Summary, discussion and conclusions

The group of alkylphospholipids (APLs) represents a heterogeneous class of synthetic lipids that has been studied as anti-cancer agent for more than 2 decades. These drugs seem to be particularly promising to target leukemic malignancies. Edelfosine has been used as a purging agent for over a decade [1,2]. Recently, Mollinedo and coworkers reported promising selective activity of perifosine and edelfosine against patient-derived and cultured multiple myeloma cells, while normal bone marrow cells were spared [3]. Although most APL analogues have shown potent anti-tumor activity in pre-clinical models, clinical use has been limited, mainly due to gastrointestinal side effects. Perifosine (D-21266) is a promising APL analogue, being suitable for oral application [4]. Because the mode of action of APLs is distinct from classical anti-cancer agents that generally target the DNA, these lipids have been considered attractive candidates for combined use with radiation [5]. Targets underlying the rationale of combining APLs with radiotherapy include survival and proliferation signaling through PKB/Akt and MAPK pathways, which are blocked by APLs [5-7]. These pathways are often upregulated in tumor cells and may contribute to radioresistance. In addition, treatment with APLs results in the activation of the SAPK pathway. This stress-induced pathway was recently shown to play a crucial role in the induction of apoptosis after treatment with APLs, both as single modality and combined with radiation [6]. For perifosine and other APLs, an enhanced apoptotic response in leukemic cells was shown after combined treatment with radiation [6]. This thesis builds on these results and describes the stepwise process of testing perifosine as radiosensitizer, from in vitro mechanistic investigations via in vivo proof-of-concept studies to a clinical phase I trial.

Chapter 1 gives a general introduction of this thesis and provides an overview of clinical applications of APLs to date. Furthermore, it discusses the molecular targets of APLs that underlie the rationale to combine these agents with radiotherapy.

Chapter 2 covers the majority of the in vitro studies of this thesis. Prior to their cytotoxic action, APLs need to be internalized by tumor cells. Chapter 2.1 focuses on raft-dependent endocytosis of APLs in lymphoma cells. This mode of drug uptake was previously identified to be essential for edelfosine to induce apoptosis in S49 cells [8,9]. Here we show similar results for the uptake of a panel of APLs. However, the relative importance of raft-dependent endocytosis seems tumor type-
dependent. We studied drug uptake in a second tumor model, the human squamous cell carcinoma KB, which was shown to be highly dependent on metabolic energy, but independent from lipid rafts (Chapter 2.2). In Chapter 2.3, we describe the use of in vitro models to characterize the anti-angiogenic potential of APLs. The sensitivity of 3 types of vascular endothelial cells to APLs was dependent on their proliferation status, because apoptosis was induced in proliferating, but not in confluent endothelial cells. In addition, all tested APLs inhibited the formation of capillary-like structures in a dose-dependent manner. These results suggest a novel mode of action of APLs that may contribute to their anti-tumor effect.

Chapter 3 describes the pharmacokinetics, tissue distribution in mice after oral administration and the in vivo anti-tumor activity of perifosine as single agent and in combination with radiotherapy. In Chapter 3.1, we report on the pharmacokinetic parameters after oral administration. We observed a slow pharmacokinetic profile and a high degree of drug stability. Drug accumulation was measured in 3 squamous cell carcinomas, and a correlation was established between both in vitro and in vivo uptake of perifosine, and drug sensitivity. In Chapter 3.2, we used the KB carcinoma model to further study the activity of perifosine, as single agent and combined with radiation. Several in vitro assays demonstrated enhanced cytotoxicity after combined treatment. Both single modalities induced dose-dependent tumor growth delay of KB xenografts, whereas combined treatment resulted in complete and sustained tumor regression. Histopathological analysis of tumor sections stained for the presence of active-caspase 3-positive cells, showed a clear induction of apoptosis after single agent treatment and more prominently, after combined treatment.

This thesis is concluded with a phase I study in patients with advanced solid tumors (Chapter 4.1). Patients received daily perifosine, combined with radiotherapy. The dose limiting toxicity was gastrointestinal, and a 150 mg daily dose was recommended for further phase II testing, to be started 1 week prior to radiation treatment.

The results presented in this thesis indicate that perifosine might be an effective agent to enhance the anti-tumor effect of radiation. Previously it was shown that the APL analogues edelfosine and miltefosine could enhance radiation-induced cell kill [10,11]. More recently, APLs were identified as potent enhancers of radiation-induced apoptosis in various leukemic cell lines [5]. Perifosine is one of these
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compounds, and has recently been evaluated as monotherapy in clinical trials [12,13]. We focused on the treatment of solid tumors, since carcinomas include the majority of cancers and are frequently treated with radiotherapy. In contrast to leukemic cells, no supra-additivity in apoptosis induction was found in carcinoma cells. This might be due to the fact that carcinoma cells in general are less prone to undergo apoptosis. Moreover, apoptosis is not necessarily the main mode of cell death in these systems. Radiosensitization by perifosine was demonstrated in the clonogenic survival assay, a long term in vitro assay which takes into account all types of cell kill. We found reduced clonogenic survival after radiation by perifosine in both KB and A431 cells. Although the mechanism of radiosensitization by perifosine remains unclear, we observed a strong exposure time-dependency. In view of this observation, survival signaling pathways such as MAPK and PKB/Akt remain relevant targets in perifosine-induced radiosensitization. In this context, it has recently been suggested that inhibition of the PKB/Akt pathway reduces DNA-PK activity, thereby interfering with DNA damage repair [14]. This may in part explain the radiosensitizing effect of perifosine.

The mechanism of action of APLs is not yet fully understood. It has been suggested that inhibition of angiogenesis could contribute to the antitumor effect of edelfosine [15]. Underlying this hypothesis is a selective induction of apoptosis by APLs in proliferating endothelial cells [16]. We studied the anti-angiogenic properties of edelfosine, perifosine and miltefosine in more detail using 2 well-established in vitro assays. Indeed, a dose-dependent inhibition of capillary-like structures was observed for all tested compounds. Whether APLs exert anti-angiogenic effects in vivo remains to be determined.

In addition to proliferation-dependent cytotoxicity in endothelial cells, APLs have been described to selectively target certain tumors. In this respect, the KB carcinoma is a tumor model which responds to APL treatment both in vitro and in vivo [17]. Importantly, in KB cells we observed an enhanced radiation response in vivo after oral perifosine treatment. This could, to a large extent, be explained by the high degree of drug uptake by these cells. We tested in vitro and in vivo perifosine accumulation in 3 human squamous cell carcinomas (KB, A431, and HNXOE). Drug uptake of these tumor models in vitro correlated both with uptake when grown as xenografts and with perifosine sensitivity. The high drug uptake, sensitivity and enhanced tumor response after combined treatment in KB cells indicate a crucial role of drug internalization both in vitro and in vivo. This is corroborated by the fact that most APL-resistant tumors display reduced drug
uptake [8,18,19]. Measurement of drug concentrations in (tumor) tissues from patients, which has not yet been feasible, would therefore be of great value to place our results obtained in the lab in clinical context.

We studied in more detail the role of endocytosis in uptake of perifosine and prototype edelfosine. Previous studies revealed a role of lipid rafts in the uptake of edelfosine by mouse lymphoma S49 cells [8]. We hypothesized that perifosine was internalized in a similar fashion. Indeed, edelfosine-resistant S49AR and S49siSMS1 cells, which lack sphingomyelin synthesis due to downregulated sphingomyelin synthase 1 expression [9], show a general resistance to the other APL analogues we tested, albeit to different extents. A clear tumor type dependency for raft-mediated uptake of APLs was demonstrated using the KB/KBr carcinoma model. The extensive drug accumulation by KB cells was shown to be severely compromised by ATP depletion and low temperature. This energy-dependent cellular uptake seems not to be related to endocytosis, since the basal endocytic pathways in the APL-resistant KBr cells, were unimpaired. Alternatively, it could be mediated by an unknown ATP-driven transporter. Identifying the mode of uptake in KB cells might allow the prediction of the response of other tumor types to APL treatment, both as single and multimodality treatment regimens.

Perifosine has been evaluated as single agent in multiple phase II studies but unfortunately, results are in general disappointing [20-27]. Therefore, instead of using perifosine as single agent, we focused on its potential radiosensitizing properties. This is a fundamentally different approach and usually requires lower, and thus less toxic drug doses. Furthermore, structure-activity studies might lead to the generation of APL analogues with an improved therapeutic index. In any case, their mechanism of action, distinct from classical anticancer regimens, makes APLs potentially most useful in combined modality strategies. Indeed, preclinical data is mounting that perifosine enhances not only the anti-tumor effect of radiotherapy, but also of other anticancer agents [28-32].

In conclusion, accumulating evidence suggests that APLs can complement conventional anti-cancer treatment in the clinic. The results presented in this thesis suggest that clinical use of perifosine in the treatment of solid tumors might be most effective in a combined modality approach. More efforts must be made to come to an evidence-based tumor treatment strategy. When there is a role of APLs beyond the experimental use as anti-cancer agents, this role will be limited to distinctive tumor types as is the case with most available anti-cancer treatments. To achieve a patient-tailored anti-cancer treatment, more preclinical data need to
be generated concerning markers predicting tumor response in vivo. In our research, we found a one-to-one relationship between APL uptake and response in multiple tumor models, suggesting that components in pathways involved in uptake of amphiphilic molecules are in this respect attractive candidate markers. Evidence is accumulating that the uptake routes of these types of molecules include both endocytic internalization pathways and more specific ATP-driven transporters, as appears to be the case in the KB tumor model. A possible identification of this transporter in KB cells and subsequent screening for the presence and expression of this and other (genetically) related transporters in radioresistant tumor cell lines and patient-derived tumor tissue might be informative on the applicability of APL treatment in clinical anti-cancer therapy. When the mechanism of entry into tumor cells is better understood, unraveling of the complex mechanism of APL-induced cytotoxicity will be the next challenge.

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