Effects of anticancer alkyl-lysophospholipids on cell death and survival
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Summary and Discussion
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Alkyl-lysophospholipids (ALPs) represent a heterogeneous group of unnatural lysophospholipids with anticancer properties. ALPs have been shown to preferentially target malignant cells, leaving normal cells relatively unaffected. Unlike most classical chemotherapeutic drugs that target the DNA, ALPs mainly exert their effects on membranes where they interfere with the biosynthesis and turnover of phospholipids. As a consequence, mitogenic signaling via the mitogen activated protein kinase (MAPK/ERK) pathway is inhibited. In addition, in combination with other cytostatic regimens, such as ionizing radiation, ALPs cause an enhancement of the cytotoxic effect.

This thesis describes the studies that were undertaken to better understand the mechanism of action of ALPs and to investigate their interaction with ionizing radiation. We found that three clinically relevant ALPs: Et-18-OCH₃ (Edelfosine), HePC (Miltefosine) and the recently developed HePC analogue octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate (Zentaris compound D-21266, Perifosine) greatly enhanced radiation-induced apoptosis. Furthermore, ALPs not only inhibited MAPK/ERK activity, but like ionizing radiation, stimulated the stress-activated protein kinase (or c-Jun N-terminal kinase) SAPK/JNK cascade within minutes after treatment. A dominant-negative mutant of c-Jun (natural substrate of SAPK/JNK) inhibited both radiation- and ALP-induced apoptosis, indicating a requirement for the SAPK/JNK pathway in the tested cell types (Chapter 2).

When we investigated the effects of ALPs on MAPK/ERK activity in more detail, we unexpectedly found in the human vulva carcinoma cell line A431 that subapoptotic, nanomolar concentrations of ALPs induced internalization of the epidermal growth factor receptor (EGFR) and a transient MAPK/ERK activation. We found no evidence for ALPs acting via G-protein coupled receptors and/or transactivation of EGFRs. Hence, we propose that ALPs induce subtle changes in the lipid microenvironment of the EGFR, resulting in clustering and internalization of the EGFR and concomitant MAPK/ERK activation (Chapter 3).

Another pathway that more recently became implicated in the regulation of apoptosis is the PI 3K-Akt/PKB survival pathway. This pathway, originating from the plasma membrane, is activated by a number of growth factors and can be inhibited by a variety of stimuli that induce apoptosis. We therefore
investigated the effect of ALPs on this pathway and demonstrated for the first time that ALPs inhibit the PI 3K-Akt/PKB pathway in a dose-dependent manner (Chapter 4).

When we further explored the differential cytotoxic effect of ALP between tumor and normal endothelial cells, we found that the sensitivity of endothelial cells to undergo ALP-induced apoptosis was dependent on the proliferative status of these cells. Whereas confluent, non-dividing endothelial cells failed to undergo apoptosis, proliferating endothelial cells showed significant levels of apoptosis after ALP-treatment. Based on these observations, we hypothesized that ALPs interfere with new blood vessel formation. By using an in vitro angiogenesis model, we studied the effect of ALPs on the ability of endothelial cells to invade a three-dimensional human fibrin matrix and form capillary-like tubular networks. We demonstrated that ALPs, but not their structural analogues such as Platelet Activating Factor 18 (PAF-18), inhibit both vascular endothelial growth factor/tumor necrosis factor α (VEGF/TNFα) and basic fibroblast growth factor (bFGF)/TNFα mediated tubule formation in a dose-dependent manner (Chapter 5).

More studies are currently being directed at elucidating the mechanism of the anticancer effect of ALPs. In particular, how these compounds affect the formation of lipid second messengers, that are involved in anti- and pro-apoptotic signaling, and how this initiates apoptosis are subjects of study in our lab. Using an ALP-sensitive mouse T-lymphoma cell line (S49) and a ALP-resistant variant (S49AR), it was found that ALPs inhibited phosphatidylcholine (PC) biosynthesis at the CTP:phosphocholine citidyltransferase (CT) step. ALP-induced apoptosis in S49 cells could be prevented by providing these cells with an alternative pathway to synthesize PC, i.e. the acylating of exogenously supplied lysoPC. PC synthesis was unaffected in S49AR cells, which did not undergo apoptosis after ALP-treatment. These results indicate that the continuous rapid PC synthesis is essential for cell survival and that it is an important target for ALPs. By using 3H-labeled ALP it was found that ALPs are rapidly taken up by endocytosis and that this process is impaired in the S49AR cells. In addition, it was shown that the endocytosis of ALPs, the inhibition of PC synthesis and the subsequent apoptosis were fully dependent on the presence of intact lipid cholesterol- and sphingomyelin-rich membrane microdomains, known as lipid rafts1.
Summary and Discussion

The results that are presented in this thesis have identified ALPs as attractive candidates for introduction in clinical radiotherapy. They have formed the basis of the concept that a combined treatment of ALPs and radiotherapy results in improvement of the tumor response and the clinical outcome. Several ALPs have already been evaluated in clinical studies. The first clinical trials that were performed with Miltefosine showed that oral administration of this drug resulted in severe gastrointestinal toxicity, in particular nausea, anorexia, vomiting and diarrhoea. Promising results have been obtained with the topical application of Miltefosine, which appeared to be very effective in the treatment of skin metastasis of breast cancer and malignant lymphoma. In addition, Edelfosine was found to be effective as a purging agent in autologous bone marrow transplantation. The structural analogue of Miltefosine, D-21266 (Perifosine) was found to be more active and better tolerated than Miltefosine upon systemic administration in preclinical studies. For this reason, phase I studies with this compound have been performed in patients with advanced solid tumors in our institute and by others in patients with refractory neoplasms. The results of the study in our institute indicated that Perifosine was tolerable at doses ranging from 50-200 mg given daily for 21 consecutive days every 4 weeks. The dose-limiting toxicities included fatigue, nausea and vomiting, while no bone marrow toxicity was observed. Interestingly, the plasma concentrations that were measured in these patients fall within the same micromolar ranges as those that were applied in the in vitro studies described in this thesis.

Considering the potential interaction between Perifosine and radiotherapy, the efficacy and safety of Perifosine in combination with locoregional radiotherapy is currently being evaluated in a phase I study in patients with locally advanced inoperable solid tumors. In this study, also the incidence of apoptosis will be determined in pre- and post-treatment tumor biopsies and correlated with the biological response. In addition, ALP concentrations will be measured in post-treatment tumor biopsies to determine intratumoral drug levels and to assess drug delivery. In parallel studies performed in experimental animal tumor models, including nude mice bearing solid tumor xenografts, the mechanisms of the synergy between ALP and radiation-induced cytotoxicity are currently being investigated.

In summary, we have shown that ALPs initiate pro-apoptotic signaling (through SAPK/JNK) and inhibit anti-apoptotic signaling (through MAPK/ERK and PI 3K-Akt/PKB). Because ALPs appear to preferentially target malignant cells and enhance radiation-induced apoptosis, this type of modulation
Summary and Discussion

may provide a basis for selective and efficient tumor cell kill. The additional inhibitory effect on angiogenesis may significantly contribute to the anti-tumor effect of these compounds.


