Combining radiotherapy with death ligands in cancer treatment: feasibility and molecular mechanisms

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General Introduction
Cancer and apoptosis

Apoptosis is a tightly controlled physiological ‘cell suicide program’ that plays a critical role in both embryonic development and adult tissue homeostasis. It is important for maintaining appropriate cell numbers in tissues, serving as a balance for cell proliferation [1]. Cells that are no longer needed or have acquired severe damage, e.g. to their cytoskeleton or DNA are eliminated by apoptosis [1, 2]. Disturbances in mechanisms that direct abnormal cells to undergo apoptosis critically contribute to cancer development. For instance, genomic aberrations such as p53 mutation often drive tumor cells towards apoptosis [1]. However, as part of their malignant evolution, tumor cells can acquire blocks in apoptosis by upregulating anti-apoptotic proteins such as Bcl-2, which facilitate their uncontrolled cell growth [2]. Targeting apoptosis in cancer treatment is therefore interesting, as overcoming blocks in apoptosis may render cancer cells more susceptible to death than normal cells [3], creating a therapeutic window for cancer treatment.

Caspases: Executioners of the apoptotic program

Caspases are a specialized family proteolytic enzymes that initiate and execute the apoptotic program [4, 5]. Caspases are synthesized as inactive zymogens (procaspases), which require proteolytic processing to become fully active. The caspases that are involved in apoptosis can be categorized as initiator caspases (2, 8, 9, 10) or effector caspases (3, 6, 7). Once switched on, initiator caspases activate the effectors, which then execute apoptosis [6].

There are principally two separate, yet interlinked signaling pathways that lead to apoptotic execution: the intrinsic pathway, which is activated by mitochondrial signals from within the cell, and the extrinsic pathway, which is activated by pro-apoptotic receptor signals at the cell surface. The two pathways converge at the level of effector caspases that ultimately execute the apoptotic program [6].

The intrinsic apoptosis pathway

The intrinsic apoptosis pathway is triggered by severe cellular stress such as DNA damage [7] (induced by for instance ionizing radiation and conventional chemotherapeutics in cancer treatment) and growth factor withdrawal. This pathway is controlled by interactions between pro-and anti-apoptotic members of the Bcl-2 protein family. Ionizing radiation induces DNA damage, which can activate the p53 pathway. This induces a cell cycle arrest allowing for the DNA damage to be repaired. If repair fails, cells can irreversibly remain in cell cycle arrest, die by mitotic catastrophe, but apoptosis may also be initiated by activation of the intrinsic apoptosis pathway [8]. This occurs by induction – both p53 dependent and independent – of BH3-only members of the Bcl-2 family, such as PUMA and Noxa [7, 9-11]. These proteins allow their relatives Bak and Bax to mediate permeabilization of the mitochondrial outer membrane [12]. Upon release from the mitochondria into the cytoplasm, Cytochrome c (Cyt c) binds to the scaffold protein Apaf-1, which allows recruitment and activation of the inducer Caspase-9 in the ‘apoptosome’ formed by these molecules. Additionally released Smac/DIABLO and Omi/HtrA2 promote caspase activation by displacing inhibitor of apoptosis proteins (IAPs). Collectively, these events result in effector caspase activation and consequent execution of the apoptotic program (Figure 1) [6]. The mitochondrial apoptosis pathway can be blocked by inhibitory Bcl-2 family members (e.g. Bcl-2, Bcl-X-), which sequester their pro-apoptotic relatives [12, 13] and whose expression can be impaired by DNA damaging regimens e.g. [14, 15].

The extrinsic apoptosis pathway

Death receptor ligands of the tumor necrosis factor (TNF) family, such as CD95 (Fas/APO-1) ligand (L), TNF-related apoptosis-inducing ligand (TRAIL), and under certain circumstances TNFα initiate the extrinsic apoptosis pathway by triggering their death receptors (CD95, TRAIL-receptor (R)1/-R2 and TNFR1 respectively) on the cell surface.

Death receptor signaling occurs both in normal and cancer cells and has been implicated in a variety of physiological processes (reviewed in e.g. [16]). For example, endogenous TRAIL expression on natural killer cells plays an important role in tumor immune surveillance [17-21] and by studying TRAIL receptor deficient mice, TRAIL-TRAIL receptor interactions were implicated in suppressing tumor metastases to lymph nodes [22].

Ligand binding to death receptors induces their oligomerization and recruitment of the adapter molecule Fas-associated protein with death domain (FADD) through homotypic death domain interactions in FADD and in the cytoplasmic tail of death receptors. FADD in turn, recruits – through homotypic death effector domain (DED) interactions – both procaspases 8/10 and c-FLIP molecules [23, 24] that also contain a DED. In the death-inducing signaling complex (DISC) thus formed at the receptor tail, Caspase-8/-10 are activated and self-processed by proteolysis, after which they are released into the cytoplasm. Here, they find effector caspases 3, 6, and 7 as their targets, which upon their activation continue to execute apoptosis (Figure 1). The activation of effector caspases by inducer caspases in the extrinsic pathway is the direct pathway
for effector caspase activation.

Death receptors also connect to the mitochondrial pathway for caspase activation via the BH3-only protein Bid. Caspase-8/-10 can cleave and activate Bid, resulting in its mitochondrial translocation and subsequent mitochondrial permeabilization [25]. Activation of downstream caspases induces processing of additional procaspase 8 and 10 molecules, leading to a mitochondrial amplification loop for effector caspase activation (Figure 1).

The efficacy of mitochondrion-independent apoptosis induction by death receptors depends on the cell type. Cells have been classified as Type I or Type II by their ability to induce apoptosis via CD95 in the presence of Bcl-2 overexpression or Bid deficiency. Whereas Type II cells rely mostly on the mitochondrial pathway for apoptosis induction, Type I cells can execute apoptosis through CD95 independent of mitochondrial permeabilization [26, 27].

**Modulation of apoptosis execution**

Both caspase activation and caspase activity is tightly regulated by several cell-endogenous factors. Inducer caspase activation in the DISC is regulated by FLIPs and FLIP isoforms. FLIP, contains two DEDs with which it can interfere with the recruitment and activation of Caspase-8/-10 in the DISC. FLIP shares structural similarity with Caspase-8/-10, since it contains in addition to a DED, a catalytically inactive caspase-like domain. At high levels, FLIPs inhibit inducer caspase activation by competing with Caspase-8/-10 molecules for binding (via FADD) to the death receptor tail (e.g. [28]). At low FLIP levels however, Caspase-8/-10 activation is facilitated due to the ability of FLIP to associate with Caspase-8/-10 and its ability to form heterodimers more efficiently than Caspases-8/-10 forms homotrimers. Upon induced proximity, Caspases-8/-10 in these heterotrimers are fully activated and released into the cytosol (reviewed in [29]).

As mentioned earlier, inhibitory Bcl-2 family members (e.g. Bcl-2, Bcl-XL) block mitochondrial permeabilization, preventing Caspase-9 and subsequent downstream effector caspase activation [12]. Members of the IAP family can also directly inhibit activation and activity of effector caspases. Caspases-3/-7/-9 can be directly inhibited by XIAP, cIAP1 and cIAP2 (Figure 1). These IAPs bind the effector caspases by one of their baculovirus IAP repeats (BIR), thereby masking their active site [30-33]. In addition, XIAP and cIAP1/2 contain a RING domain with ubiquitin ligase (E3) activity. The ubiquitin-conjugating enzymes (E2) use the RING domain as an adaptor to direct the transfer of ubiquitin to effector caspases and thereby mediate their proteasomal degradation [34-36]. Mitochondrial permeabilization following activation of the intrinsic apoptosis pathway promotes effector caspase activation in a number of
Improving therapeutic outcome of radiotherapy with death receptor agonists

Radiotherapy is often complemented with conventional chemotherapy to improve treatment outcome and prognosis of cancer patients [42]. However, since radiotherapy and chemotherapy act by largely the same molecular mechanisms, the additional therapeutic effects remain limited and side effects will determine the maximally tolerable doses of both radiotherapy and chemotherapy. Therefore, radiotherapy should be combined with drugs that achieve cytotoxic activity by inducing cell death using (partially) distinct molecular mechanisms than those activated by ionizing radiation. This would enable additive or synergistic interactions and reduce the risk of therapy resistance. Death receptor agonists, such as CD95L, TRAIL and agonistic antibodies targeting TRAIL-R1/-R2 are attractive agents for combined modality treatment with radiotherapy. Whereas ionizing radiation exerts its therapeutic effects by inducing cell cycle arrest, mitotic catastrophe and mitochondrion-dependent apoptosis, all resulting in reduced clonogenicity of tumor cells, death receptor agonists operate irrespective of the p53 status of cells and can activate effector caspases independently of the mitochondria [24]. Because these (partially) distinct routes of cell death are triggered, it can be envisioned how combined treatment can increment the anti-tumor response and improve therapeutic outcome of radiotherapy. In addition, enhanced responses can be expected by upregulation of TRAIL-R1/-R2, CD95 and Caspase-10 [43-46], as well as increased CD95 transport to the cell surface [47] in response to p53 activation by radiotherapy. Even in the absence of death receptor upregulation at the cell surface, combined effects are also observed [48-51].

Death receptor agonists could be combined with either conventional (systemic) chemotherapy or radiotherapy to improve outcome of cancer therapy, since these regimens induce cell death by largely the same molecular mechanisms. However, since radiotherapy induces (local) DNA damage only at the tumor site, the systemic administration of death receptor agonists imposes a local combined effect of the treatment at the tumor site. This could enhance the therapeutic window and limit normal tissue toxicity.

Clinical development of death receptor agonists in cancer therapy

Since the discovery of death receptors and their mechanism of apoptosis induction, there has been great interest in (pre) clinical development of death receptor agonists in cancer therapy. Soluble recombinant TRAIL and agonistic antibodies directed against TRAIL-R1/-R2 in particular are promising anti-cancer therapeutics, because TRAIL induced apoptosis in a large proportion of long-term established tumor cell lines, but was not toxic to normal tissue [52, 53]. Presently, several Phase I and II clinical trials with TRAIL receptor agonists have been completed and corroborated low toxicity (see ref [54] and references therein).

To achieve optimal efficacy of treatment, death receptor agonists must be combined with conventional or novel therapeutics. These agents have been shown to sensitize tumor cells to TRAIL-induced apoptosis by a variety of mechanisms ([55] and references therein), resulting in additive or even synergistic interactions in cell death induction. These interactions have been shown both in vitro and upon xenografting of tumor cells in mice (e.g. [56, 57]). For certain cell lines, combined effects became more apparent when death receptor ligands were added after irradiation or chemotherapy (e.g. [58, 59]), indicating that DNA-damaging regimens condition cells to more effectively undergo death receptor-induced apoptosis. Clinical trials will have to point out whether these encouraging experimental observations are predictive of the patient response to combined modality treatment.

Although TRAIL receptors and CD95 induce apoptosis by highly similar mechanisms [24], CD95 agonists have not been pursued for cancer therapy. Such application was discouraged by the early observation that systemic treatment of mice with anti-CD95 antibody resulted in acute, lethal hepatotoxicity [60]. Recently, however, a novel form of soluble recombinant CD95L has been generated. This so-called MegaFas Ligand – currently named APO010 – is composed of a fusion protein of the extracellular domain of FasL/CD95L and the collagen domain of the 30 kDa adipocyte complement-related protein (ACRP30). Whereas CD95L naturally forms a trimer, the ACRP30 domain imposes a dimeric state allows APO010 to crosslink two adjacent CD95 trimers, which renders it highly agonistic as compared to other available forms of soluble recombinant CD95L or anti-CD95 antibodies [61-63]. It could be envisioned that in a combined treatment setting with radiotherapy a local combined therapeutic effect might be achieved at APO010 concentrations that have no or acceptable toxicity. Currently, APO010 is in a Phase I clinical trial at TopoTarget (ClinicalTrials.gov identifier: NCT00437736) to establish its safety and tolerability.
What determines a cell’s response to death receptor agonists?

Not all tumor cells expressing death receptors undergo apoptosis upon their triggering by death receptor agonists, potentially limiting their therapeutic application. In order to predict which (tumor) cells are responsive to death receptor therapy, numerous studies have addressed the underlying molecular mechanisms that determine sensitivity to death receptor agonists. A full understanding of tumor cell sensitivity to death receptor agonists could also allow for mechanisms of (re) sensitizing tumor cells to these agents.

Besides the expression of cytoplasmic pro- and anti-apoptotic proteins discussed previously, there are several other factors that determine how a cell will respond to death receptor agonists and these are important considerations for future clinical development.

Quality and formulation of death receptor ligand

Like all membrane bound TNF family members, TRAIL and CD95L are type II transmembrane proteins. A variety of soluble, pro-apoptotic formulations of the (trimeric) ligands have been produced. They include – but are not limited to – a native recombinant form of human TRAIL [52], Leucine-zippered (LZ) [53] or Isoleucine-zippered (IZ) TRAIL formulations [64]. In addition, CD95L has been produced as soluble trimeric molecule [65] or in soluble form as a dimer of CD95L trimers [61]. Critical for their pro-apoptotic effect is the trimerization of the molecules. Further crosslinking of CD95L trimers appears necessary for apoptosis induction in Type I cells, but not in Type II cells [66]. For TRAIL, activation of TRAIL-R1 occurs both by native trimeric soluble forms of TRAIL as well as membrane-bound or crosslinked TRAIL formulations, whereas activation of TRAIL-R2 apparently requires membrane-bound TRAIL or secondarily crosslinked soluble TRAIL formulations [67]. In addition, we have found differential apoptosis sensitivity of Type I breast cancer cells to different cross-linked versions of TRAIL (crosslinked FLAG-tagged TRAIL with anti-FLAG antibody versus IZ-TRAIL), indicating that crosslinking per se is not the only determinent as to whether cells undergo apoptosis in response to the ligand (Figure 2A).

Initial studies demonstrated a 79% overall homology at the protein level between human and mouse TRAIL and cross-species reactivity with their receptors [69]. Upon careful titration of a different TRAIL formulation (the SuperKiller version of both human and mouse TRAIL), we found a certain degree of species specificity; human SuperKiller TRAIL appeared to be more effective in apoptosis induction in human cells compared to mouse cells and vice versa (Figure 2B). These results are consistent with previous findings of LZ-TRAIL-induced apoptosis in both human and mouse TRAIL-sensitive tumors cell lines; showing a certain degree of species specificity, although both human and mouse versions of LZ-TRAIL were capable of killing both human and mouse TRAIL-sensitive tumor cell lines [53]. A recent report studying the interaction between TNF and TNF receptor family members in the mouse and human [70] also indicated that human TRAIL interacts most strongly with human TRAIL receptors. Collectively, these results indicate that although there is certainly cross-species reactivity for a number of ligand formulations, the highest reactivity is achieved by activating receptors by their species specific ligands.

Therefore, both the species and specific formulation determine to a large extent how well tumor cells undergo apoptosis in response to death receptor ligands.

Expression of decoy receptors

In addition to TRAIL death receptors, cells may also express TRAIL decoy receptors (TRAIL-R3, -R4 and Osteoprotegerin (OPG) that can bind TRAIL, but do not convey an apoptotic signal. Potentially, decoy receptor expression on normal cells could explain the tumor-selectivity of TRAIL-induced apoptosis. To date however, no correlation has been established between levels of TRAIL-R3/-R4 compared to TRAIL-R1/-R2 and relative sensitivity to TRAIL [71, 72].

Figure 2: Apoptotic responses in tumor cells are partly determined by formulation and species specificity of death ligands. (A) Apoptotic responses of human breast carcinoma cells (MCF7-Caspase-3, black lines) and human T-leukemic cells (Jurkat, clone J16, gray lines) to either isoleucine-zippered (IZ)- or crosslinked FLAG-tagged recombinant (human) TRAIL. (B) Apoptotic responses of human leukemia cells (J16, left panel), and mouse XhoC3 tumor cells [68] (right panel) to human or mouse (SuperKiller®) TRAIL formulations.
Alternative pathways induced upon death receptor engagement

Although CD95 and TRAIL-R1/-R2 are viewed primarily as a death-inducing receptor, like TNFR1 they can induce a variety of cellular responses via different signaling routes. Alternative signaling pathways in particular the NF-κB survival pathway [73-75], all three major MAP kinase pathways [73, 76, 77] and the PI-3 kinase pathway [77, 78] can be induced by both CD95 and TRAIL-R1/-R2. Activation of these pathways results in cell survival or proliferation, rather than apoptosis induction. Studies in TRAIL receptor deficient mice indicate that TRAIL receptor inhibits metastases via detachment-induced inactivation of the ERK survival pathway, which sensitized tumor cells to TRAIL-induced apoptosis [22]. NF-κB can activate a potent anti-apoptotic pathway that involves the new synthesis of inhibitory Bcl-2 family members, c-FLIP and IAPs and can also induce pro-inflammatory mediators [79]. How is the decision made between apoptosis induction and NF-κB activation? As further discussed below, it appears that the subcellular localization of death receptors greatly determines the capacity to induce either pro- or anti-apoptotic signals. In addition, there is evidence that NF-κB activation requires a lower level engagement of death receptors [80].

Receptor status and aggregation in microdomains

The precise plasma membrane localization of death receptors determines – at least in part – their ability to induce apoptosis. Localization of CD95 and TNFR1 to cholesterol and sphingolipid rich domains (‘lipid rafts’) within the plasma membrane is important for apoptotic signal transduction [81, 82]. In Type I cells, CD95 is preferentially localized to lipid rafts, whereas in Type II cells CD95 relocalizes to rafts upon ligand binding [83]. In these rafts, sphingomyelin to ceramide conversion facilitates receptor clustering and promotes apoptosis induction [82, 84]. There is evidence that ligation of TRAIL death receptors localized to lipid rafts induces a proapoptotic signal, whereas TRAIL receptors not associated with lipid rafts mediate the activation of survival pathways [85].

Apart from their membrane localization, post-translational modifications of death receptors also affect receptor functionality. A recent report indicates that O-glycosylation of TRAIL death receptors are important for apoptosis induction by TRAIL [71]. Inhibition of O-glycosylation did not affect TRAIL receptor surface levels, but suppressed TRAIL-mediated apoptosis. Palmitoylation of CD95 is required for apoptosis signaling, by facilitating the formation of SDS-stable receptor aggregates [86].

Death receptor internalization and signaling

Following receptor activation, receptors and ligands can internalize from the cell surface by various routes (e.g. clathrin- or lipid raft-dependent endocytosis). Recent data indicate that the subcellular localization of death receptors determines the capacity to induce either pro- or anti-apoptotic signals. Triggering TNFR1 results in the formation of a primary (membrane-associated) complex in lipid rafts [81], which comprises TNF, TNFR1, TNFR1-associated protein with death domain (TRADD), TNF receptor-associated factor 2 (TRAF-2) and receptor interacting protein (RIP) and induces NF-κB activation. Upon (subsequent) internalization into endosomes, the primary complex is dissociated, allowing FADD to bind TRADD or possibly RIP through their death domains, which in turn recruits and activates Caspase-8 [87, 88], initiating the apoptosis cascade.

Internalization (by actin polymerization) of CD95 also appears to be essential for apoptosis induction in Type I cells, as upon ligand binding DISC assembly and subsequent Caspase-8 activation occurred at the endosomal membrane. Blocking internalization abrogated apoptosis-induction and switched the response to NF-κB activation [89]. In Type II cells however, CD95 was internalized (independent of actin polymerization) after ligand binding, but no overt co-localization of CD95 DISC components with endosomal markers was observed. In addition, blocking internalization did not prevent DISC formation, nor apoptosis induction, indicating that for Type II cells, CD95 internalization is not required for apoptosis induction [89]. Upon ligand binding, TRAIL-R1/-R2 also internalize in Type I cells, via clathrin dependent and independent routes. However, internalization of TRAIL or its receptors was not required for TRAIL DISC formation and for the initiation of a full apoptotic signal [90]. (for a review of death receptor compartmentalization and signaling see [91])

The exact molecular mechanisms that drive death receptor internalization have not yet been fully elucidated, but ubiquitination of the cytoplasmic tail may be important. For a number of transmembrane glycoproteins, such as the epidermal growth factor (EGF) receptor, mono-ubiquitination of the cytoplasmic tail is essential for clathrin-mediated endocytosis (e.g. [92-94]). CD95 has been shown to interact with E2 ubiquitin conjugating enzyme UBC9 [95] and ubiquitination of TNFR1 has also been observed [96]. Recently, a novel family of transmembrane E3 ubiquitin ligases of the MARCH protein family were shown to target membrane receptors (including CD95) for (clathrin-mediated) endocytosis via ubiquitination of the cytoplasmic tail [97].

In conclusion, death receptors can induce a variety of cellular responses on a wide range of tissues. Therefore, the anti-tumor responses and normal tissue toxicity may vary widely, depending on the tumor type and death receptor agonist used. The levels, post-translational status and membrane localization of death receptors at the cell surface, ligand delivery, duration of the signal,
agonistic activity of the death receptor agonists and cell type will determine whether the relative strength of the apoptotic signal outweighs the strength of the anti-apoptotic signal (also reviewed in [98]).

Scope of this thesis

Combining death ligands to improve the therapeutic outcome of radiotherapy is an elegant and rational strategy, since these regimens induce partially distinct routes to cell death. Radiotherapy impairs clonogenicity of tumor cells by mitotic catastrophe, cell cycle arrest or mitochondrion-dependent apoptosis pathways and can upregulate death receptors at the cell surface, whereas death receptor ligands induce apoptosis – irrespective of the p53 status of the cell – and by pathways that are able to bypass the mitochondrial route for caspase activation. In addition, radiotherapy (as opposed to chemotherapy) is applied locally; therefore systemic administration of death receptor agonists will have a local combined effect, enhancing the therapeutic window and limiting systemic toxicity.

In this thesis, we (1) evaluated the effectiveness and toxicity of combined treatment with radiotherapy and death receptor ligands, (2) investigated the molecular mechanisms underlying improved sensitivity to cell death induction upon combined treatment and (3) investigated intracellular transport of (TRAIL) death receptors in relation to apoptosis sensitivity.

In a preclinical setting, we have tested whether radiotherapy in combination with a novel isoleucine zippered (IZ) recombinant form of TRAIL (IZ-TRAIL, Chapter 2) or APO010 (MegaFas Ligand, Chapter 4) showed enhanced therapeutic efficacy. This work involved evaluating apoptosis induction and clonogenic survival in vitro and evaluating tumor responses of subcutaneously and orthotopically transplanted tumors in vivo. Using the human leukemic cell line Jurkat overexpressing Bcl-2 (Jurkat-Bcl-2), we demonstrated that radiotherapy and TRAIL synergize for apoptosis induction, which translated in a strong combined therapeutic effect in vivo, without systemic toxicity (Chapter 2). In Chapter 4 we showed that although APO010 and radiation had a clear combined cytotoxic effect on Jurkat-Bcl-2 cells and a variety of solid tumor cells in vitro, a combined therapeutic effect was not achieved on the same cells subcutaneously grafted in mice. In this setting, APO010 doses were approximating the maximally tolerable level and reversible liver toxicity was observed.

Next, we investigated the mechanism underlying the enhanced response to combined treatment with DNA-damaging regimes and TRAIL (Chapter 3) or APO010 (Chapter 5) using a variety of genetic and biochemical approaches. We found that Jurkat-Bcl-2 cells, which are Type II cells and largely reliant on a mitochondrial contribution in apoptosis induction by death receptor ligands, no longer require this pathway for apoptotic execution after irradiation or treatment with DNA-damaging anti-cancer drugs. For the combination TRAIL and ionizing radiation, this effect was explained by an increase in recruitment and activation of inducer caspase activation at the DISC (Chapter 3), without c-FLIP downregulation. Sensitization to APO010-induced apoptosis by a wide range of stimuli however, was strongly correlated with decreased c-FLIP levels in cell lysates and in the DISC. Upon deliberate downregulation by RNA interference, cells were similarly sensitized to APO010 and (consequently) the capability of the sensitizers to increment apoptotic execution was largely overruled (Chapter 5).

In Chapter 6 we undertook a variety of cell biological and genetic approaches to investigate how TRAIL receptor expression is regulated in relation to apoptosis sensitivity. We found that internalization of TRAIL death receptors was not required for TRAIL-induced apoptosis. In addition, we found that cell surface expression of TRAIL-R1 was differentially regulated to that of TRAIL-R2. Blocking internalization resulted in increased TRAIL-R1 (but not TRAIL-R2) surface levels, indicating that homeostatic regulation of TRAIL-R1 surface levels is mediated in a dynamin-dependent manner. In addition, we found that overexpression of members of the MARCH protein family of ubiquitin ligases promoted downregulation of TRAIL-R1 surface levels by targeting a distinct lysine residue in its cytoplasmic tail. Finally, the results of all chapters and potential future perspectives are discussed in Chapter 7.

References

8. Brown, J.M. and B.G. Wouters,


