CHAPTER 1

GENERAL INTRODUCTION AND SCOPE OF THE THESIS

GENERAL INTRODUCTION

Cancer is one of the leading causes of death in the world and the number of new cases and deaths continues to rise as the world population is aging, growing, and increasingly adopting cancer-associated lifestyle choices, such as smoking and physical inactivity [1].

For decades, the primary method for cancer treatment consists of surgical resection and/or radiotherapy that is combined with chemotherapy in case of more extensive disease. Conventional radio- and chemotherapies inflict DNA damage and cellular stress in proliferating cells predominantly leading to the activation of the intrinsic, or mitochondria-dependent, apoptotic pathway [2]. Their rather unspecific mode of action is a major drawback of these treatments since all multiplying cells in the body, including fast-dividing healthy tissues such as present in the bone marrow and gut, are targeted causing unwanted side effects. Moreover, signalling via the intrinsic pathway is often hampered in cancer cells due to inactivation of the tumour suppressor protein p53 by mutations in the *TP53* gene or through modulators such as the MDM2 protein [2, 3]. Consequently, tumours that lost p53 function become resistant to therapy, leaving radiation and chemotherapy approaches rarely curative. Biological therapeutics have been developed as alternative strategies to selectively target molecular mechanisms in tumour cells, such as the activation of apoptosis, while minimizing toxic side effects.

Tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) has gained much attention as a promising biological anti-cancer agent as it was demonstrated that recombinant human (rh) TRAIL selectively induced apoptosis in a variety of tumour cells *in vitro* and *in vivo* regardless of p53 status [4, 5]. Moreover, clinical grade rhTRAIL (dulanermin) has been tested in several phase I and II single agent and combination studies and proved to be well tolerated in patients [6-8].

TRAIL, also known as Apo2 ligand (Apo2L), is a member of the TNF superfamily and was first discovered independently by Wiley *et al*. and Pitti *et al*. [9, 10]. TRAIL is a cytokine that plays a physiological role in the immune system, including anti-tumour immune surveillance [11] and transcripts were found in various tissues, most predominantly in spleen, lung and prostate [9]. TRAIL is a type-II transmembrane glycoprotein that can be proteolytically cleaved at the stalk domain to give rise to soluble TRAIL. Crystallographic studies of soluble TRAIL revealed that TRAIL has a zinc binding site in the core of its homotrimeric subunit, which is essential for stabilization and optimal biological activity [12, 13].

TRAIL is the most promiscuous ligand of the TNF superfamily and is known to bind to five different receptors; the membrane-bound death receptors (DRs) DR4 (TRAIL-R1) and DR5 (TRAIL-R2), the membrane-bound decoy receptors (DcRs) DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4), and the soluble decoy receptor osteoprotegerin (OPG). Trimeric TRAIL activates the extrinsic, or death receptor-dependent, apoptotic pathway by binding to DR4 and DR5, leading to the formation of the death-inducing signalling

complex (DISC) consisting of Fas-associated death domain (FADD) and pro-caspase-8 [14-16] and/or pro-caspase-10 [17, 18]. At the DISC, these initiator caspases are activated by proteolytic cleavage and on their turn they cleave the effector caspases-3 and -7 resulting in apoptosis [19]. The extrinsic pathway can cross activate the intrinsic pathway by caspase-8-dependent cleavage of Bid into truncated Bid (tBid) [20], leading to activation of the pro-apoptotic Bcl-2 family members Bax and Bak that form pores in the outer mitochondrial membrane causing the release of mitochondrial factors, such as cytochrome c [21, 22]. In the presence of dATP or ATP, cytochrome c and Apaf-1 form a complex called the apoptosome, which facilitates caspase-9 cleavage and further activation of effector caspases [23]. Depending on whether mitonchondrial apoptotic events are needed for TRAIL-induced apoptosis, tumour cells have been categorized into two types; cells in which TRAIL-induced apoptosis is independent (Type I) or dependent (Type II) of mitochondrial amplification of the apoptotic signal [19].

A disadvantage of TRAIL is its ability to bind DcR1, DcR2 and OPG, that diminish apoptosis activation by sequestering TRAIL from the DRs [24, 25]. In order to improve anti-tumour efficacy, DR-selective rhTRAIL variants [26-31] and monoclonal antibodies (mAbs), such as mapatumumab and lexatumumab (Human Genome Sciences), have been generated to specifically target one DR and reduce DcR-binding. Moreover, DR-selective agonists demonstrated faster DR binding and more rapid and potent initiation of TRAIL-induced apoptosis [32, 33].

Unfortunately, TRAIL resistance is often seen in cancer cells, especially in highly malignant tumours. It is also feasible that resistance could develop after repeated exposure to TRAIL therapeutics. Therefore it is important to understand mechanisms underlying resistance that can occur at different steps in the TRAIL signalling pathways, such as dysfunctions of the DRs, DISC inhibition, overexpression of anti-apoptotic proteins or loss of pro-apoptotic proteins [34]. It can be envisioned that the most effective use of TRAIL is in combination therapy in order to circumvent TRAIL resistance.

Beside apoptosis, which can be regarded as the canonical route of TRAIL signalling, TRAIL-induced DR activation can also promote the formation of the intracellular RIP1/TRAF2/NEMO complex, called complex II, which is located downstream of the DISC [35]. This secondary signalling complex can activate non-canonical signalling pathways that may stimulate NF-κB and kinases, including RIP1, IκB/ NF-κB, MAPK p38, JNK, ERK1/2, MAP3K TAK1, PKC, PI3K/Akt and Src [36, 37]. The activated intracellular kinase cascades can be involved in cell proliferation, survival, migration/invasion and angiogenic properties in TRAIL-resistant tumour and normal non-transformed cells [36, 37]. For example, TRAIL-mediated activation of JNK and p38 was demonstrated to depend on RIP1 and TRAF2, whereas IKK activation required NEMO [35]. Furthermore, TRAIL-induced kinase activation increased chemokine secretion and macrophage attraction [35], and TRAIL enhanced migration and invasion of TRAIL resistant tumour cells [38, 39], which

was shown to be RIP1-dependent in resistant NSCLC cells [40]. Clearly, non-canonical signalling of TRAIL is an unwanted effect when aiming to induce apoptosis and might be prevented by inhibition of the involved kinases.

The research described in this thesis is aimed at exploring effective strategies to enhance TRAIL-induced apoptosis and to investigate the contribution of either DR4 and DR5 in this context. We examined the molecular mechanisms influencing TRAIL resistance in several cancer types and used this knowledge to examine rational combination strategies to enhance rhTRAIL-induced apoptotic signalling.

SCOPE OF THE THESIS

Studies in which DR4- and DR5-selective agonists were used, indicated that certain cancer types demonstrate DR preference and/or differ in apoptotic and non-apoptotic signalling ability via DR4 or DR5. The possible underlying molecular grounds of receptor dominance include DR cell surface expression levels, post-translational modifications, DISC formation and downstream signalling. These differences in function and regulation of DR4 and DR5 are extensively reviewed in **Chapter 2**.

Since approximately 50% of cancer cell lines have been reported to be TRAIL resistant [41-43], research has been focussed on establishing rational combination therapies of rhTRAIL or mAbs with other agents to provide an effective strategy to enhance TRAIL-induced apoptosis. In **Chapter 3** we investigated the combined anti-tumour effects of TRAIL with the endoplasmic reticulum (ER) stress-inducing agent 2,5-dimethyl-celecoxib (DMC) in glioblastoma multiforme (GBM) cells. ER stress has been reported to enhance DR5 expression, and decrease the expression of anti-apoptotic proteins, including c-Flip, Bcl-2 and survivin, all of which have been demonstrated to be deregulated in GBM cells. Underlying mechanisms of synergy between rhTRAIL WT and DMC, and the role of DR5 cell surface expression levels and survivin in this synergy, were examined using overexpression of DR5 and siRNA-dependent knockdown of survivin in a TRAIL sensitive and resistant cell line. **Chapter 4** describes the way in which fucosylation enhanced TRAIL-induced apoptosis via both DRs in DR4-sensitive colon adenocarcinoma cells. Fucosylation, the attachment of fucose residues to glycans or glycolipids on cell-surface and secreted glycoproteins [44, 45], is an important type of post-translational modification in colon cancer [46] and is essential in numerous biological processes [44, 47]. Fucosylation by L-fucose treatment and ectopic overexpression of fucosyltransferase enzymes FUT3 and FUT6 re-sensitized DLD-1 and HCT116 cells mainly via activation of DR5. Moreover, the mechanism by which fucosylation enhanced TRAIL-induced apoptosis in DR5 resistant cells was studied. In **Chapter 5** we examined the synergistic effect of rhTRAIL with the thymidine analogue trifluorothymidine (TFT) in a panel of NSCLC cell lines. TFT can be activated by thymidine kinase (TK) and inhibits thymidylate synthase (TS) in its monophosphate form, or can be incorporated into the DNA causing DNA damage and

subsequent cell death in its triphosphate form [48]. The mechanisms of synergy between rhTRAIL and TFT were investigated by focussing on cell-cycle progression and kinases, the intrinsic and extrinsic apoptotic pathways, and DR expression levels after TFT exposure.

In addition to investigating the canonical TRAIL signalling route by exploring rational combination therapies of TRAIL with other agents to enhance TRAIL-induced apoptosis, we also focussed on the non-canonical signalling route by examining TRAIL-induced protein kinase signalling. **Chapter 6** describes the opposing effects of TRAIL-activated p38 and JNK in sensitive and resistant non-small cell lung cancer (NSCLC) cells. It was previously demonstrated that TRAIL stimulation phosphorylated JNK and p38 through the formation of the secondary complex, which consists of FADD, caspase-8, RIP1 and TRAF2 [35]. The influences of these two MAPK kinases in modulating TRAIL-induced apoptosis have been evaluated using selective chemical kinase inhibitors and siRNA strategies. The effect of RIP1 and caspase-8 on p38 and JNK activation was examined in more detail by shRNA-mediated depletion of RIP1 and ectopic overexpression of the caspase-8 inhibitor CrmA in H460 cells.

Finally, a summary of all study results described in this thesis is presented in **Chapter 7** (English) and **Chapter 8** (Dutch).

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