Beyond chemotherapy

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CHAPTER 7

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

SUMMARY

Ovarian cancer has the highest mortality rate amongst the gynecological malignancies.[1] Patients usually present with advanced stage disease, in which the cancer has already spread beyond the ovaries.[2] This late presentation is largely due to the aspecific nature and late onset of the symptoms accompanying ovarian cancer, like abdominal bloating or pain and common digestive tract complaints like constipation. Attempts to develop early detection strategies have been unsuccessful to date. The prognosis faced by ovarian cancer patients is an inevitable consequence of having advanced stage disease at the time of diagnosis, but the widespread occurrence of chemoresistance is also a major contributor to poor patient outcome.[2] Initial response rates to standard carboplatin *plus* paclitaxel following surgical debulking can be as high as 80%, with 40-60% designated as complete responses, but platinum resistant recurrences often develop.[3] Altogether, this results in a disappointing 25-30% 5-year survival for patients with advanced ovarian cancer.

Finding ways of improving therapeutic efficacy in ovarian cancer has been avidly pursued. Numerous chemotherapeutic regimens have been explored, as well as intraperitoneal administration of chemotherapy, but impact on patient survival has been limited.[4] Consequently, these efforts have not brought much change to the treatment algorithm since the introduction of the current frontline regimen over a decade ago. Since chemotherapy efficacy seems to have reached its ceiling in ovarian cancer, interest has shifted towards the application of molecular targeted drugs to interfere with hallmark biological phenomena crucial to ovarian cancer development and progression. Amongst these, the capacity of ovarian cancer cells to form a supportive vascular system (angiogenesis) and to evade programmed cell death (apoptosis) provide important pharmacological targets for therapy.[5]

An important hurdle for the implementation of molecular targeted drugs in ovarian cancer treatment is posed by its heterogeneity. There are no generic driver mutations present in ovarian cancer which can be universally targeted, and the genetic and molecular profiles differ markedly amongst ovarian cancers.[6] As a consequence, only a selected subgroup of ovarian cancer patients may benefit from using a specific targeted agent. This notion is backed up by ample evidence from clinical trials which explored the value of different targeted agents in ovarian cancer, demonstrating modest response rates and limited survival benefits. It is therefore likely that the implementation of targeted agents in ovarian cancer could benefit from the discovery of patient selection strategies. This could be facilitated by finding upfront predictive

biomarkers or early predictive biomarkers to predict drug efficacy during treatment. Understanding the mechanisms that determine sensitivity or resistance to these agents is therefore of importance.

This thesis describes studies with antiangiogenic and proapoptotic targeted agents in preclinical ovarian cancer models and analysis of potential predictive biomarkers in human ovarian cancers.

Following a short introduction and outline of this thesis in **Chapter 1**, the first chapters are devoted to angiogenesis regulation and inhibition in ovarian cancer. Ovarian cancers are characterized by extensive vascularization and are considered highly angiogenic. Angiogenesis is required for locoregional tumor growth, but also facilitates metastasis.[7] Since angiogenesis is a crucial contributor to tumor development and progression, interest has turned to the development of drugs that inhibit this process.

In **Chapter 2**, an overview is presented on the currently available clinical experience with antiangiogenic therapies in ovarian cancer. The majority of the antiangiogenic agents that have been evaluated in clinical trials target members of the vascular endothelial growth factor (VEGF) family of proangiogenic cytokines or their receptors (VEGFRs), which are regulators of angiogenesis.[8] Ovarian cancer cells produce VEGF ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor). These can distinctly activate three receptor tyrosine kinases (VEGFR1, VEGFR2, VEGFR3) present on the cell membrane of vascular endothelial cells, promoting their proliferation, migration and survival. VEGF(R)-targeted agents that have been evaluated thus far in ovarian cancer include the VEGF-A-neutralizing antibody bevacizumab, the VEGFR1/2 fusion protein VEGF-Trap and several small-molecule tyrosine kinase inhibitors (TKIs) capable of inhibiting VEGFR kinase activity. Available experience from phase II and III clinical trials is summarized in this chapter. Although these agents show some activity when incorporated into frontline therapy and when given as monotherapy for recurrent disease, response rates and survival gains are too modest to justify their use in general clinical practice.[9] This underlines the need for patient selection strategies and has fuelled a search for alternative antiangiogenic therapies. Biomarker data as well as preclinical data obtained with alternative angiogenesis inhibitors are also described in **Chapter 2**.

Some of the clinical trials published to date have included exploratory biomarker analyses. However, the number of patients included in these analyses has been small and circulating biomarkers have been the focus of attention.[10,11] Currently, no markers have been discovered to aid the selection of patients which are likely to respond to VEGF(R)-targeted drugs. Insight into the expression of multiple vascular endothelial growth factor (VEGF) family members can support the implementation of antiangiogenic therapy, considering that these cytokines are the main therapeutic targets. In **Chapter 3**, VEGF family member expression was studied in ovarian cancers and their related omental metastases. Tissue microarrays (TMAs) encompassing 270 primary cancers and 112 paired metastases were immunostained for VEGF-A, VEGF-B, VEGF-C and VEGF-D. Protein expression profiles were related to clinicopathological characteristics and survival. Immunohistochemical positivity was observed for VEGF-A in 90%, VEGF-B in 4%, VEGF-C in 41% and VEGF-D in 55% of the primary ovarian cancers. VEGF-A expression correlated with VEGF-C and VEGF-D expression. Simultaneous positivity for VEGF-A and VEGF-C was observed in 38% of the cancers, and for VEGF-A and VEGF-D in 54%. Metastases showed positivity for VEGF-A in 78%, VEGF-B in 5%, VEGF-C in 26% and VEGF-D in 45% of cases. VEGF family member expression was independent of common clinicopathological parameters and showed no independent prognostic significance in multivariate analysis. The frequent simultaneous expression of VEGF-A, VEGF-C and VEGF-D in ovarian cancers observed may contribute to bevacizumab resistance. It has been previously demonstrated that prolonged exposure of colorectal cancer and glioma cells to bevacizumab results in compensatory upregulation of VEGF-C and VEGF-D which stimulate the proliferation of endothelial cells despite effective VEGF-A blockade, highlighting their potential role in bevacizumab resistance.[12,13] Combinatorial analysis of VEGF family member expression could support a rational, individualized choice of antiangiogenic therapy and might be used to predict antiangiogenic drug efficacy.

Several molecular targeted agents are being evaluated for their antiangiogenic potential. In ovarian cancer, inhibitors of the mammalian target of rapamycin (mTOR) provide an interesting alternative to the aforementioned VEGF(R)-targeted agents.[14] The serine/threonine kinase mTOR controls the translation of several proteins which contain specific regulatory sequences in their messenger RNA (mRNA), including VEGF-A and its transcriptional regulator hypoxia inducible factor-1α (HIF-1α).[15] Inhibitors of mTOR, rapamycin (sirolimus) and its analogs (rapalogs, like everolimus) can thus

potentially reduce VEGF-A production by ovarian cancer cells. Changes in tumor VEGF-A levels might be used as an early read-out for response to these agents. If so, longitudinal monitoring of tumor VEGF-A levels would greatly facilitate response assessment early on after starting treatment with a mTOR inhibitor. Positron emission tomography (PET) using radiolabeled bevacizumab provides a tool for non-invasive monitoring of tumor VEGF-A levels in response to treatment.[16] In **Chapter 4**, we investigated whether early changes in tumor VEGF-A levels after everolimus treatment could be monitored with ^{89}Zr-bevacizumab PET in mice. First, we showed a decrease in VEGF-A secretion by everolimus in a panel of ovarian cancer cell lines. BALB/c mice were subsequently xenografted subcutaneously with A2780^{luc+} ovarian cancer cells and were given daily everolimus (10 mg/kg intraperitoneally) for 14 days. ^{89}Zr-bevacizumab PET scans were performed before (baseline) and after treatment, from which mean standardized uptake values (SUV_{mean}) were calculated. For *ex vivo* analyses, control animals were sacrificed after the baseline scans. Everolimus treatment decreased ^{89}Zr-bevacizumab uptake by 21.7 ± 4.0% (SUV_{mean} 2.26 ± 0.18 *versus* 2.89 ± 0.20, $P < 0.01$). *Ex vivo* biodistribution confirmed decreased tumor uptake of the tracer in treated animals compared to control animals (7.78 ± 0.84 %ID/g *versus* 14.02 ± 1.68 %ID/g, $P < 0.01$), while no differences were observed in other tissues. VEGF-A protein expression was indeed lowered in treated versus untreated tumors ($P < 0.05$), which resulted in a diminished vascular density. Our data show that ^{89}Zr-bevacizumab PET can visualize and quantify reduced tumor VEGF-A levels in response to everolimus therapy. We therefore propose clinical testing of ^{89}Zr-bevacizumab PET as an early predictor of antiangiogenic efficacy in response to mTOR inhibitor therapy.

Chapter 5 and **Chapter 6** are devoted to *in vitro* studies assessing the potential of death ligand-based therapies targeting the extrinsic apoptosis pathway. We studied the apoptosis inducing potential of two drugs related to members of the tumor necrosis factor (TNF) superfamily of death ligands: trimeric recombinant human TNF-related apoptosis-inducing ligand (rhTRAIL) and a hexameric form of Fas ligand (MegaFasL, or APO010).

Like TNF, rhTRAIL and APO010 are capable of binding to death receptors (DRs) present on the outer membrane of ovarian cancer cells. Upon binding of TRAIL to either TRAIL receptor 1 (DR4) or 2 (DR5), or binding of APO010 to Fas, the extrinsic apoptosis pathway is activated via assembly of a membrane-associated death inducing signaling complex (DISC).[17] The DISC is a multiprotein complex, encompassing the

death receptor, the adaptor molecule Fas-associated death domain (FADD), the proapoptotic signal transduction protein (pro)caspase-8 and the inhibitory protein cellular Flice-like inhibitory protein (c-FLIP). Cleavage of procaspase-8 to its active form initiates the caspase cascade making up the extrinsic apoptosis pathway. Whether or not apoptosis is induced depends on the balance between pro- and antiapoptotic signals.

Triggering apoptosis using death ligands like rhTRAIL might overcome the problem of chemoresistance in ovarian cancer. *In vitro*, conventional chemotherapeutic drugs can act synergistically with death receptor-targeted agents, enhancing cell death in both chemosensitive and -resistant ovarian cancer cell lines.[18,19] The mechanisms underlying the sensitization to rhTRAIL by conventional chemotherapy can be best studied in isogenic cell line models with differential chemosensitivity. In **Chapter 5** we aimed to find molecular determinants of rhTRAIL sensitivity in an isogenic ovarian cancer cell line model of platinum resistance as well as the mechanism of synergy between rhTRAIL and cisplatin. To this end, we exposed A2780 ovarian cancer cells and their platinum resistant CP70 counterparts to rhTRAIL alone or in combination with cisplatin. A2780 cells showed moderate sensitivity to rhTRAIL-induced apoptosis, while CP70 cells were resistant. Combining rhTRAIL with cisplatin strongly increased levels of apoptosis in both cell lines, which could be explained by elevated caspase-8 protein and mRNA levels upon cisplatin exposure, in particular in CP70 cells. Untreated CP70 cells expressed less caspase-8 protein compared to A2780, while mRNA levels were similar. Caspase-8 mRNA turnover and protein stability did not differ between both cell lines in the presence or absence of cisplatin. By downregulating caspase-8 with small interfering RNA (siRNA) and by using constructs to overexpress caspase-8, we showed that the induction of caspase-8 protein levels by cisplatin is essential for sensitizing ovarian cancer cells to rhTRAIL. Additional c-FLIP and p53 siRNA experiments showed that neither an altered caspase-8/c-FLIP ratio nor a p53-dependent increase in DR5 membrane expression following cisplatin is involved in sensitization to rhTRAIL. We conclude that cisplatin enhances rhTRAIL-induced apoptosis in platinum sensitive and resistant ovarian cancer cells, and that induction of caspase-8 protein expression is the key factor in rhTRAIL sensitization.

In **Chapter 6** we aimed to study the apoptosis-inducing potential of APO010 in 12 human solid tumor cell lines with various resistance profiles, including platinum

sensitive and resistant ovarian cancer cell lines, to assess its ability to circumvent resistance to chemotherapy and death ligands. These cell lines together comprised three isogenic resistance models, in which we determined cell survival, apoptosis induction, caspase-3 activation, Fas membrane expression and the expression of DISC components in a panel of human solid tumor cell lines. Several solid tumor cell lines showed reduced cell survival and apoptosis induction upon APO010 exposure, including A2780 and CP70 ovarian cancer cells. Sensitivity to APO010 was independent of inherent resistance to chemotherapy. Fas membrane expression, determined by flow cytometry, correlated qualitatively with sensitivity to APO010. Fas membrane expression was highest in the ovarian cancer cell lines compared to cell lines derived from other solid cancers. Interestingly, pretreatment with cisplatin resulted in upregulation of Fas membrane expression and sensitization of cancer cells to APO010 independently of inherent cisplatin resistance or initial insensitivity to APO010 monotherapy. These data show that APO010 is a potent inducer of apoptosis in various drug sensitive and resistant solid tumor cell lines, with no consistent cross-resistance observed between APO010 and classical chemotherapeutic agents, indicating APO010 can be used to circumvent chemoresistance. In addition, combining APO010 with platinum-based chemotherapy can enhance its antitumor efficacy.

DISCUSSION AND FUTURE PERSPECTIVES

Because of the marked molecular heterogeneity observed among ovarian cancers, finding tools for patient selection is key to (cost-)effective implementation of molecular targeted agents. Molecular profiling studies have provided evidence that different ovarian cancer subtypes arise from precursor lesions of different histological origin, implying different mechanisms in their carcinogenesis. Differences in (epi)genetic background or (micro)environmental signaling cues may result in activation of specific oncogenic signaling pathways.[20] Targets for therapy are therefore likely subtype-dependent. For example, the presence of a BRCAness profile in serous ovarian cancers renders them more sensitive to PARP inhibitors.[21] Also, inhibitors of mTOR or phosphoinositide-3-kinase (PI3K) seem more potent in clear cell ovarian cancers, which display stronger phosphorylation of Akt.[22] Clear heterogeneity also exists among ovarian cancers with the same histological subtype. This is illustrated by the discovery of three distinct serous ovarian cancer gene expression signatures, characterized mainly by differences in expression of angiogenesis-related genes.[23] High-throughput analyses like deep sequencing, microarrays and proteome/kinome

profiling tools have readily become available and can offer valuable insight into subtype-specific ovarian cancer biology and targeted drug sensitivity. This provides clues as to which molecular targeted therapy certain ovarian cancers might respond best.

Ultimately, however, patient-tailored therapy will rely on the discovery of biomarkers that can be applied at the level of the individual patient. Analyzing upfront target expression in patients receiving molecular targeted agents seems to be a logical first step in the quest for biomarker discovery, and should preferentially be included in every phase II or III clinical trial investigating their efficacy. In this respect, for example, analysis of tumor VEGF-A expression when using antiangiogenic drugs could provide a primary rationale for either giving or withholding therapy. However, as we have demonstrated in **Chapter 5** and **Chapter 6**, numerous other (non-target) factors can be important determinants of sensitivity to targeted drugs. Their relevance in different tumor types may vary, which makes it difficult to pinpoint universal biomarkers that can be used for patient selection. Moreover, adaptive responses in reaction to molecular targeted drugs may influence (initial) sensitivity, as has been demonstrated for bevacizumab which induces compensatory upregulation of VEGF-C and VEGF-D by cancer cells.[12] More preclinical models that reflect the existing biological diversity between and among ovarian cancer histological subtypes are needed to understand tumortype-specific mechanisms responsible for drug sensitivity.

Preclinical studies aimed at unraveling mechanisms of targeted drug resistance might provide insight in potential biomarkers as well as providing a rationale for effective combination therapies. Since the nature of these mechanisms is likely to differ between cytotoxic (*e.g.* proapoptotic) and cytostatic (*e.g.* antiangiogenic) therapies, different experimental approaches and techniques should be adopted in their preclinical investigation.

Exposure to chemotherapeutic or death receptor-targeted drugs will result in the death of ovarian cancer cells sensitive to these treatments, making it likely that clonal selection plays an important role in (acquired) resistance. Genetic or epigenetic screening of cell lines or tissue samples before treatment and after resistance has occurred may therefore be the best initial approach. Data from such studies can be compared to data obtained when analyzing sensitive *versus* primarily insensitive cancers, to assess overlap between intrinsic and acquired resistance.

In the case of other targeted drugs which do not (primarily) induce cell death, like mTOR inhibitors, resistance could occur in the same cells which were initially

sensitive to the study drug. This could be a consequence of rescue or feedback mechanisms activated in response to treatment. Examples of such mechanisms are emerging for many molecular targeted agents. Induction of HIF-1α activity in response to angiogenesis inhibition could paradoxically cause a more aggressive tumor phenotype upon treatment with, for example, the VEGFR-targeted TKI sunitinib.[24] Also, for mTOR inhibitors compensatory feedback mechanisms have been described, mainly resulting in Akt activation.[25] In some instances, these feedback mechanisms may induce a state comparable to oncogene addiction, without the need for activating mutations or other underlying (epi)genetic aberrations. Proteomic or kinomic analyses might serve to understand (acquired) resistance to cytostatic molecular targeted therapy. Creation of isogenic models for resistance to molecular targeted agents, which has also been done in efforts investigating resistance to chemotherapy, could be of value. It is of interest to develop inducible knock-out systems for *in vivo* studies to further explore the importance of feedback-mediated drug resistance, for example by generating models stably transfected with tetracycline-inducible insulin receptor substrate-1 (IRS-1) and HIF-1α short hairpin RNA (shRNA) expression vectors.

As upfront biomarkers often fail, it is of interest to see whether the early effect of the study drug on the tumor can be measured. This can be done with repeat biopsies, but this necessitates multiple invasive procedures. An alternative could be to longitudinally collect circulating tumor cells (CTCs), although this does not take into account the influence of interactions between cancer cells and the tumor microenvironment.[26] Molecular imaging has the advantage of providing insight into the actual tumor biology, if the putative biomarker is accessible for tracer binding (*i.e.* membrane- or matrix-associated) or intracellular trapping. ^{89}Zr-bevacizumab is an interesting candidate biomarker, considering that solely blocking VEGF-A with bevacizumab has shown clinical efficacy in ovarian cancer trials (see **Chapter 2**).[27-29] This tracer could be of value as an early predictive biomarker for angiogenesis inhibitors capable of reducing VEGF-A levels, including mTOR inhibitors as demonstrated in **Chapter 4**, or heat shock protein 90 (HSP90) inhibitors. Ongoing clinical studies will provide answers concerning the clinical utility of ^{89}Zr-bevacizumab. Similarly, tracers are being developed to assess DR4/5 expression to select patients for death receptor-targeted therapy.[30]

To conclude, implementation of antiangiogenic and proapoptotic drugs is likely to depend on the discovery of biomarkers for patient selection. Establishing solid biomarkers will rely on understanding ovarian cancer biology and the mechanistic

basis of drug sensitivity and resistance. Therefore, an effort should be made in improving and developing preclinical models that accurately reflect the existing biological diversity between and among ovarian cancer histological subtypes.

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