.05	5.31	0.87	1.51	0.26
kidney	199.20	40.69	126.60	49.68	43.00	7.82	12.73	2.47	2.06	0.40
spleen	23.84	5.85	9.88	1.64	13.65	1.20	5.01	0.71	1.85	0.28
stomach	7.86	2.09	11.77	1.65	46.34	13.54	30.99	7.40	9.07	2.93
pancreas	7.27	1.54	11.98	6.87	8.11	0.88	3.77	0.62	1.12	0.23
small intestine	12.05	3.99	5.84	1.69	10.44	1.30	3.88	0.47	1.30	0.32
large bowel	6.69	1.60	2.95	1.30	6.88	1.27	2.82	0.20	0.62	0.24
tumor	5.34	1.19	4.84	1.19	6.85	1.29	3.09	0.43	2.30	1.69
tumor/blood	0.13	0.04	0.20	0.07	0.30	0.18	0.42	0.08	0.55	0.67

Table 1 B. Biodistribution of IP administered 125I-rhTRAIL

	t = 15 min (n = 5)		t = 30 min (n = 5)		t = 60 min (n = 5)		t = 90 min (n = 4)		t = 360 min (n = 3)	
organ	% ID/g ± SEM		% ID/g ± SEM		% ID/g ± SEM		% ID/g ± SEM		% ID/g ± SEM	
heart	4.62	0.39	3.48	0.35	4.18	0.40	2.63	0.37	2.04	0.25
blood	25.30	5.04	15.33	3.78	23.74	6.85	22.04	7.98	8.26	1.74
lung	10.65	1.30	6.02	0.64	9.64	1.38	7.01	1.48	4.20	0.28
liver	12.47	1.16	7.91	0.71	9.93	2.06	4.66	1.00	3.05	0.17
kidney	19.83	1.38	21.45	1.90	27.98	6.82	17.87	1.28	4.45	0.31
spleen	11.03	1.24	9.75	3.37	9.24	1.86	15.71	10.68	4.19	0.29
stomach	13.88	1.94	8.71	2.80	36.12	3.40	17.90	6.19	34.10	13.34
pancreas	21.71	3.55	7.27	1.30	13.43	2.48	12.11	6.46	5.45	0.83
small intestine	12.62	1.46	6.88	0.98	13.19	1.92	4.82	1.12	7.67	1.99
large bowel	14.78	2.83	10.92	4.49	13.19	3.00	3.79	0.40	3.30	0.20
tumor	11.30	1.51	5.09	0.92	12.91	3.29	5.91	0.84	5.02	0.77
tumor/blood	0.48	0.04	0.50	0.32	0.55	0.13	0.44	0.32	0.57	0.14

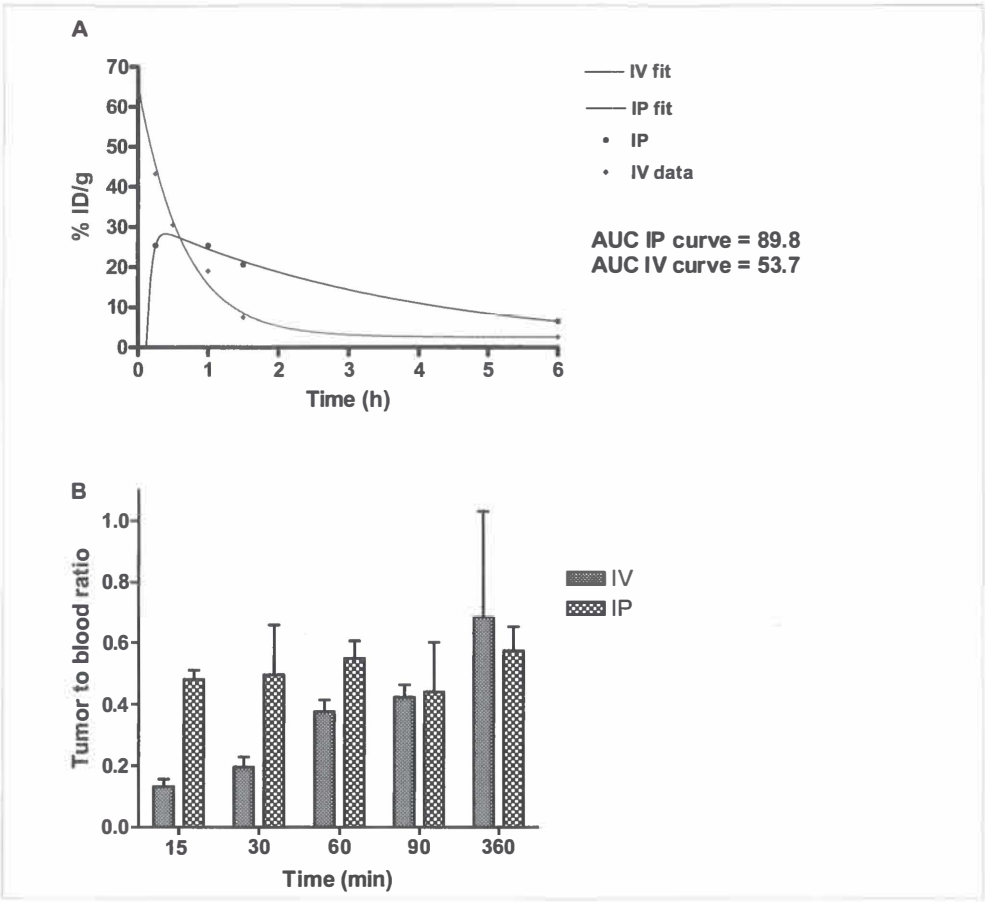

Figure 3

(A) Area under the blood activity versus times curves for IP and IV ^{125}I-rhTRAIL. The blood activity was determined at 15, 30, 60, 90 and 360 min after administration of ^{125}I-rhTRAIL. The % ID/g were calculated and averaged for 3-5 mice per time point and pharmacokinetic profiles were fitted with a two-compartment model.

(B) Tumor-to-blood ratio versus time of 125I-rhTRAIL administered intravenously or intraperitoneally. Tumor to blood ratios were calculated by dividing the average tumor activity in %ID/g per time point through the average blood activity in % ID/g per time point.

Assessment of caspase 3 activity in tumors

To determine whether the higher tumor uptake after IP administration resulted in enhanced efficacy of ^{125}I-rhTRAIL, paraffin embedded tumor tissue obtained at 15 and 360 min after ^{125}I-rhTRAIL injection was stained for cleaved caspase 3. While almost no cleaved caspase 3 was detected in samples obtained at 15 min (Figure 4A+B), tissue obtained at 360 min showed increased cleaved caspase 3 staining. IP administration resulted in caspase 3 activation near the surface of the tumor and near small blood vessels (Figure 5A), whereas IV administration induced detectable caspase 3 activity near blood vessels but not near the tumor border (Figure 5B).

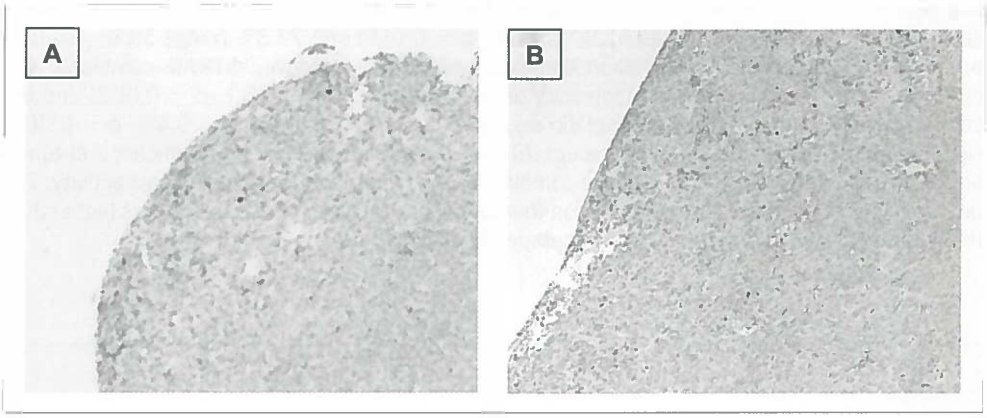

Figure 4 Ovarian cancer xenograft tissue excised at 15 min after IP (A) and IV (B) administration of ^{125}I-rhTRAIL, stained for cleaved caspase 3. *See appendix, page 187 for color figure.*

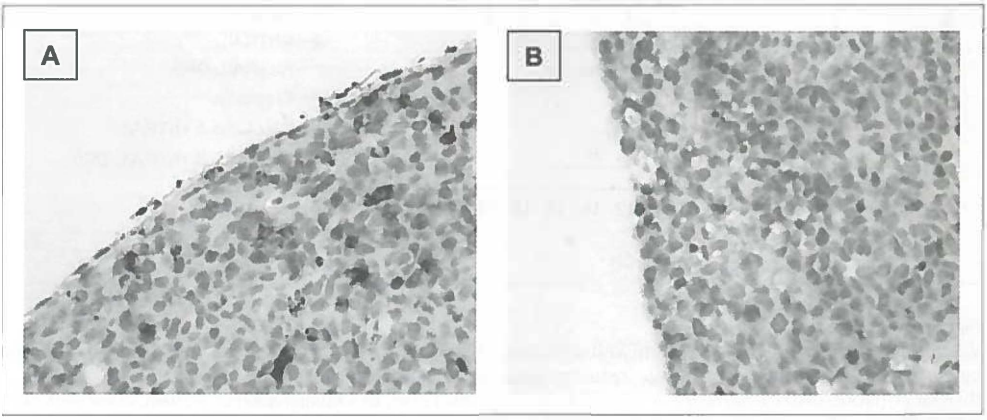

Figure 5 Ovarian cancer xenograft tissue excised at 360 min after IP (A) and IV (B) administration of ^{125}I-rhTRAIL, stained for cleaved caspase 3. *See appendix, page 187 for color figure.*

In vivo efficacy of rhTRAIL, rhTRAIL-DR5 and cisplatin on IP xenografts

The response of IP A2780-Luc xenografts to treatment with rhTRAIL, rhTRAIL-DR5 and cisplatin or a combination of either rhTRAIL or rhTRAIL-DR5 with cisplatin was assessed by bioluminescence imaging. Tumor regression was not visible within the first 48 h after treatment initiation at day 5, but was clearly evident at the end of the first treatment period (day 9), with the largest signal reduction seen after combination of rhTRAIL or rhTRAIL-DR5 with cisplatin (Figure 6A+B). Signals rose in the days between both treatments. All treatment groups except the rhTRAIL-treated arm had significantly smaller tumors at day 16 than the vehicle treated group. This is reflected in the mean signal reduction as to vehicle treated mice; whereas rhTRAIL therapy alone did not result in a significant decrease (48.8% - range 32.8 – 64.6%, $p = 0.097$); rhTRAIL-DR5 and cisplatin gave a reduction of 68.3% (range 61.8 – 74.8%, $p = 0.015$) and 72.3% (range 59.8 – 84.9%, $p = 0.009$) respectively. Combination therapies were highly effective; rhTRAIL combined with cisplatin caused a decline in signal intensity of 84.8% (range 73.5 – 96.1, $p = 0.003$) and the combination of rhTRAIL-DR5 with cisplatin resulted in 96.5% (range 93.7 – 99.4%, $p = 0.002$) signal reduction. Thus, all therapies, except rhTRAIL monotherapy exhibited significant anti-tumor activity at the end of treatment, with the combination therapies displaying the highest activity. The decline in signal intensity after combination therapy of rhTRAIL-DR5 with cisplatin was higher than the mean light reduction after cisplatin therapy alone ($p = 0.027$).

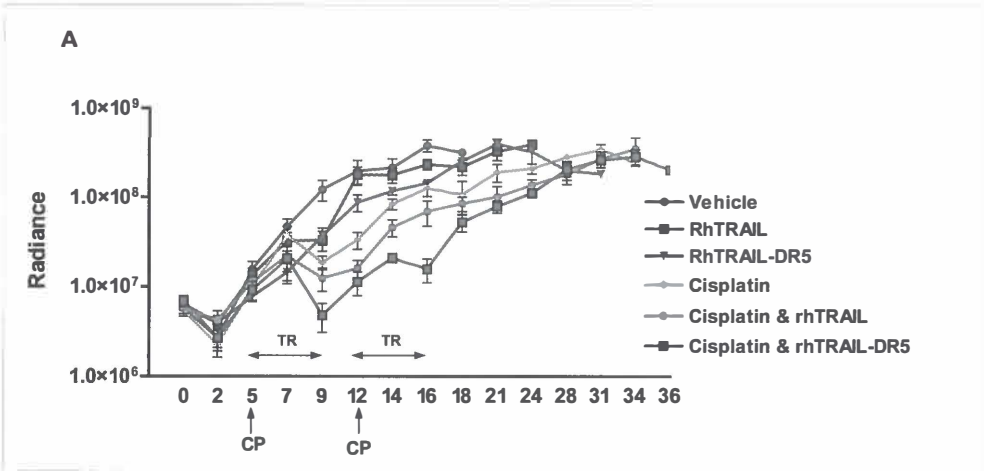

Figure 6
Visualization of response to rhTRAIL, rhTRAIL-DR5, cisplatin and to the combination of either ligand with cisplatin by means of bioluminescence imaging. Treatment consisted of cisplatin (4 mg/kg IP) or vehicle at day 5 and 12, rhTRAIL, rhTRAIL-DR5 (5 mg/kg IP) or vehicle at day 5-10 and 12-16, or a combination of rhTRAIL or rhTRAIL-DR5 with cisplatin. CP = cisplatin, TR = rhTRAIL/rhTRAIL-DR5.

A. Change in light emission (in radiance units) over time per treatment arm. Bioluminescent signals at each time point were averaged per treatment group and are represented by means ± SEM. Differences at day 16 were ($4.6 \times 10^8 \pm 6.7 \times 10^7$) vs. ($2.3 \times 10^8 \pm 3.1 \times 10^7$) $p = 0.097$ between the vehicle group and rhTRAIL; vehicle vs. rhTRAIL-DR5 ($1.4 \times 10^8 \pm 1.3 \times 10^7$) $p = 0.015$; vehicle vs. cisplatin ($1.3 \times 10^8 \pm 2.4 \times 10^7$) $p = 0.009$; vehicle vs. cisplatin and rhTRAIL ($6.7 \times 10^7 \pm 2.1 \times 10^7$) $p = 0.003$; vehicle vs. cisplatin and rhTRAIL-DR5 ($1.6 \times 10^7 \pm 4.6 \times 10^6$) $p = 0.002$.

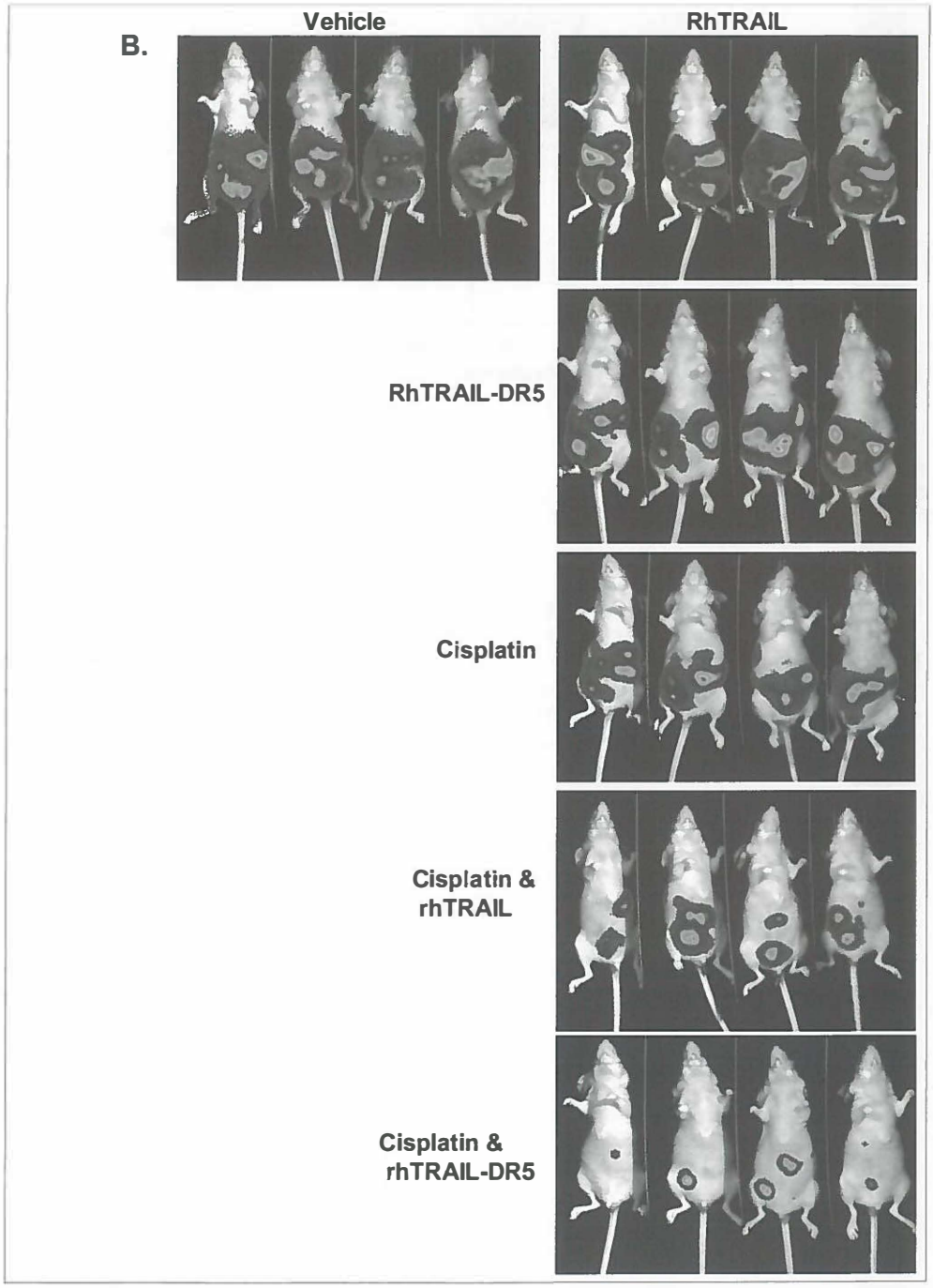

Figure 6
B. Bioluminescent images at the end of treatment (day 16) of each 4 mice representative for 10 mice per experimental arm. Images are displayed and quantified in log radiance (photons/sec/cm^2/sr). *See appendix, page 188-189 for color figure.*

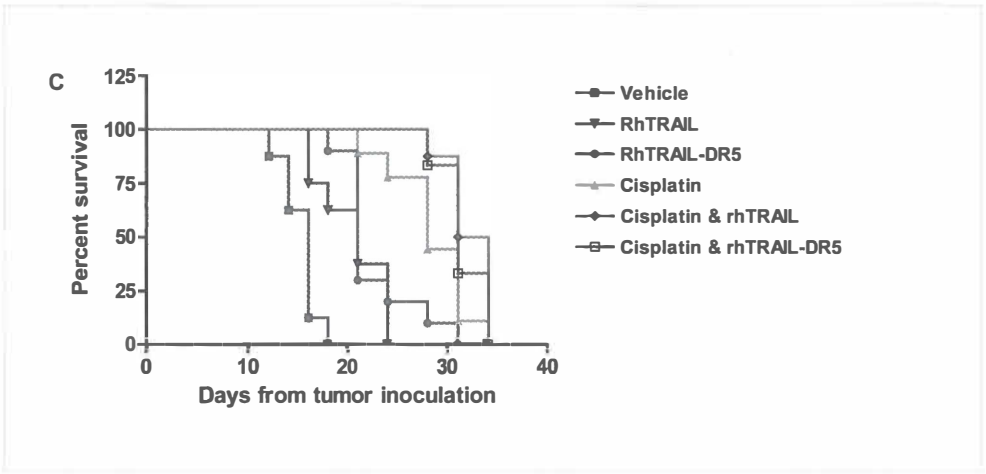

Figure 6
C. Kaplan Meier survival analysis of all mice. A bioluminescent signal of > 3.1 x 10^8 was used as surrogate endpoint for survival, as described in the materials and methods.

In general, light intensity at the end of treatment was inversely associated with survival (Figure 6C). Animals were sacrificed when a bioluminescent signal ≥ 3.1 x 10^8 photons/sec/cm^2/sr was reached, as a surrogate marker for survival. The median survival of the vehicle controls was 16 days, with no mice surviving after 18 days. Monotherapy with rhTRAIL and rhTRAIL-DR5 prolonged median survival to 21 days ($p < 0.001$) and monotherapy with cisplatin to 28 days ($p < 0.0001$). Combination of rhTRAIL-DR5 with cisplatin resulted in a median survival of 31 days ($p < 0.0001$), and rhTRAIL combined with cisplatin in 32.5 days ($p < 0.0001$). The latter was also significant as compared to cisplatin monotherapy ($p = 0.038$). Liver histology at sacrifice did not show any signs of liver damage.

DISCUSSION

In this study we show that a rhTRAIL-variant designed to specifically bind DR5 induced higher levels of apoptosis and growth inhibition in ovarian cancer cells than rhTRAIL, in a time and dose-dependent manner. Pre-treatment with low dose cisplatin enhanced apoptosis and cytoxicity induced by rhTRAIL-DR5 or rhTRAIL, with the combination of cisplatin and rhTRAIL-DR5 being more effective than cisplatin and rhTRAIL. IP administration of these drugs in an orthotopic bioluminescent mouse model of human ovarian peritonitis carcinomatosis delayed tumor growth, with superior efficacy of the combination of cisplatin and rhTRAIL-DR5 over cisplatin alone.

Whereas rhTRAIL can bind four membranous receptors of which two may act as inhibitory receptors, agents that specifically target one agonistic death receptor are in various stages of development. Recent studies with receptor-selective rhTRAIL mutants have defined that cancer cells can display a preference for either DR4 or DR5 for apoptosis signaling, resulting in enhanced apoptosis when the dominant receptor is targeted. Colon carcinoma and breast carcinoma cell lines were reported to prefer to signal through DR5 (10), whereas primary lymphoid malignancies do so through DR4 (25,26). Interestingly, selective DR5-mutants caused higher levels of apoptosis than wild type rhTRAIL in Jurkat expressing only DR5 (10). In the present study, a DR5-selective mutant of rhTRAIL induced apoptosis more effectively than wild type rhTRAIL in DR5 expressing A2780 ovarian cancer cells in vitro. Moreover, pretreatment of A2780 cells with low dose cisplatin (2.5 μM –IC20) augmented rhTRAIL-DR5- or rhTRAIL-induced cytotoxicity and apoptosis, with higher efficacy of the combination of cisplatin with rhTRAIL-DR5 over the combination with rhTRAIL. Combinations of anticancer agents with receptor targeted drugs are often more effective than the single agents in preclinical models. Death receptor upregulation induced by chemotherapeutic drugs or irradiation in a p53-dependent (27-29) or -independent (30) matter is regarded as one of the mechanisms contributing to enhanced effects of combinatory regimens. In our model, cisplatin induced DR5 and DcR2 upregulation. DR5 upregulation is more frequently reported than DR4 upregulation (22,27,31). In ovarian cancer specimens obtained before and after cisplatin treatment, DR5 expression increased from 37% to 74% after chemotherapy, whereas DR4 staining was unaltered (32). This might imply that combinatory strategies with DR5 targeted agents are more effective than combinational regimes with DR4 targeted drugs.

Additionally, agents binding only one receptor will cause exclusively the formation of DR4 or DR5 homotrimers, whereas TRAIL binding causes homo-and heterotrimer formation. Whether this affects apoptosis induction is not well established. Immunoprecipitation of death inducing signaling complexes after treatment with TRAIL showed fewer heterotrimeric than homotrimeric complexes, which might indicate that homotrimers are favored over heterotrimers (33). Moreover, targeting a single receptor may induce enhanced apoptosis due to a lack of competition with decoy receptors (34,35). A2780 ovarian cancer cells express DR5 and low levels of DcR2, whereas DR4 and DcR1 are not expressed on the cell surface. Blocking antibodies against DcR2 did not enhance rhTRAIL-induced apoptosis, indicating that another mechanism is responsible for the superior efficacy of rhTRAIL-DR5 over rhTRAIL. Increased binding capacities for DR5 of the mutant over rhTRAIL might be involved, although the exact kinetics of receptor binding of rhTRAIL-DR5 need to be established in further detail (11). Other death receptor mutants showed that increased affinity for the targeted receptor might play a role, although a 1:1 correlation of increased affinity with increased efficacy was not shown (10).

Loss of affinity for decoy receptors may result in toxicity to normal tissues, as decoy receptor expression was suggested to be involved in resistance of normal tissues to rhTRAIL. However, a clear correlation between decoy receptor expression and resistance to rhTRAIL has not been established (36) and monoclonal antibodies devoid of binding capacity to decoy receptors can be safely administered in clinical trials.

The rationale behind IP drug administration is to increase local drug exposure, while lowering plasma clearance (37). We show that IP rhTRAIL-administration resulted in a higher area under the curve and a reduced clearance. The high kidney activity confirms the function of the kidney as main site of rhTRAIL-clearance, which is not influenced by IP administration. Activity in most organs followed that of blood pool activity, suggesting that distribution to normal tissues was limited, which corresponds to previous studies (39).

In this study, specific tumor retention of ^{125}I-TRAIL takes place. This is demonstrated by maximal tumor activity at 60 min after IV administration, while activity in all other well perfused organs is maximal at 15 min. IP administration resulted in even higher activity in the tumor and a higher cumulative tumor to blood ratio, which shows that IP rhTRAIL administration results in higher tumor drug exposure compared to IV administration in this model. Moreover, 6 h after IP injection of a low dose of rhTRAIL, cleaved caspase 3 was detected in the superficial layers of the tumors, suggestive of rhTRAIL penetration by free-surface diffusion. This is a possible advantage of rhTRAIL and -variants over monoclonal antibodies, which show limited IP tumor penetration after IP administration (38).

Our in vitro results could be translated into in vivo efficacy in an A2780-Luc xenograft model. We used BLI to assess tumor response, since no reliable methods based on clinical features exist to accurately evaluate IP tumor proliferation over time. Our BLI data clearly visualized the differences in response to the applied treatments. Furthermore, we used bioluminescence to define an endpoint for survival. As survival of mice with IP xenografts is mostly based on assessment of clinical condition, our method ensured the definition of a uniform and objective early endpoint. The response to treatment in each arm reflected the in vitro results with high accuracy. At the end of treatment, tumor burden in mice treated with low dose cisplatin together with rhTRAIL-DR5 was lower than after cisplatin alone. These results were associated with a survival advantage, although this advantage was limited. Our study comprising only two cycles of therapy, was however not designed to primarily assess survival. Further studies in patients are warranted to define optimal dosage schedules for maximal survival benefit.

In summary, or data indicate that a receptor selective variant of rhTRAIL, rhTRAIL-DR5, displays better anti-tumor efficacy than rhTRAIL. IP administration of these drugs results in a higher tumor drug exposure than IV administration and should be considered as the optimal route of administration for IP situated tumors. Thus, the combination of rhTRAIL-DR5 together with cisplatin might offer a new strategy for more effective ovarian cancer treatment.

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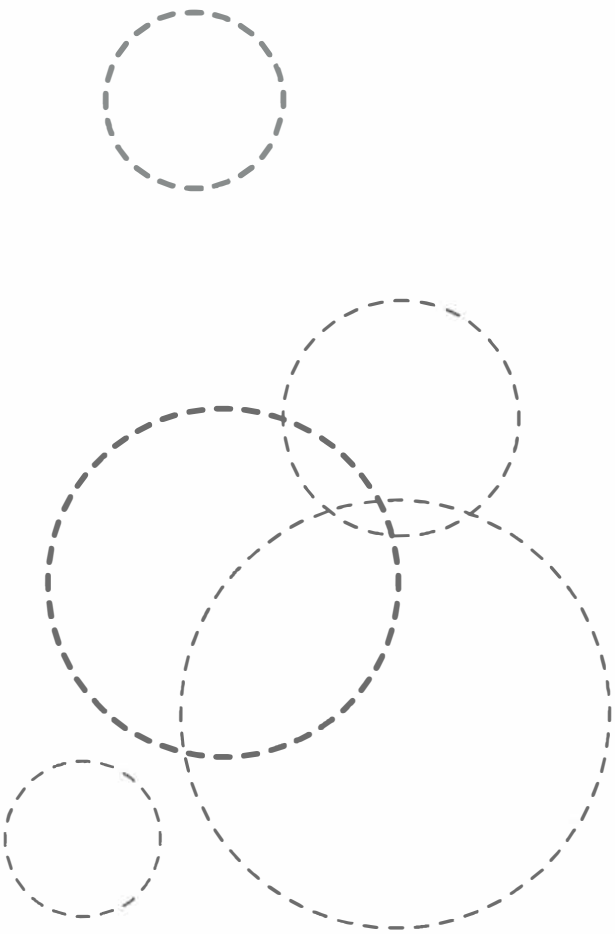

CHAPTER 9

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

SUMMARY

Ovarian cancer is among the most lethal of all malignancies in women. In The Netherlands its incidence is about 10 times lower than for breast cancer, whereas its mortality rate is three times higher (1). The main cause of this high lethality is the advanced stage of disease present in the majority of patients at the time of initial diagnosis. As the disease is generally asymptomatic early in its progression and the molecular pathogenesis is poorly understood, strategies for early detection and prevention have not yet been successful. Treatment of advanced stage ovarian cancer, consisting of combined modality treatment with surgery and chemotherapy, yields initial response rates of over 80%, with 40-60% complete responses (2). However, the vast majority of patients dies within five years after disease manifestation with drug-resistant cancers.

The possibility to escape apoptosis is defined as one of the hallmarks of carcinogenesis (3) and is a major factor involved in chemotherapy resistance (4). Apoptosis results from caspase activation brought upon through two separate pathways. Since the intrinsic pathway is commonly disrupted in cancer cells, targeting the extrinsic pathway by ligand-activation of cell-surface death receptors is regarded as a promising strategy to overcome apoptosis resistance. The recombinant human form of the TNF-family member TRAIL and other drugs directed at its agonistic receptors DR4 or DR5 induce apoptosis in a wide variety of human cancer cell lines and their xenografts, including ovarian carcinoma cells, without being toxic to normal tissues. Furthermore, combination of these agents with conventional chemotherapeutics and radiotherapy results in enhanced cytotoxicity. RhTRAIL and monoclonal antibodies targeting DR4 or DR5 are currently evaluated in early phase clinical studies. The aim of this thesis was to explore the potential of rhTRAIL and DR4 or DR5-targeting drugs as anticancer agents, with the focus on ovarian cancer.

Following a brief introduction and outline of this thesis in **chapter 1**, an overview of clinically assessed drugs directed at molecular pathways of importance in ovarian carcinogenesis was presented in **chapter 2**. This was followed by a discussion of strategies identifying new relevant targets for tumor based therapies. Substantial genetic heterogeneity of ovarian cancer underlies heterogeneity in the response to conventional treatment strategies (5). Most attempts to identify patients that are most likely to benefit from particular therapies have focused on key components of apoptotic and pro-survival pathways. These strategies have not resulted in single markers that reliably predict treatment response and survival, but alterations in these pathways were shown to be negatively associated with disease outcome (6). Numerous new drugs targeting these pathway-alterations have reached the phase of clinical testing. The literature on these strategies was discussed in this review. Modulation of the intrinsic apoptosis pathway through viral based-therapies and targeting of the extrinsic pathway with rhTRAIL and monoclonal antibodies directed at its receptors were discussed first. In addition, the attack on pro-survival pathways through monoclonal antibodies or small molecule drugs directed at members of the epidermal growth factor receptor family or components of downstream signaling pathways was discussed. Another approach, based on the inhibition of tumor angiogenesis and finally strategies designed to hit multiple targets simultaneously were considered. Some of these therapies have shown encouraging results in preclinical models and early phase clinical trials. It is however highly likely that tumor cells possess redundant pathways to maintain their homeostasis and growth potential. Thus, identification of the different deregulated oncogenic pathways most essential to these cells offers the possibility to rationally combine targeted drugs and develop new therapeutic options. This can be achieved through molecular profiling techniques, especially DNA microarrays, which enable determination of the relationships between thousands of genes and clinical phenotypes. The different DNA microarray studies conducted in ovarian cancer were also reviewed in this chapter.

In **chapter 3** the status quo on TRAIL-research is presented. Data on the physiological role of TRAIL and its receptors in tumor immune surveillance and autoimmunity were summarized. Furthermore, the many levels at which sensitivity to TRAIL can be regulated were described. Initial studies with various TRAIL-preparations raised concerns about its safety, as toxicity occurred in freshly isolated primary human hepatocytes. Yet, optimized versions proved to be nontoxic (7). These results, together with the selective anti-tumor efficacy of rhTRAIL both as single agent and in combination with conventional chemotherapeutics, boosted the development of alternative strategies targeting DR4 or DR5, such as monoclonal antibodies. Data regarding the efficacy and safety of rhTRAIL or antibody administration were summarized as well as the preliminary results of clinical studies with monoclonal antibodies. As stated in chapter 2, combinatory regimens that target different oncogenic pathways hold potential as novel anti-tumor strategies. Possible combinations of pathway-specific drugs with death receptor-based therapies were therefore discussed.

While the intrinsic and extrinsic apoptosis pathway can act as two separate routes in apoptosis induction, crosstalk exists between both pathways. The tumor suppressor gene p53 which is mutated in approximately 50% of ovarian cancers is an important player in the intrinsic pathway. A major mechanism through which p53 fulfills its function is as a transcription factor that positively or negatively regulates the expression of a large group of target genes, including constituents of the extrinsic pathway such as DR4, DR5 and caspase 8. In **chapter 4** the effect on TRAIL-sensitivity of one of the most common p53 mutations in ovarian cancer was investigated in an isogenic model of the ovarian cancer cell line A2780 stably transfected with a control vector (A2780/cmv) or with a mutant p53His273 expression vector (2780/m273). The latter was highly sensitive to rhTRAIL, while A2780/cmv was resistant to rhTRAIL. In addition, the mechanism of acquired rhTRAIL-resistance was determined in A2780/m273TR, which was obtained by exposure of A2780/m273 to increasing rhTRAIL-concentrations. The procaspase 8 protein level was higher in A2780/m273 and A2780/m273TR than in A2780/cmv. Membranous death receptor expression was similar for the three cell lines. Exposure of A2780/m273 and its resistant counterpart to rhTRAIL resulted in caspase 8 activation, while Bid and caspase 9 were not activated in A2780/3273TR. This implies that the mechanism of rhTRAIL resistance was located at the mitochondrial level, which was supported by the loss of Bax protein expression in A2780/m273TR. Genes containing simple repeat sequences, such as the Bax gene, are prone to frame-shift mutations in mismatch repair deficient cells (8). Mismatch repair deficiency results in microsatellite instability, which was confirmed in the three transfected cell lines and in A2780. Sequence analysis revealed the presence of a mutant and wild type Bax sequence in A2780/m273, whereas A2780/m273TR expressed two mutated alleles. Conversely, culturing A2780/m273TR in the absence of rhTRAIL resulted in partial rhTRAIL-sensitivity, indicating that other mechanisms besides Bax-loss contributed to acquired rhTRAIL-resistance. Conclusively, these results indicate that mutation of p53 does not influence cell surface death receptor expression and may be accompanied by increased caspase 8 protein levels, with concomitant sensitization to rhTRAIL. Acquired rhTRAIL resistance is partially due to a loss of Bax protein expression and can be reverted by withdrawal of rhTRAIL.

Ovarian cancer is in general initially a chemosensitive disease. However, upon treatment the vast majority of cancers develop chemoresistance. In vitro, conventional chemotherapeutic drugs synergize with death receptor targeted agents, causing enhanced cell death in chemosensitive and -resistant ovarian cancer cell lines. However, most of the established mechanisms involved in this synergy were determined by comparison of cell lines with different genetic background. Isogenic cell lines with differential sensitivity to conventional chemotherapy allow the detection of causal factors implicated in synergy with chemotherapeutics and in sensitivity to rhTRAIL. In **chapter 5**, the molecular determinants of rhTRAIL sensitivity were studied in an isogenic model of cisplatin resistance together with the mechanism of synergy between cisplatin and rhTRAIL. The cisplatin sensitive cell line A2780 was moderately sensitive to rhTRAIL induced apoptosis, whereas its cisplatin resistant counterpart CP70 was resistant to rhTRAIL. CP70 expressed low procaspase 8

protein, while expressing higher membranous DR5 and DcR2. Pre-exposure of CP70 to cisplatin or the proteasome inhibitor MG132 resulted in a signficant increase in caspase 8 protein levels, while caspase 8 mRNA levels slightly increased. This resulted in an enhanced apoptotic effect of cisplatin or MG132 in combination with rhTRAIL. Caspase 8 mRNA turnover and levels before and after cisplatin exposure, as well as caspase 8 protein stability did not differ between the cell lines. Therefore, a decrease in protein translation is responsible for these low caspase 8 protein levels in CP70. Downregulation of the caspase 8 inhibitor c-FLIP with siRNA decreased apoptosis induction in CP70 in response to cisplatin and rhTRAIL. This indicated that the cisplatin induced caspase 8 expression contributed to rhTRAIL sensitization and not an altered caspase 8/c-FLIP ratio. Additionally, p53 siRNA revealed that a p53-dependent increase in membrane expression of DR5 following cisplatin was not involved in sensitization to rhTRAIL. In conclusion, cisplatin enhances apoptosis induction by rhTRAIL in cisplatin resistant ovarian cancer cells without requirement of functional p53 or DR5 upregulation. An induction of caspase 8 protein expression is the key driver of sensitization to rhTRAIL, which is likely caused by lifting a blockade in caspase 8 protein translation.

Cell surface DR expression on tumors is a necessary prerequisite for death receptor targeted drugs to be effective as anticancer agents. As the extrinsic apoptosis pathway is involved in tumor immune surveillance, alterations in the expression of key proteins of the extrinsic pathway are involved in carcinogenesis and may hamper future therapies directed at death receptors. Furthermore, because response to chemotherapeutic drugs can be mediated through death ligand dependent and independent activation of caspase 8, these alterations may cause resistance to chemotherapeutic agents. To determine protein expression of key components of the extrinsic pathway in ovarian cancers, in **chapter 6**, immunohistochemical staining of Fas, FasL, TRAIL, DR4, DR5, caspase 8 and c-FLIP was studied in a tissue microarray containing 382 ovarian tumors. Protein expression profiles were correlated with clinicopathologic variables, response to chemotherapy and survival. The majority of early and late stage ovarian tumors expressed at least one death receptor, as well as the initiator caspase 8 and the anti-apoptotic protein c-FLIP. FasL expression was expressed by approximately one third of the tumors. Expression of TRAIL and Fas was less abundant and more frequently observed in early than late stage tumors. Fas expression was lower in post-chemotherapy samples, but was not associated with a response to chemotherapy, nor were other proteins. Fas and TRAIL expression were associated with a higher tumor grade and with better progression free and disease-specific survival. These results indicate that loss of Fas and TRAIL is associated with dedifferentiation and a worse prognosis in ovarian cancers. Expression of pro-apoptotic DR4, DR5, caspase 8 and the anti-apoptotic c-FLIP by the majority of cancers does not correlate with survival, but high c-FLIP expression should be taken into account for future death receptor targeted therapies in ovarian cancer.

Monoclonal antibodies directed at DR4 and DR5 and rhTRAIL are currently evaluated for their safety, tolerability and pharmacokinetics in clinical phase I-II studies. So far, results show that these agents can be administered safely and are well tolerated (9-13). A major difference between antibodies and rhTRAIL, besides differences in receptor specificity and affinity, is their pharmacokinetic profile. The profile of rhTRAIL is characterized by fast renal clearance and a short plasma half life. In contrast, long plasma half life and catabolic degradation in tissues throughout the body, with a high contribution of the liver and spleen characterises antibody pharmacokinetics. Both rhTRAIL and antibodies displayed substantial anti-tumor efficacy in numerous animal models, showing that tumors can be targeted by the different agents. Radiolabeled rhTRAIL and antibodies offer the possibility of molecular imaging in humans to get a better insight in the biodistribution and pharmacokinetics of these agents. Additionally, imaging might enable the visualization of efficient drug targeting. In **chapter 7**, the development of radiolabeled rhTRAIL and antibodies was described, along with their in vivo biodistribution in mice bearing xenografts of human tumor cell lines. Because the objective was to develop radiopharmaceuticals suitable for imaging in patients,

special attention was paid to optimization and validation of the labeling process, to stability testing and to the assurance of unchanged receptor binding capacities. RhTRAIL iodination was optimized with chloramine T as oxidizing agent. ^{125}I-rhTRAIL was stable in buffer solution and human serum for 24 hours, although free iodine and high molecular weight compounds gradually developed. Considering the short half life of rhTRAIL these impurities are acceptable. The immunoreactive fraction was 0.80, which shows that radioiodinated rhTRAIL retained excellent receptor binding capacities. The antibodies were labeled with ^{111}In after conjugation to the chelator ITC-DTPA. High stability of the ^{111}In-antibodies as determined in ammonium acetate and serum ensures optimal measurement and imaging during 1 week. Receptor binding properties of the labeled antibodies were unaltered. Biodistribution of ^{111}In-HGS-ETR1 directed at DR4 and ^{111}In-HGS-TR2J directed at DR5 was similar. Blood clearance was characterized by a slow decline between 24 hours and 168 hours after injection. Liver and spleen displayed high activity, which is consistent with known antibody metabolism. Unexpectedly, high kidney activity occurred which might be indicative of specific antigen-binding with subsequent metabolism. ^{111}In-HGS-TR2J displayed specific tumor uptake, whereas uptake of ^{111}In-HGS-ETR1 was rather low. This could be explained by necrosis, caused by a large tumor volume in the DR4 and DR5-positive tumor exposed to ^{111}In-HGS-ETR1. ^{125}I-rhTRAIL biodistribution was in accordance with previous results, showing rapid renal clearance, illustrated by very little detectable kidney activity 4 hours after administration and no signs of distribution to normal tissues. The ratio of tumor to blood activity increased in time, which is suggestive of specific uptake of rhTRAIL in the tumor. In conclusion, antibodies and rhTRAIL can be efficiently radiolabeled. The radiopharmaceuticals retain receptor binding properties and can be used to study pharmacokinetics, biodistribution and tumor targeting in humans.

Translation of in vitro results into solid clinical research protocols is facilitated by animal models which mimic the human course of disease. Intraperitonal xenografts of ovarian cancer cells accurately model advanced ovarian cancer as the disease is initially confined to the peritoneal cavity. Due to its localization, disease progression of an intraperitoneal xenograft model is however difficult to monitor. Labeling of ovarian cancer cells with luciferase reporters that are propagated along with each cell division enables to monitor their proliferation. Emitted light can be detected through tissue, which allows temporal and sequential evaluation of tumor growth in response to various therapeutic approaches in animal models. In **chapter 8**, a bioluminescent human ovarian cancer model in nude mice was described, in which the in vivo efficacy of rhTRAIL, a designed variant of rhTRAIL specifically directed at DR5 (rhTRAIL-DR5), cisplatin and the combination of cisplatin with rhTRAIL or rhTRAIL-DR5 was compared. In addition, the results were presented of a biodistribution study with ^{125}I-rhTRAIL, performed to assess the optimal administration route i.e. intravenous or intraperitoneal of rhTRAIL and its variants in ovarian cancer. In vitro, long term exposure to low dose rhTRAIL-DR5 and rhTRAIL induced a dose dependent loss of viability in A2780 cells. Pre-incubation with low dose cisplatin followed by rhTRAIL or rhTRAIL-DR5-exposure resulted in a greater loss of cell viability, with rhTRAIL-DR5 showing greater efficacy than rhTRAIL (27.8 ± 4.0 % vs. 52.6 ± 4.5 % respectively). Apoptosis induction after short term exposure to rhTRAIL-DR5 or rhTRAIL was consistent with these results. Again, rhTRAIL-DR5 was more effective than rhTRAIL with or without pre-exposure to cisplatin. Co-incubation with anti-DcR2 antibodies did not enhance apoptosis induction by rhTRAIL, showing that the differential activity of rhTRAIL-DR5 and rhTRAIL was not caused by competition of DcR2. The biodistribution of ^{125}I-rhTRAIL administered intravenously or intraperitoneally in mice bearing ovarian xenografts, showed that intraperitoneal administration of rhTRAIL increases the area under the blood activity versus time curve and leads to a higher tumor exposure compared to intravenous administration. In addition, following intraperitoneal rhTRAIL administration caspase 3 activity was detected near the surface of tumors, indicative of free surface drug penetration. Intraperitonal administration of all agents according to a two-week treatment schedule to mice with intraperitoneal xenografts of A2780-luc, confirmed the efficacy-results obtained in vitro. At the end of this schedule rhTRAIL-DR5 and cisplatin had caused a significant delay in tumor growth, represented by the bioluminescent signal.

This was even greater for the combination of cisplatin with rhTRAIL or rhTRAIL-DR5, with superior efficacy of the latter over the former. In addition, treatment with cisplatin or the combination of TRAIL-DR5 or rhTRAIL with cisplatin resulted in a survival advantage. These results indicate that intraperitoneal treatment of ovarian cancers with rhTRAIL-DR5 or rhTRAIL together with cisplatin might offer a new strategy for more effective ovarian cancer treatment, with even better perspectives for rhTRAIL-DR5 than for rhTRAIL.

DISCUSSION AND FUTURE PERSPECTIVES

Deregulated apoptosis is a major causative factor in the development and progression of cancer and in the development of resistance to widely applied cytotoxic drugs. The low 5-year survival rate of advanced stage ovarian cancer patients results from the failure of conventional therapies to eradicate all ovarian tumor tissue present at initial treatment, together with the occurrence of drug resistance. A rational approach for new anticancer strategies in ovarian cancer is therefore to shift the apoptotic imbalance towards cell death by targeting the extrinsic apoptosis pathway.

The intrinsic and extrinsic pathways are interrelated at several levels of the apoptosis cascade and converge at the level of caspase 3 activation. Therefore, chemoresistance due to defects in the intrinsic pathway may directly affect sensitivity to the death receptor pathway. P53, which is mutated in approximately half of the ovarian cancers (14), can upregulate DR4 and DR5 expression through direct transactivation of the respective genes (15,16). Additionally, caspase 8 gene expression can be induced in a p53-dependent fashion (17). In **chapter 4** we have shown that mutation of p53 does not influence the surface expression of death receptors in ovarian cancer cells and may even result in increased rhTRAIL-sensitivity, possibly due to increased caspase 8 protein expression. In **chapter 6**, expression of DR4 or DR5 in human ovarian cancer tumors was not altered with aberrant p53 status, nor was the expression of caspase 8. Other reports have also demonstrated that rhTRAIL can induce apoptosis independently of p53-status. However, not all ovarian cancer cells tested are sensitive to rhTRAIL and resistance to chemotherapy often results in cross-resistance to rhTRAIL. This was also shown in **chapter 5**, where cells with acquired cisplatin-resistance were resistant to rhTRAIL. Understanding the basis of sensitivity and resistance to rhTRAIL is important not only to anticipate on the effect of rhTRAIL and to find ways to modulate rhTRAIL-resistance, but also in future selection of patients that can benefit from rhTRAIL-treatment. Cancer cells have evolved numerous ways to evade rhTRAIL-induced apoptosis. One side of the resistance spectrum consists of changes in the expression of pro-apoptotic members of the pathway due to mutations, epigenetic gene silencing and posttranscriptional modifications. The other side embodies the upregulation of anti-apoptotic factors such as c-FLIP, Bcl-2, Bcl-XL, Mcl-1, and the inhibitors of apoptosis proteins. Each of the constituents of the spectrum may cause rhTRAIL-resistance and current data indicate that there is not one uniform mechanism accounting for resistance. The general anti-apoptotic equilibrium existing in cancer cells therefore seems to contribute to rhTRAIL-resistance as well. Importantly, synergy between rhTRAIL and conventional anti-cancer treatments was shown to overcome resistance to either treatment in cancer models of diverse origin, including ovarian cancer. The molecular mechanisms that account for this synergy cover changes in the entire resistance spectrum. In **chapter 5**, combination of cisplatin and rhTRAIL caused caspase 8 protein upregulation in CP70 resulting in the induction of apoptosis. Studies that examine whether combinational regimes are also feasible as clinical anticancer strategies are currently undertaken. First results of combinations of antibodies directed at DR4 (10) or DR5 (18) with conventional chemotherapeutics show that no additional toxicities are induced by the antibodies and that these combinations are safe to administer. These results offer good perspectives for the safety of combinatory strategies with rhTRAIL and justify progression into studies evaluating clinical efficacy.

The challenge for future therapies is to determine which patients will benefit most of which treatment. Advances in molecular profiling techniques such as DNA microarray analysis have already resulted in definition of profiles with independent prognostic significance for outcome and chemotherapy response (19-21). Recently, a strategy was defined to individualize treatment of advanced stage ovarian cancer patients (5). By combining prognostic gene expression profiles for platinum response with expression signatures of activated oncogenic pathways, identification of patients with platinum resistance that could benefit from specific targeting of deregulated pathways was achieved. Although these studies confirm the great potential of profiling strategies, they currently lack power to be generally applied in clinical stratification of ovarian cancer patients. Improving the design of these studies, as stated in **chapter 2**, will be necessary before the ambition of personalized medicine through expression profiling can become reality. A different approach that may allow assessment of sensitivity of individual tumors to various therapies is the use of precision cut tissue slicing systems (22). These systems, which are generally applied in pharmacotoxicogical studies (23), allow creation of tissue slices with a thickness of several cell layers that can be cultured ex vivo for up to 96 hours without losing viability and their original architecture. Feasibility of this system was confirmed in several human tumor models (24,25), including ovarian cancer, in which efficacy of oncolytic viruses (26) and of a monoclonal anti-DR5 antibody (27) was determined. To establish whether this simple method can enable patient selection for individualized treatment in the near future, correlative studies that compare ex vivo slice-sensitivity with clinical responses are required.

In contrast to the other ligands of the TNF-family, TRAIL shows a high degree of promiscuity as it can bind to five different receptors. The reason for this receptor redundancy is currently unknown. Whereas the existence of decoy receptors was originally considered as the main cause of insensitivity of normal tissues to TRAIL signaling, this is now disputed since decoy receptor expression also occurs in cancer cells without a clear correlation to TRAIL resistance. Agonistic DR4 and DR5, which have a high degree of structural homology (28), are co-expressed in many normal tissues and tumors (29), including ovarian cancers as shown in **chapter 6**. Recent data suggest that in spite of their structural homology, DR4 and DR5 may be functionally different. Studies with receptor-selective mutants of rhTRAIL or with DR4/DR5 blocking antibodies have shown that DR4 and DR5 can have a different contribution in rhTRAIL-induced apoptosis depending on the cell line used. Whereas colon, breast (30,31) and prostate carcinoma (32) cell lines preferentially signaled for apoptosis through DR5, lymphocytic leukemic cells (33), primary lymphoid malignancies (34) and melanoma cell lines (35) signaled through DR4. Furthermore, differential regulation of sensitivity to rhTRAIL via DR4 or DR5 was reported. In Jurkat cells, interaction of the anti apoptotic protein c-FLIP$_L$ occurred in a FADD- independent manner with DR5, but not with DR4, which inhibited DISC formation (36). DISC analysis in HeLa cells overexpressing DcR2 showed that DR5 was co-recruited with DcR2 into the DISC, whereas DR4 was not (37). These results show that we are only just beginning to appreciate the differential function of DR4 and DR5, which calls for further investigation. The concept of a preferential death receptor for apoptosis induction can have significant clinical implications for death receptor based therapies. Prior in vitro establishment of a tumor's sensitivity to DR4 or DR5 gives way for true targeted therapy. In addition, as was shown in **chapter 8**, antibodies or rhTRAIL variants exclusively directed at one death receptor can be more effective than rhTRAIL. This can be due to their design to bind the respective receptor with high affinity, but the exact mechanisms still need to be established.

Besides the role of DR4 and DR5 in TRAIL-signaling, DR5 is implicated in delay of tumorigenesis and TRAIL-independent sensitivity to chemotherapy. Inducible in vivo silencing of DR5 promoted colon xenograft growth and resulted in resistance to 5-FU (38). Apoptosis of colon carcinoma cell lines in response to 5-FU, oxaliplatin and CPT-11 was mediated through DR5 (39). **In chapter 5**, we have shown that DR5 might be involved in cisplatin induced cell death in the ovarian cancer cell line A2780. If DR5 can act as a tumor suppressor and contributes to chemosensitivity,

dysfunctional DR5 may be involved in tumor progression and resistance to chemotherapy. In concordance, downregulation of c-FLIP could increase the response to chemotherapy in colon carcinoma cell lines (39). These results support further research into the role of DR5 in TRAIL-independent signaling and stress the importance of the development of in vitro assays that can determine the functionality of death receptors in individual tumors.

Phase I studies have shown that rhTRAIL and antibodies directed at DR4 and DR5 can be administered safely and are well tolerated. Currently, several combinations of death receptor based strategies and classical chemotherapeutics or other targeted therapies are evaluated (10). These studies will be most important for the clinical use of targeted therapies in the near future, as chemotherapy will remain the mainstay in the treatment of advanced cancers. Whether TRAIL-based therapies and monoclonal antibodies will display the same efficacy remains to be established. The rapid clearance and short half life of rhTRAIL, as illustrated in **chapter 7**, might affect its applicability as anticancer agent. Continuous infusion or frequent administration may be required to sustain a plasma concentration sufficient for anti-tumor efficacy. On the other hand, the short half life may give less rise to acquired rhTRAIL-resistance than longer circulating drugs. If the pharmacological behavior of rhTRAIL narrows its therapeutic window, drug modeling techniques could be applied to alter its pharmacological profile. Conjugation to polyethylene glycol (PEG) is a well established method to delay protein clearance that has already successfully been applied for interferon-α2b in the treatment of chronic hepatitis C (40) and may also be used to delay the half life of rhTRAIL or -variants. Intraperitoneal administration can also positively affect its half life and may additionally exert local anti-tumor efficacy, as was shown in **chapter 8.** This is a possible advantage for rhTRAIL or -variants over antibodies in treatment of intraperitoneal localized tumors, since it was shown that intraperitoneal antibody administration will not have local effects, because their size severely limits tissue penetration (41). Altogether, these results show that many questions regarding the clinical application of rhTRAIL-based therapies and monoclonal antibodies are still open and ought to be addressed in future clinical trials.

The way forward in ovarian cancer is combined modality treatment that includes conventional chemotherapeutics and drugs directed at specific molecular targets. Identification of targets and thereby of patients which will benefit from therapies directed at these targets will be crucial in this approach. This is a major challenge, especially in ovarian cancer where its relative rarity asks for uniform research procedures and cooperation to obtain adequate data. Combination of chemotherapy with death receptor based strategies may prove to be one of the strategies that can achieve substantial clinical benefit. Further research into the molecular mechanisms underlying sensitivity or resistance to death receptor signaling combined with data from clinical studies may give rise to true patient tailored cancer therapy.

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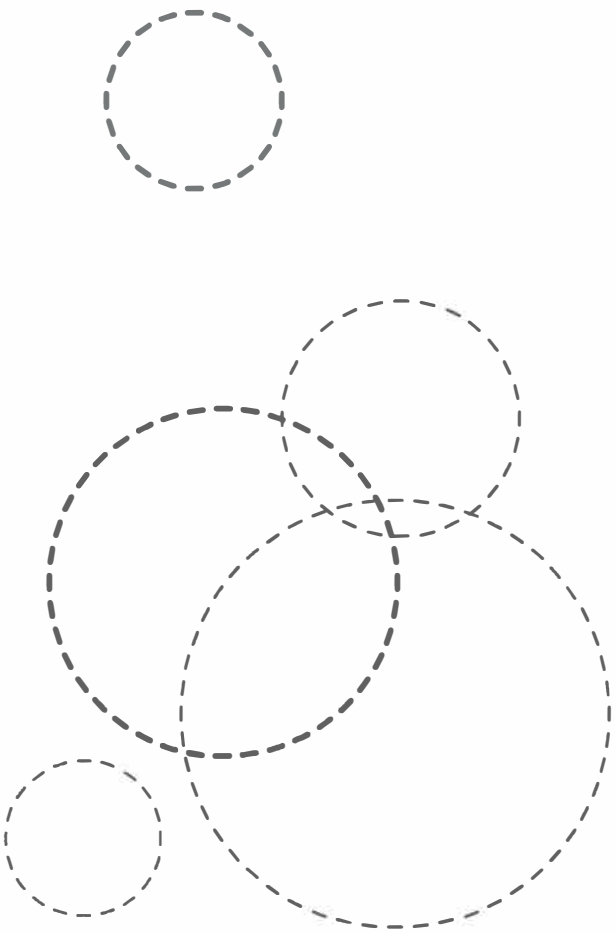

CHAPTER 10

SUMMARY IN DUTCH

NEDERLANDSE SAMENVATTING

NEDERLANDSE SAMENVATTING

Eierstokkanker is een belangrijke oorzaak van kankergerelateerde sterfte onder vrouwen. In Nederland is het aantal nieuw vastgestelde gevallen van eierstokkanker in een jaar 10 keer kleiner dan het aantal nieuwe gevallen van borstkanker, terwijl het relatieve sterftecijfer 3 keer hoger is (1). De voornaamste oorzaak van dit hoge sterftecijfer is het gevorderde stadium waarin de ziekte in de meeste gevallen wordt vastgesteld, met uitzaaiingen in de buikholte buiten het kleine bekken of elders in het lichaam. Omdat de ziekte in vroege stadia geen kenmerkende symptomen veroorzaakt en de moleculaire ontstaansmechanismen nog niet goed zijn begrepen, zijn strategieën gericht op vroege opsporing en preventie tot nu toe niet succesvol gebleken. De behandeling van eierstokkanker in een gevorderd stadium bestaat uit een operatie gevolgd door chemotherapie. Dit leidt aanvankelijk bij meer dan 80 % van de patiënten tot een substantiële verkleining van de tumor en bij 40 – 60 % van de patiënten tot het verdwijnen van de tumor, waarbij zowel klinisch als met beeldvorming geen tumormassa meer wordt vastgesteld (2). De meerderheid van de patiënten overlijdt echter binnen 5 jaar na de initiële diagnose door een recidief van tumorweefsel dat ongevoelig is geworden voor behandeling.

Chirurgie, chemotherapie en radiotherapie vormen de hoeksteen van oncologische therapie. De werking van chemo- en radiotherapie berust op het feit dat deze behandelingen ingrijpen op het proces van celdeling, met als secundair gevolg dood van de cel. Kankercellen vermenigvuldigen zich ongecontroleerd, waardoor ze relatief gevoeliger zijn voor chemotherapie dan normale cellen. Het feit dat normale cellen echter eveneens in hun proliferatie worden geremd verklaart de toxiciteit die sneldelende weefsels, zoals de bekleding van de darm, het beenmerg en het haar van chemotherapie ondervinden. Naast toxiciteit vormt al aanwezige of verworven resistentie een probleem bij de behandeling van kanker met chemotherapie. Onderzoek naar nieuwe therapieën richt zich daarom zowel op het vinden van behandelingen die resistentie kunnen voorkomen of omzeilen als op mogelijkheden om te voorkomen dat therapieën toxiciteit aan gezonde weefsels veroorzaken. Op basis van toegenomen kennis over de moleculaire ontstaansmechanismen van kanker is de laatste jaren een nieuwe klasse van geneesmiddelen ontwikkeld, de zogenaamde doelgerichte medicijnen of "targeted drugs", die specifiek ingrijpen op gedereguleerde processen in kankercellen.

Een van de karakteristieken van kanker (3) en een belangrijke factor in resistentie voor chemotherapie (4) is de mogelijkheid om te ontsnappen aan apoptose of geprogrammeerde celdood. Apoptose kan via verschillende intracellulaire routes tot stand komen. De zogenaamde intrinsieke of mitochondriële route is vaak verstoord in kankercellen, maar kan worden omzeild door activering van de extrinsieke of "death receptor" gemedieerde route. Hierbij worden apoptose-inducerende receptoren op de celmembraan geactiveerd door specifieke liganden. In het menselijk lichaam spelen deze liganden een rol in het immuunsysteem bij bijvoorbeeld de eliminatie van kankercellen of van virus geïnfecteerde cellen. De recombinante humane (rh) vorm van TRAIL en andere medicijnen gericht tegen de TRAIL receptoren DR4 en DR5 kunnen apoptose induceren in kankercellijnen van uiteenlopende origine, zonder toxisch te zijn voor normale cellen. Daarbij is gebleken dat deze medicijnen in combinatie met conventionele chemo- of radiotherapieën effectiever zijn dan de afzonderlijke therapieën. RhTRAIL en monoclonale antilichamen gericht tegen DR4 of DR5 worden momenteel in patiënten met uitbehandelde kanker getest. Het doel van dit proefschrift was de geschiktheid van rhTRAIL en middelen gericht tegen DR4 en DR5 als nieuwe medicijnen tegen kanker te evalueren, in het bijzonder tegen eierstokkanker.

Na een korte introductie en beschrijving van het proefschrift in **hoofdstuk 1** is in **hoofdstuk 2** een overzicht geschetst van klinisch geëvalueerde medicijnen gericht op moleculaire routes die van belang zijn bij het ontstaan van eierstokkanker. Dit werd gevolgd door een discussie van strategieën gericht op het identificeren van nieuwe aangrijpingspunten voor potentiële antikankermedicijnen.

Substantiële genetische heterogeniteit tussen ovarium tumoren ligt aan de basis van een heterogene reactie op de conventionele behandelingsstrategieën (5). Veel studies hebben derhalve getracht om meer homogene groepen van patiënten te identificeren die op doelgerichte therapieën kunnen reageren. Deze studies hebben zich voornamelijk gericht op enkele belangrijke componenten van apoptose- en overlevingsroutes. Onder deze componenten zijn geen individuele voorspellers gevonden voor respons op therapie of overleving, hoewel veranderingen in de routes wel negatief geassocieerd bleken met verloop van de ziekte (6). Verschillende nieuwe medicijnen gericht op deze veranderingen hebben het stadium van klinische studies bereikt. De beschikbare literatuur over deze studies is in dit hoofdstuk besproken. Als eerste werden modulatie van de ontregelde intrinsieke apoptose route door middel van therapieën met gemodificeerde virussen en activering van de extrinsieke route door rhTRAIL en monoclonale antilichamen gericht tegen TRAIL receptoren besproken. Vervolgens werden medicijnen gericht tegen abnormaal geactiveerde overlevingsroutes in kankercellen bediscussieerd. Verder kwamen strategieën gericht op tegengaan van vaatgroei in kankercellen en op simultaan aanpakken van verschillende aberrante processen aan bod. Sommige van deze geneesmiddelen hebben bemoedigende resultaten geboekt in preklinische modellen en vroege fase klinische studies. Omdat het echter zeer waarschijnlijk is dat in tumorcellen meerdere routes tegelijkertijd ontregeld zijn, is het van belang te identificeren welke van de verschillende oncogene routes essentieel zijn voor de overleving van een kankercel. Hierdoor kunnen doelgerichte medicijnen weloverwogen gecombineerd en nieuwe therapieën ontwikkeld worden. Dit kan bereikt worden door middel van technieken die een moleculair profiel van kankercellen kunnen maken. Een DNA "microarray" of DNA-chip is in staat om genen te identificeren die in kankercellen actiever zijn dan in normale cellen. De verschillende DNA microarray studies die op eierstoktumoren zijn uitgevoerd werden eveneens in dit hoofdstuk beschreven.

In **hoofdstuk 3** is de huidige stand van zaken in het TRAIL-onderzoek gepresenteerd. De beschikbare gegevens over de fysiologische rol van TRAIL en zijn receptoren in eliminatie van kankercellen en de preventie van auto-immuunziekten werden samengevat. Verder werd een overzicht geschetst van de verschillende niveaus waarop gevoeligheid van een cel voor TRAIL kan worden geregeld.

De eerste studies waarin verschillende vormen van rhTRAIL als potentieel kankermedicijn werden geëvalueerd riepen vragen op over de veiligheid van rhTRAIL, omdat in vers geïsoleerde menselijke levercellen toxiciteit optrad. Nieuwe geoptimaliseerde rhTRAIL-vormen bleken echter niet schadelijk (7). Mede door de selectieve antitumoreffectiviteit van rhTRAIL als mono- en combinatietherapie met conventionele middelen, resulteerde dit in ontwikkeling van alternatieve strategieën gericht tegen DR4 en DR5, zoals monoclonale antilichamen. De beschikbare gegevens over de effectiviteit en veiligheid van rhTRAIL en antilichamen werden samengevat, evenals de voorlopige resultaten van klinische studies met monoclonale antilichamen. Zoals in hoofdstuk 2 werd geschetst, vormen combinaties tussen doelgerichte therapieën gericht op verschillende oncogene routes veelbelovende antitumor strategieën. De mogelijke combinaties met medicijnen gericht tegen TRAIL receptoren werden bediscussieerd.

Hoewel apoptose afzonderlijk via de intrinsieke en extrinsieke route tot stand kan worden gebracht, bestaan niveaus waarop beide routes elkaar beïnvloeden. Het tumorsuppressorgen p53 dat in 50% van de eierstokkankers is gemuteerd, speelt een belangrijke rol in de functionaliteit van de intrinsieke route. Een belangrijk mechanisme waardoor P53 zijn rol als tumor suppressor uitoefent, is als transcriptiefactor die positief of negatief de expressie van een grote groep van doelwitgenen kan beïnvloeden, waaronder ook componenten van de extrinsieke route zoals DR4, DR5 en caspase 8. In **hoofdstuk 4** is het effect op rhTRAIL-gevoeligheid onderzocht van een van de meest in eierstoktumoren voorkomende mutaties in p53. Hiervoor werd een isogeen model gebruikt, bestaande uit de eierstokkankercellijn A2780 stabiel getransfecteerd met een controle vector (A2780/cmv) of met een expressie vector van mutant p53 (A2780/m273). De laatste was sterk gevoelig voor rhTRAIL, terwijl A2780/cmv resistent was voor rhTRAIL. Ook werd het mechanisme van verworven rhTRAIL-resistentie in dit model onderzocht door A2780/m273 bloot te stellen aan oplopende concentraties van rhTRAIL, resulterend in de TRAIL-resistente cellijn A2780/m273TR.

Tussen de cellijnen werd geen verschil in TRAIL receptor expressie op de celmembraan vastgesteld, terwijl het eiwit caspase 8 hoger tot expressie kwam in de p53-gemuteerde A2780/m273 en A2780/m273TR. Blootstelling van A2780/m273 en A2780/m273TR aan rhTRAIL leidde in beide cellijnen tot caspase 8 activering, terwijl de eiwitten Bid en caspase 9, die belangrijk zijn in de intrinsieke route niet werden geactiveerd. Dit impliceert dat rhTRAIL resistentie in A2780/m273TR gelokaliseerd is op het niveau van de mitochondrieën, wat ondersteund werd door verlies van expressie van het eiwit Bax. Genen zoals Bax die herhalende sequenties in hun DNA bevatten, zijn gevoelig voor mutaties wanneer ze voorkomen in "mismatch repair" deficiënte cellen, die niet in staat zijn kleine foutjes in hun DNA te herstellen (8). Dit leidt tot zogenaamde microsatelliet instabiliteit (MSI), wat werd vastgesteld in A2780 en de drie getransfecteerde cellijnen. Sequentie analyse toonde de aanwezigheid van een mutant en gemuteerd Bax allel in A2780/m273, terwijl A2780/m273TR twee gemuteerde Bax allelen bezat. Hoewel deze Bax-mutatie belangrijk is voor verworven TRAIL-resistentie van A2780/m273TR, bleken andere mechanismen ook nog een rol te spelen. Dit werd aangetoond door A2780/m273TR te kweken zonder toevoeging van rhTRAIL, wat leidde tot het herkrijgen van gedeeltelijke gevoeligheid voor rhTRAIL. Concluderend wijzen deze resultaten erop dat mutatie van p53 niet de expressie van TRAIL receptoren beïnvloedt en gepaard kan gaan met een hogere eiwit expressie van caspase 8, resulterend in verhoogde gevoeligheid voor rhTRAIL. Verlies van het eiwit Bax door een genmutatie leidt tot verworven rhTRAIL-resistentie, hoewel die weer kan afnemen door rhTRAIL niet meer continu toe te dienen.

Hoewel eierstokkanker beschouwd kan worden als een ziekte die in eerste instantie gevoelig is voor conventionele chemotherapie zoals cisplatine, worden de meeste tumoren in de loop van de behandeling resistent. In eerder onderzoek met zowel chemotherapiegevoelige als -resistente cellijnen resulteerden combinaties van conventionele chemotherapie met middelen gericht tegen TRAIL receptoren in een synergistisch effect. Om de mechanismen te bepalen die betrokken zijn bij deze synergie werden echter vaak cellijnen met elkaar vergelijken die niet alleen een verschillende gevoeligheid voor rhTRAIL of chemotherapie, maar ook een verschillende genetische samenstelling hadden. Isogene cellijnen die van elkaar zijn afgeleid, met een differentiële gevoeligheid voor chemotherapie laten het toe om te bepalen welke mechanismen verantwoordelijk zijn voor synergie en welke bepalend zijn voor rhTRAIL-gevoeligheid. In **hoofdstuk 5** zijn in de cisplatine gevoelige cellijn A2780 en de cisplatine-resistente cellijn A2780/CP70 de moleculaire determinanten van rhTRAIL gevoeligheid evenals het mechanisme van synergie tussen rhTRAIL en cisplatine onderzocht. A2780 was matig gevoelig voor apoptose-inductie door rhTRAIL, terwijl A2780/CP70 resistent was. CP70 bracht caspase 8 eiwit laag tot expressie, terwijl het een hogere membraanexpressie van de TRAIL receptoren DR5 en DcR2 had. Apoptose-inductie door rhTRAIL kwam wel tot stand in CP70 door voor te behandelen met cisplatine of de proteasoomremmer MG132. Deze voorbehandeling leidde tot substantiële verhoging van caspase 8 eiwit, terwijl

het caspase 8 mRNA-niveau slechts lichtjes toenam. De snelheid waarmee caspase 8 mRNA werd omgezet en de mRNA hoeveelheden voor en na cisplatine voorbehandeling verschilden niet tussen beide cellijnen. Hetzelfde gold voor de stabiliteit van het caspase 8 eiwit. Hieruit kan geconcludeerd worden dat de lagere caspase 8 eiwit hoeveelheden in CP70 worden veroorzaakt door een verminderde vorming van caspase 8 eiwit uit mRNA. Verder leidde verlaging van de endogene caspase-8 remmer c-FLIP door middel van een techniek genaamd "short interfering RNA" (siRNA) niet tot meer apoptose in CP70, maar zelfs tot minder. Dit betekent dat na cispl atinevoorbehandeling enkel de toegenomen hoeveelheid caspase 8 eiwit in CP70 van belang is voor toegenomen rhTRAIL-gevoeligheid en niet de veranderde verhouding tussen caspase 8 en c-FLIP, zoals in eerdere studies wel is aangetoond. Voorts werd met siRNA tegen p53 aangetoond dat een p53-gemedieerde verhoging van de DR5 membraanexpressie na cisplatine blootstelling niet bijdroeg aan sensibilisering van CP70 voor rhTRAIL. Samenvattend kan gesteld worden dat cisplatine voorbehandeling van cisplatine-resistente eierstokkankercellen apoptose-inductie door rhTRAIL versterkt, zonder dat daarbij functioneel p53 of verhoging van de membraanexpressie van DR5 vereist is. Verhoging van de caspase 8 eiwitexpressie, waarschijnlijk geïnduceerd door opheffen van een blokkade op caspase 8 eiwitvorming, lijkt hierbij het mechanisme verantwoordelijk voor sensibilisering te zijn.

Opdat doelgerichte middelen tegen TRAIL-receptoren effectief kunnen zijn, is het een vereiste dat tumoren deze receptoren op hun celmembraan tot expressie brengen. Omdat de extrinsieke apoptose-route gebruikt wordt door het immuunsysteem om tumoren tegen te gaan, kunnen veranderingen in de expressie van essentiële eiwitten in deze route betrokken zijn bij tumor progressie. Voorts kunnen dergelijke veranderingen het succes van toekomstige behandelingen met doelgerichte medicijnen beperken. Omdat "death ligand" afhankelijke en onafhankelijke caspase 8 activering verder een rol kan spelen bij een respons op chemotherapie, kunnen deze veranderingen bijdragen aan resistentie voor chemotherapie. In **hoofdstuk 6** is de eiwitexpressie van essentiële eiwitten uit de extrinsieke apoptose route met behulp van immunohistochemie onderzocht in ovariumtumoren. Door middel van de tissue microarray techniek werd de expressie bepaald van Fas, FasL, TRAIL, DR4, DR5, caspase 8 en c-FLIP in 382 tumoren, verkregen van patiënten die vanaf 1985 in het UMCG en samenwerkende ziekenhuizen behandeld zijn voor eierstokkanker. De expressie profielen werden gecorreleerd aan patiënt- en tumorkarakteristieken, respons op chemotherapie en overleving.

De meerderheid van de vroeg en laat stadium tumoren bracht tenminste één death receptor tot expressie, zowel als caspase 8 en zijn anti-apoptotische homoloog c-FLIP. FasL werd door een derde van de tumoren tot expressie gebracht. TRAIL en Fas expressie was minder veelvoorkomend, maar kwam vaker voor bij vroeg dan laat stadium tumoren. Fas expressie was lager in tumoren verkregen na chemotherapie, hoewel het niet geassocieerd was met een respons op chemotherapie. Dat was ook niet het geval voor de andere eiwitten. Fas en TRAIL expressie waren geassocieerd met een betere tumor differentiatiegraad en met een betere overleving en ziektevrije overleving. Deze resultaten laten zien dat verlies van Fas en TRAIL expressie geassocieerd is met dedifferentiatie en een slechtere prognose in eierstoktumoren. Expressie van de pro-apoptotische eiwitten DR4, DR5, caspase 8 en het anti-apoptotische eiwit c-FLIP is niet geassocieerd met overleving, hoewel de hoge c-FLIP expressie een beperkende factor kan zijn voor succes van toekomstige therapieën gericht op de extrinsieke apoptose route.

Monoclonale antilichamen die DR4 en DR5 kunnen activeren en rhTRAIL worden momenteel in vroeg-klinische studies geëvalueerd op veiligheid, bijwerkingen en farmacokinetiek. Tot nu toe kunnen deze medicijnen veilig en zonder grote bijwerkingen worden toegediend (9-13). Een groot verschil tussen antilichamen en rhTRAIL is hun farmacokinetisch gedrag. RhTRAIL wordt snel via de nieren geklaard en heeft een korte plasma-halfwaarde tijd, terwijl de antilichamen een lange plasma-halfwaarde tijd hebben en worden afgebroken in verschillende weefsels zoals de lever en milt. Ondanks deze verschillen zijn zowel rhTRAIL als de antilichamen effectief gebleken in

verschillende diermodellen. Beschikbaarheid van radioactief gelabeld rhTRAIL en antilichamen biedt de mogelijkheid om met behulp van moleculaire beeldvorming inzicht te krijgen in de biodistributie en farmacokinetiek in mensen. Bovendien kan moleculaire beeldvorming zichtbaar maken of het geneesmiddel daadwerkelijk zijn doel bereikt. In **hoofdstuk 7** is de ontwikkeling van radioactief gelabeld rhTRAIL en antilichamen beschreven, evenals het farmacologische gedrag van deze middelen in een diermodel. Omdat het de bedoeling is om met deze radiofarmaca beeldvorming in patiënten uit te voeren, is speciale aandacht besteed aan optimalisatie en validatie van het labelingsproces, stabiliteitsbepalingen en bestudering van de receptorbinding.

Voor de labeling van rhTRAIL met radioactief jodide (^{125}I) is gebruik gemaakt van chloramine T als oxidator. ^{125}I-rhTRAIL was gedurende 24 uur stabiel in een bufferoplossing en humaan serum, hoewel vrij jodide en aggregaten zich geleidelijk aan ontwikkelden. Gelet op de korte halfwaardetijd van rhTRAIL zijn deze verontreinigingen acceptabel. De immunoreactieve fractie was 0,80, wat betekent dat radioactief gelabeld rhTRAIL een uitstekende receptorbindingscapaciteit heeft behouden. De antilichamen werden gelabeld met ^{111}indium (^{111}In). Omdat rechtstreekse koppeling van ^{111}In aan de antichamen niet mogelijk is, werd gebruik gemaakt van de chelator ITC-DTPA. De goede stabiliteit van de ^{111}In-antlichamen, zoals aangetoond in ammonium acetaat en serum, verzekert gebruik voor beeldvorming gedurende een week na injectie. De bindingscapaciteit van de antilichamen aan de TRAIL receptoren was niet gewijzigd na labeling.

De biodistributie van de radiofarmaca werd bepaald in muizen met xenografts van de humane colontumorcellijn SW948 (met expressie van DR4 en DR5) of van de humane borstkankercellijn SKBR3 (met lage DR4 en DR5 expressie). De biodistributie van ^{111}In-HGS-ETR1, gericht tegen DR4 en ^{111}In-HGS-TR2J tegen DR5 vertoonde hetzelfde patroon. De radioactiviteit in het bloed nam tussen 24 en 168 uur na injectie geleidelijk af. In de lever en milt werden hoge signalen gedetecteerd, wat in overeenstemming is met gekend metabolisme van antilichamen. Onverwachts werd hoge activiteit in de nieren gevonden, wat kan duiden op specifieke binding van de antilichamen in de nieren met aansluitend metabolisme. ^{111}In-HGS-TR2J liet specifieke ophoping zien in de receptor positieve SW948 tumor, terwijl ophoping van ^{111}In-HGS-ETR1 betrekkelijk laag was. Dit kon verklaard worden door weefselnecrose in de receptor positieve tumoren blootgesteld aan ^{111}In-HGS-ETR1 veroorzaakt door een groot tumorvolume. Biodistributie van ^{125}I-rhTRAIL was in overeenstemming met eerdere studies. Extreem snelle klaring via de nieren werd bevestigd doordat 4 uur na injectie al geen activiteit meer in de nieren werd gezien en geen verdeling over andere weefsels werd aangetoond. De tumor-bloed ratio nam toe in de tijd, wat suggestief is voor specifieke tumor opname van ^{125}I-rhTRAIL.

Concluderend kan gesteld worden dat antilichamen en TRAIL efficiënt radioactief gelabeld kunnen worden. De radiofarmaca behouden hun receptorbindingscapaciteit en kunnen gebruikt worden om in patiënten farmacokinetiek, biodistributie en beeldvorming van tumoren te onderzoeken.

Vertaling van in vitro onderzoeksresultaten naar een gedegen opzet van klinische studies wordt vereenvoudigd door diermodellen die het humane ziektebeloop benaderen. Xenografts van eierstokkankercellijnen die in de buikholte groeien vormen een goed model voor laat stadium ovariumkanker vanwege de intraperitoneale uitbreiding die daarbij altijd is opgetreden. Overeenkomstig de humane situatie is het betrouwbaar opvolgen van groei van deze xenografts echter moeilijk vanwege hun lokalisatie. Door gebruik te maken van bioluminescentie is het mogelijk intraperitoneale tumorgroei in proefdieren op een niet-invasieve manier te meten. Hierbij wordt in het DNA van ovariumcarcinoom cellijnen het luciferase-gen ingebracht dat door vuurvliegjes wordt gebruikt om licht uit te zenden. Omdat dit gen stabiel in het DNA is geïncorporeerd, zullen na celdeling nieuwe cellen dit gen ook met zich meedragen. Licht uitgestraald door xenografts gevormd uit luciferase-dragende cellen gaat door weefsel en kan gemeten worden. Dit laat het toe om in de tijd tumorgroei vast te stellen en het effect van verschillende behandelingen te evalueren. In **hoofdstuk 8** werd een bioluminescent humaan eierstokkankermodel in naakte

muizen beschreven, waarin de effectiviteit van rhTRAIL, een variant van rhTRAIL die gemaakt is om enkel op DR5 te binden (rhTRAIL-DR5), cisplatine en de combinatie van cisplatine met rhTRAIL of rhTRAIL-DR5 is vergeleken. Eveneens werden de resultaten van een biodistributie-studie met ^{125}I-rhTRAIL beschreven, waarin werd bepaald wat de beste toedieningsweg zou zijn voor rhTRAIL of rhTRAIL-DR5 in ovariumkanker. Intraperitoneale rhTRAIL toediening gaf een betere biodistributie dan intraveneuze toediening en leidde tot een hogere tumorblootstelling. Intraperitoneale toediening volgens een therapieschema van 2 weken liet een grotere antitumor effectiviteit zien voor rhTRAIL-DR5 dan rhTRAIL. Aan het einde van het therapieschema gaf de combinatie van rhTRAIL-DR5 met cisplatine de grootste vertraging van tumorgroei, zoals bepaald met bioluminescentie. Een overlevingsvoordeel trad op voor combinatietherapie van cisplatine met rhTRAIL-DR5 of rhTRAIL. Deze resultaten laten zien dat intraperitoneale toediening van rhTRAIL-DR5 of rhTRAIL in combinatie met cisplatine een mogelijke nieuwe strategie is voor een meer effectieve behandeling van eierstokkanker, waarbij op grond van preklinisch onderzoek rhTRAIL-DR5 betere perspectieven biedt dan rhTRAIL.

CONCLUSIE EN TOEKOMSTPERSPECTIEVEN

De toekomst in de behandeling van eierstokkanker ligt in de combinatie van conventionele chemotherapie met geneesmiddelen gericht tegen specifieke moleculaire doelwitten. Cruciaal in deze benadering is identificatie van zowel deze doelwitten als van patiënten voor wie deze gerichte therapieën effectief kunnen zijn. Dit is vooral in eierstokkanker een grote uitdaging, omdat de relatieve lage incidentie vraagt om uniforme onderzoeksprotocollen en samenwerking om voldoende gegevens te genereren. Combinatie van chemotherapie met strategieën gericht tegen death receptoren kan zich mogelijk bewijzen als een van de strategieën die substantiële winst kan boeken in de behandeling van eierstokkanker. Verder in vitro onderzoek naar de moleculaire mechanismen die ten grondslag liggen aan gevoeligheid of resistentie voor death receptor signalering, gecombineerd met data uit klinische studies zal dan aanleiding kunnen geven tot een behandelingsstrategie die op de tumorkarakteristieken van de individuele patiënt is aangepast.

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"Eigentlich weiß man nur, wenn man wenig weiß. Mit dem Wissen wächst der Zweifel." *Goethe.*

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Alle (ex)-collegae van HET LAB, Alessandra, Agnes, Alice, Anne, Annemieke, Annechien, Arne, Bart, Baukje-Nynke, Bea, Bodvaël, Caroline, Coby, Corina, Derk-Jan, Deva, Diana, Dianne B, Dianne H, Dorenda, Edmond, Eli, Esther de H, Esther N, Filip, Fiona, Gerry, Gert-Jan, Gitte, Harry, Haukeliene, Hetty, Hermien, Ineke, Inge, Ingrid, Janet, Janine, Jasper, Jelmer, Jeroen, John, Karlijn, Klaske, Maaike, Margaretha, Martine, Nathalie, Nynke L, Neeltje, Nynke Z, Patrick, Paul, Pauline, Phuong, Roelof, Rudolf, Sander, Shinta, Steven, Stijn, Sophie, Wendy, Wouter, Wytske, Yang-Nan, Xiang-Yi, studenten en collegae van de Hematologie en Kinderoncologie, bedankt voor alle hulp, steun en gezelligheid op en naast het werk. Gretha en Bianca, jullie zijn de motor die de administratie draaiende doen houden, bedankt daarvoor.

Tijdens mijn promotietraject heb ik twee studenten mogen begeleiden, Neeltje Kooi en Arne van der Bilt. Neeltje, samen een project uitvoeren kostte een investering van ons beiden die zich zeker heeft uitbetaald. Ik heb je zien groeien in de maanden dat we hebben samengewerkt en ik hoop dat je met plezier terugkijkt op 10 leerzame en leuke maanden. Veel succes met je verdere carrière! Arne, blakend van ambitie kwam je via Derk-Jan bij mij terecht. Hoe je het onderzoek in je drukke agenda weet te proppen is mij soms een raadsel, maar het lukt je. Bedankt voor je inzet, enthousiasme en gezelligheid, ik hoop dat je je promotietraject met evenveel geestdrift zult uitvoeren als je mij met het mijne hebt geholpen.

Buiten HET LAB heb ik mij nog op plaatsen begeven waar ik hulp heb ontvangen van een aantal mensen, die ik hierbij wil bedanken. Geert en Henk voor alle hulp bij het Facsen. Hans, Chris en Hugo van de radiofarmacie voor het uitvoeren van en ondersteuning bij de labelingen. Robbert en Marieke van de farmaceutische biologie voor de productie van rhTRAIL-DR5. Henk van der Molen van de medische biologie voor de iv-injecties van hoofdstuk 8 en uiteraard alle medewerkers van het Centraal Dieren Laboratorium (CDL) die mij tijdens mijn project hebben geholpen.

Gelukkig bestaat er ook (soms) een leven naast het onderzoek. Beginnend in "Soest" op een vrijdag dat ik eens niet in de trein naar Brussel zat. Borrelaars Bart, Caroline, Derk-Jan, Gitte, Hilde, John, Mirjam, Patrick, Pauline en Stijn: met een drankje en sateetje erbij lijkt alles anders. Many thanks! Mirjam, echt sportief zullen we nooit worden, maar gezellig was het altijd wel. Veel succes met jouw laatste loodjes.

Mijn vrienden van "vroeger" Keetje, Marianne, Hilde, Hanine en Olga: onze vriendschap is diepgeworteld, elkaar tijden niet zien of spreken maakt niet uit. Jullie plaatsen me altijd weer met beide benen op de grond en dat mogen jullie wat mij betreft nog heel lang blijven doen. Eds, vanaf ons tweede jaar in Antwerpen is samen even een biertje doen nooit even. Nu we weer bij elkaar in de buurt wonen, moeten we dat snel weer even doen. Carla en David, menig toneelavondje werd gevolgd door een goed gesprek. Jullie deur staat altijd open, waarna de Mojito's hun gelijke niet kennen!

Caroline en Maaike, bedankt dat jullie als paranimfen aan mijn zijde zullen staan. Lieve Caroline, de stevige basis van onze vriendschap is gelegd in het eerste deel van mijn promotietraject. Ik ben heel blij dat je er weer bent, nu ik aan het eind ben gekomen. Lieve Maaike, in een Antwerpse collegebank zuchtend boven een wiskundetoets werden wij onafscheidelijk. Ik ben je met aanzienlijke vertraging naar Groningen gevolgd, waar we onze vriendschap weer hebben opgepakt. Wordt vervolgd, waar dan ook!

Familie Westerling en aanhang, sinds mijn vestiging in Groningen hebben we de banden stevig aangehaald. Wat fijn dat jullie er zijn! De wijnavonden zijn een goede traditie geworden die behouden moet blijven. Lieve Fok en Eddie: jullie hebben een speciaal plekje in mijn hart ingenomen. Fokje, vanaf ons eerste wijntje samen (en de velen die erop volgden) ben je heel veel voor me gaan betekenen. Dat zal zo blijven!

Lieve Thomas, je enthousiasme over jouw doctoraat staat mede aan de basis van het mijne. Jouw liefde, steun en vertrouwen zijn er vervolgens in verwerkt. Gelet op onze (geografische) ambities is het niet evident dat we nog samen zijn. Ik ben gelukkig dat ik je nog steeds mijn lief mag noemen. Quand on n'a que l'amour, mon amour toi et moi, nous aurons dans nos mains le monde entier (Jacques Brel).

Lieve Arjen, Bro, in de loop der jaren is onze band alleen maar hechter geworden. Hoe ver we ook van elkaar verwijderd wonen, je bent altijd bij me.

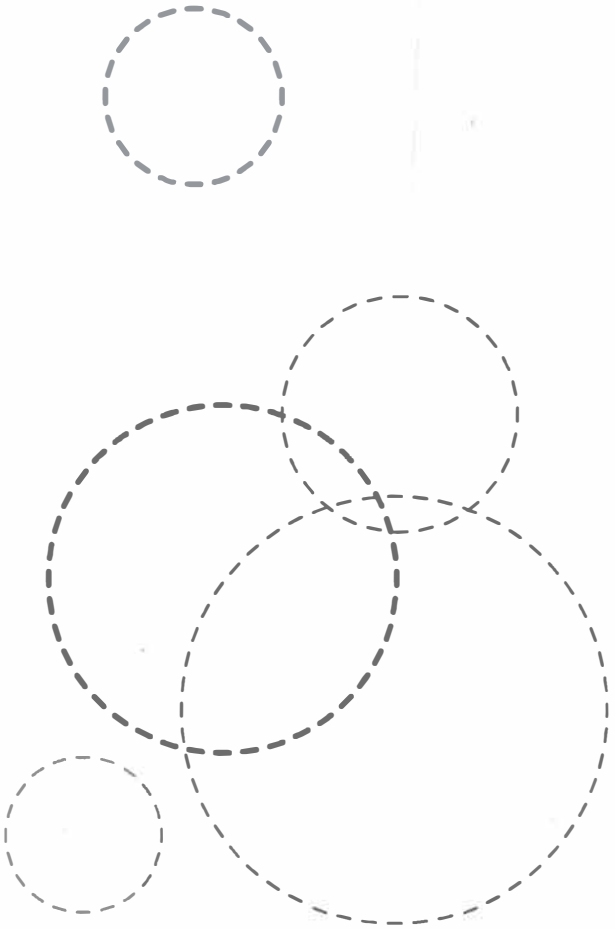
Lieve pap en mam, uit jullie warme nest ben ik op mijn achttiende ver weggevlogen. Als jullie me niet de mogelijkheid hadden geboden en me niet hadden gestimuleerd mijn dromen te volgen, vertrouwen in mezelf te hebben en door te zetten, was ik misschien dichtbij weer geland. Jullie zijn de ware grenzenverleggers van mijn bestaan. Bedankt voor alles, ik houd van jullie.

CURRICULUM VITAE

Evelien Duiker werd op 26 januari 1977 in Leeuwarden geboren. Het grootste deel van haar jeugd bracht zij door in Goutum, een dorp onder de rook van Leeuwarden. In 1995 behaalde zij haar Gymnasium diploma aan het Stedelijk Gymnasium te Leeuwarden. In verband met uitloting voor de Nederlandse studie Geneeskunde startte zij datzelfde jaar met de eerste kandidatuur Geneeskunde in Antwerpen. In 1998 behaalde zij haar kandidatuursdiploma met onderscheiding, waarna zij doorstroomde naar de doctoraatsjaren aan de Universiteit Antwerpen. Het tweede doctoraatsjaar volgde zij via het Erasmusprogramma in zijn geheel aan de Universität Wien, in Wenen - Oostenrijk. In het derde doctoraatsjaar deed zij haar wettelijke stages (co-schappen) pediatrie en gynaecologie in het Charité Krankenhaus, Campus Mitte en Virchow, te Berlijn - Duitsland. In 2002 slaagde zij met onderscheiding voor het artsexamen, waarna ze naar Amsterdam verhuisde en begon als AGNIO verloskunde in het Flevoziekenhuis in Almere. Begin 2003 ving zij in Groningen aan met haar promotietraject onder begeleiding van Prof.dr. E.G.E. de Vries, Prof.dr. A.G.J.van der Zee en dr. S.de Jong.

Op 1 oktober 2007 begon zij met de opleiding Obstetrie en Gynaecologie in het cluster Leiden. Momenteel is zij werkzaam in het Leids Universitair Medisch Centrum.

Evelien woont samen met Thomas van Schaftinghen in Den Haag. Samen delen zij de passie voor zeilen. Daarnaast is Evelien een fanatieke thuiskok en wijnliefhebster.



APPENDIX

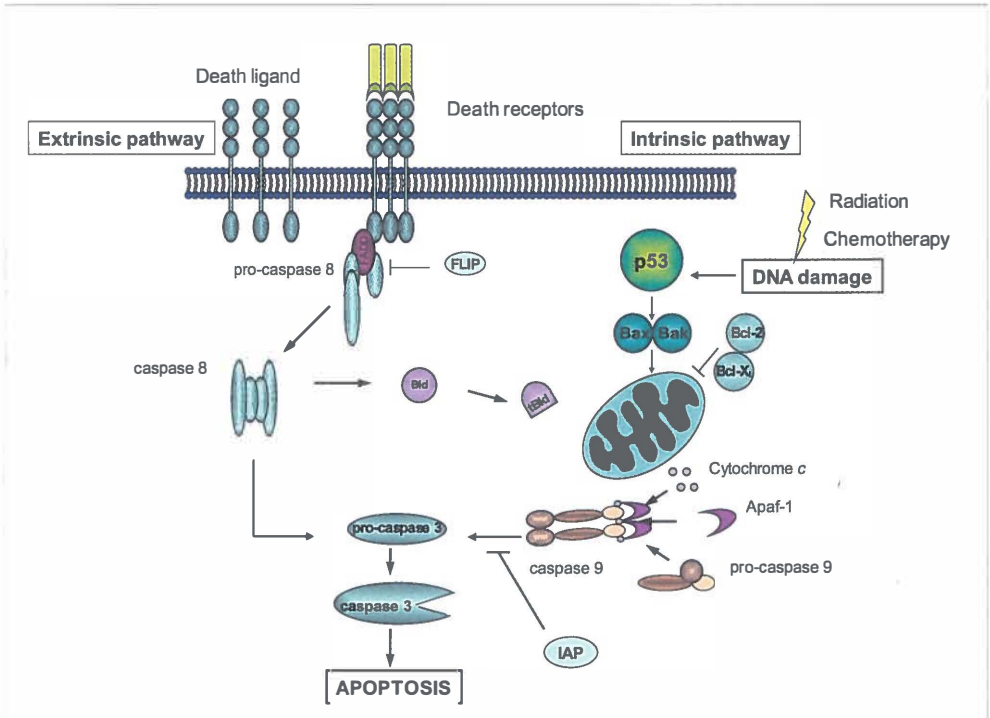


Figure 1. Apoptotic pathway. The death receptor-initiated apoptosis pathway is referred to as the extrinsic apoptosis pathway. The death ligand, TRAIL in this example, binds as a homotrimer to DR4 and DR5, which results in homo-trimeriziation of the receptors. This leads to the assembly of a death-inducing signalling complex (DISC). At the DISC, the adaptor protein FADD (Fas- associated death domain) acts as a bridge between the death receptor complex and the initiator caspase 8. Upon recruitment by FADD, caspase 8 will be activated by autocleavage and activate downstream effector caspases such as caspase 3, 6 and 7. Cross-talk exists between the extrinsic pathway and the intrinsic or mitochondria-initiated apoptosis pathway through Bid, a BH3-only protein member of the Bcl-2 gene superfamily. Activated caspase 8 will cleave Bid, which then translocates to the mitochondria to induce cytochrome c release, forming the connection between the extrinsic and intrinsic pathway. The intrinsic pathway triggers apoptosis after DNA damage, hypoxia, starvation and many other kinds of severe cellular stress. When the intrinsic pathway is activated, pro-apoptotic members of the Bcl-2-gene family translocate to the mitochondria, causing subsequent release of cytochrome c and other mitochondrial factors into the cytosol. In the cytosol, cytochrome c binds in the presence of deoxyadenosine triphosphate (dATP) the adaptor protein Apaf-1 and pro-caspase 9, forming the apoptosome-signaling complex in which caspase 9 is activated and can activate on its turn the effector caspases 3, 6 and 7.

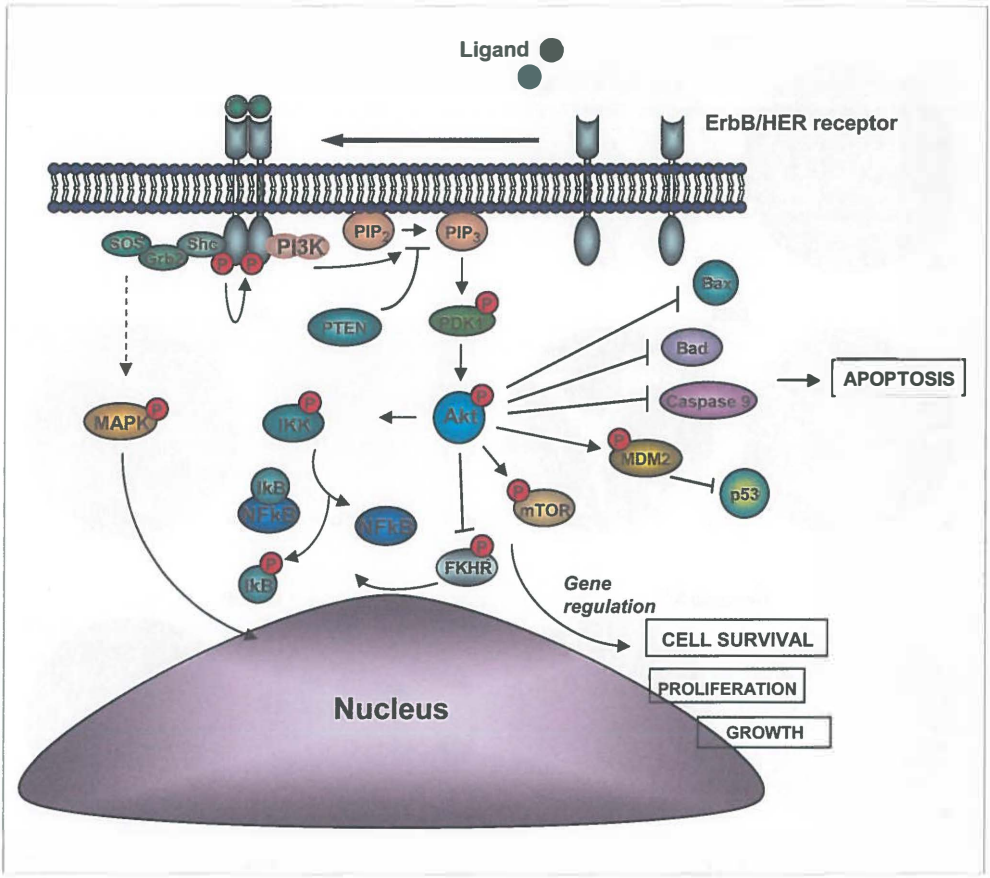


Figure 2. Prosurvival pathways; Erb/HER and PI3K-AKT. Dimerization between two growth factor receptors causes activation of the receptor tyrosine kinases (RTKs) with subsequent autophosphorylation. This leads to activation of the MAP-kinase pathway and of the PI3K-AKT pathway. Active PI3K converts phosphatidylinositol-4,5-biphosphate (PIP_2) to the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3), which is able to recruit plecktrin-homology (PH) domain containing proteins, such as AKT to the cell membrane with subsequent activation of AKT. The tumor suppressor PTEN is responsible for converting the second messenger PIP_3 to its inactive state PIP_2. Active AKT is able to control essential cellular processes, such as apoptosis, survival, proliferation and growth by phosphorylating downstream proteins. P53 degradation is increased by phosphorylation of MDM2. AKT promotes gene expression of several prosurvival genes via nuclear factor kappa B (NFkB) and inhibits gene expression of several proapoptotic genes by preventing forkhead-related transcription factors (FKHR) to enter the nucleus. Furthermore, AKT can exert effects on cell metabolism and growth through activation of the protein kinase "mammalian target of rapamycin" (mTOR). AKT can also stimulate cell proliferation by effects on mediators of the cell cycle. Numerous other AKT targets involved in different cellular reactions have been recognized.

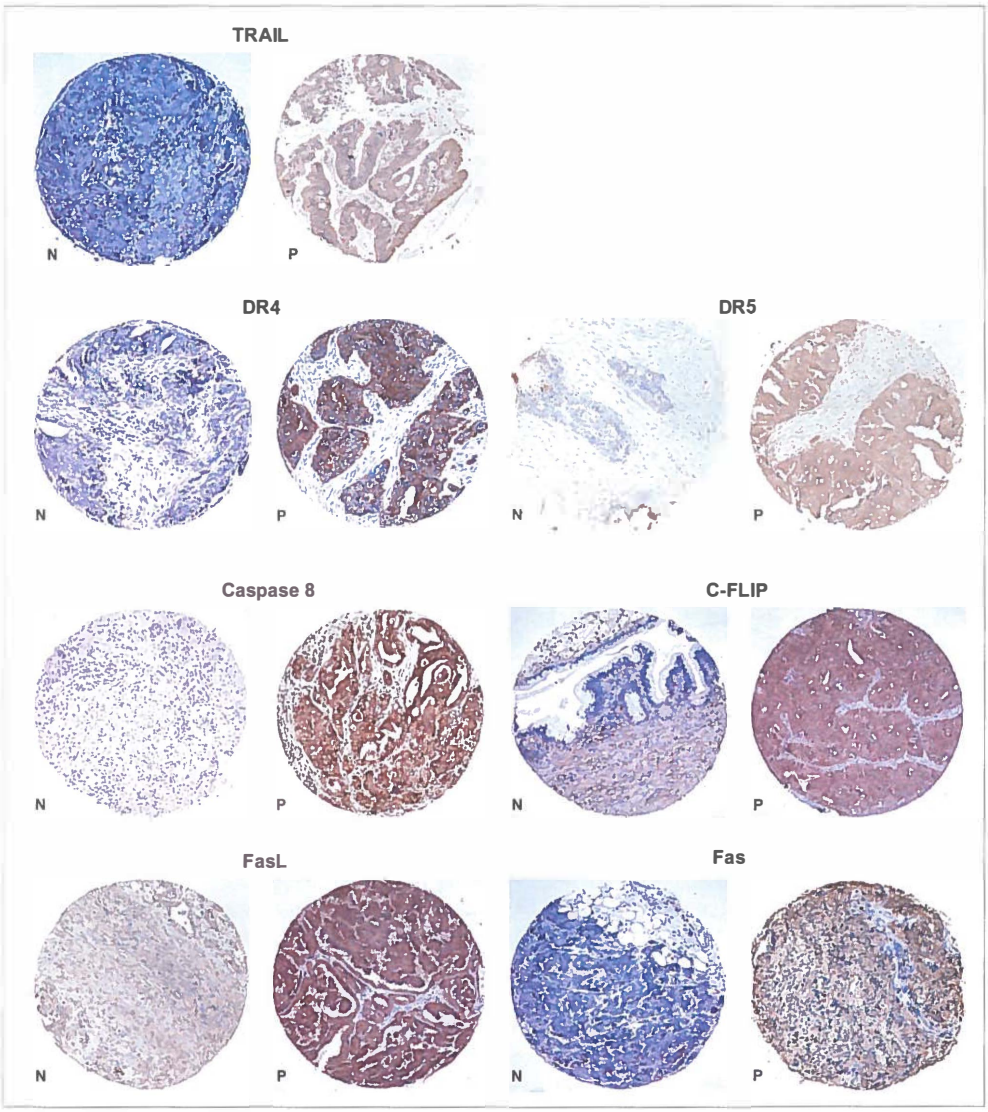


Figure 1 Results of immunostaining for TRAIL, DR4, DR5, caspase 8, c-FLIP, FasL and Fas. N stands for negative and P for positive staining.

CHAPTER 8

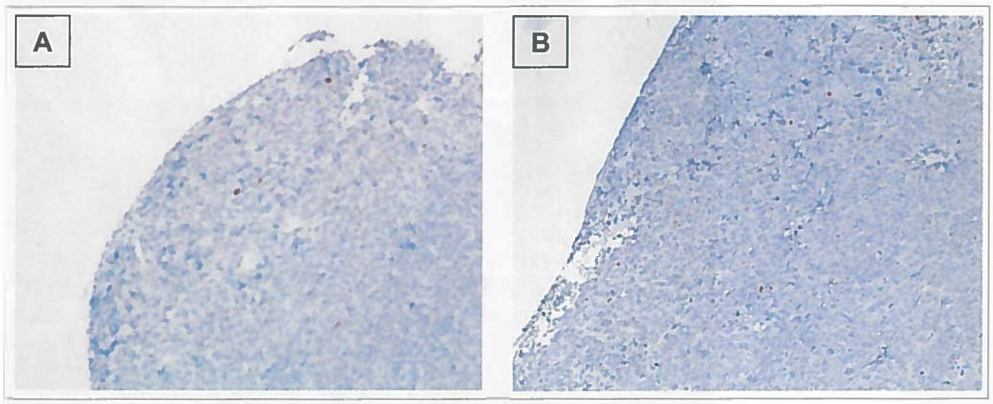


Figure 4 Ovarian cancer xenograft tissue excised at 15 min after IP (A) and IV (B) administration of ^{125}I-rhTRAIL, stained for cleaved caspase 3.

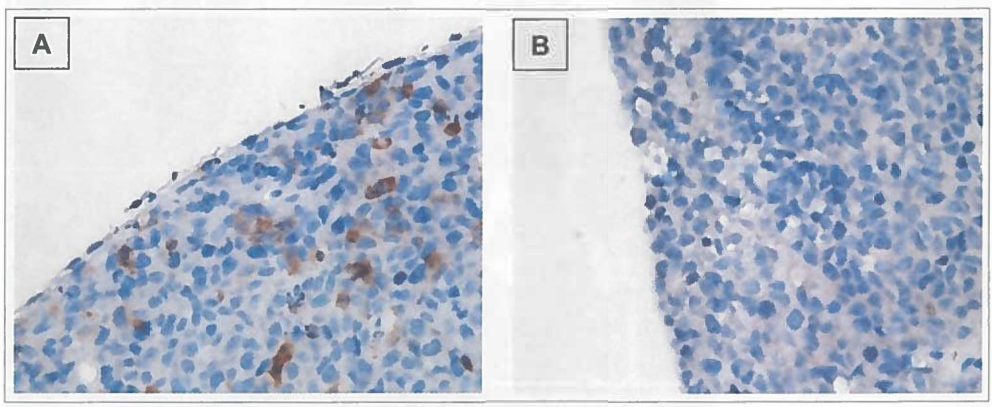
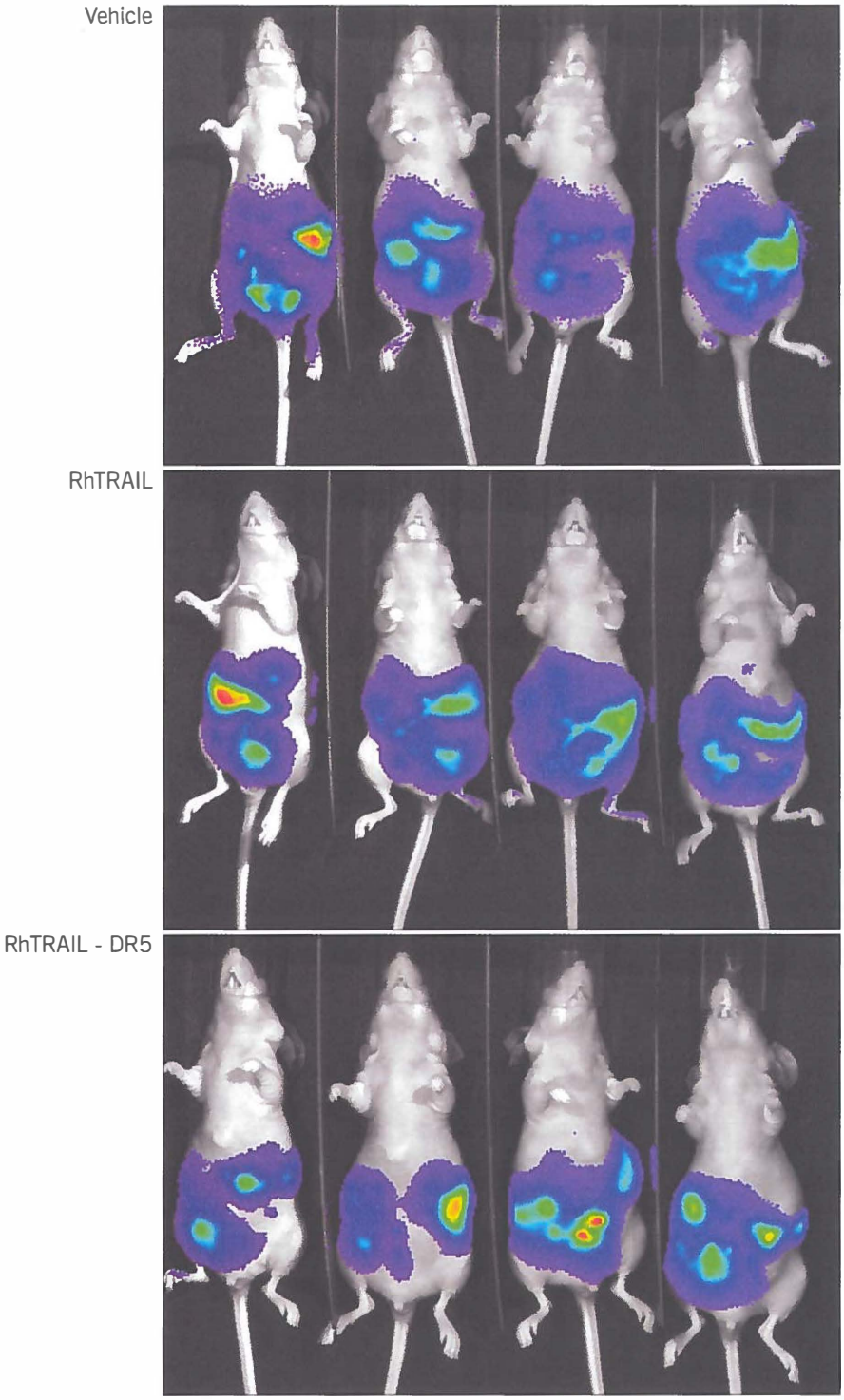


Figure 5 Ovarian cancer xenograft tissue excised at 360 min after IP (A) and IV (B) administration of ^{125}I-rhTRAIL, stained for cleaved caspase 3.


Vehicle
RhTRAIL
RhTRAIL - DR5

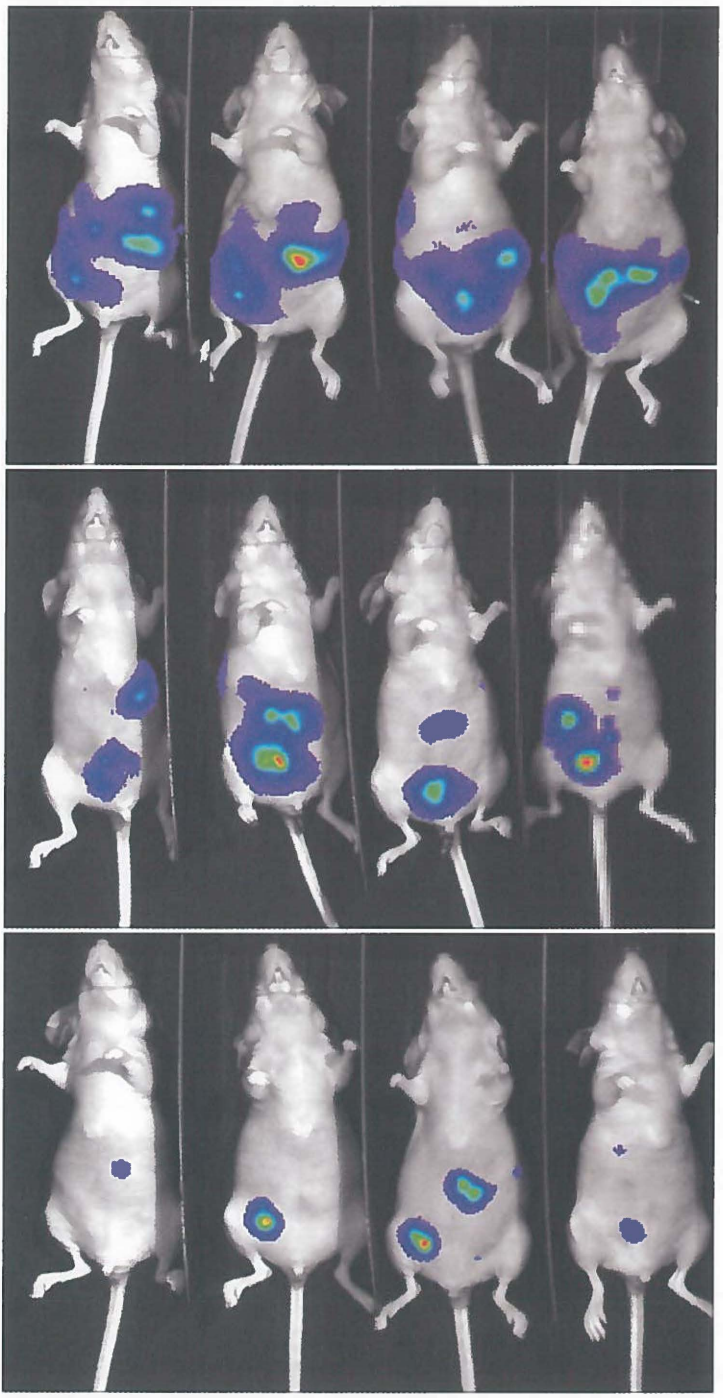


Figure 6 B. Bioluminescent images at the end of treatment (day 16) of each 4 mice representative for 10 mice per experimental arm. Images are displayed and quantified in log radiance (photons/sec/cm²/sr).