Alkylphospholipids combined with radiotherapy; translational studies

Nederlandse titel:
Alkylfosfolipiden gecombineerd met radiotherapie; translationele studies

Cover: Representation of translational research, from drug development to the targeting of cultured cancer cells and xenografts. The back cover shows notes from my lab journal and illustrates the underlying intensive experimentation at the lab (which was only rarely this sloppy).

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VRIJE UNIVERSITEIT

**Alkylphospholipids combined with radiotherapy;
translational studies**

ACADEMISCH PROEFSCHRIFT

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door

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geboren te Zaanstad

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Introduction

Chapter 1.1

General Introduction

General introduction

Combined treatment consisting of radiation and chemotherapy has improved clinical outcome after radiotherapy in a variety of tumor types [1]. Efforts have been made to further enhance tumor response and decrease normal tissue toxicity by combining radiation with more specific, molecular targeted agents [2]. The class of alkylphospholipids (APLs) is a heterogeneous group of synthetic, single-chain phospholipids that has been studied as anti-cancer agent for decades. These compounds accumulate in cellular membranes [3], thereby affecting both apoptotic and survival signal transduction pathways. Modes of action underlying the anti-tumor effect of APLs include activation of the pro-apoptotic SAPK/JNK pathway and inhibition of the MAPK/ERK and PI3K-Akt/PKB survival pathways [4-7]. One of these compounds, perifosine, was designed for oral use [8] and has been tested in clinical phase I trials as monotherapy in patients with advanced solid tumors [9,10]. Because their mechanism of action is distinct from classical anti-cancer drugs, APLs are considered as attractive compounds to combine with radiotherapy [4,11]. Indeed, in addition to potent anti-tumor properties as single agents in preclinical models, APLs have shown promising results *in vitro* when combined with radiation. For example, miltefosine reduced clonogenic survival after radiation [12], whereas an enhanced apoptotic response was observed when leukemic cells were treated with perifosine, miltefosine or edelfosine, combined with radiation [5].

Outline of this thesis

The aim of this thesis is to provide a solid basis for further clinical development of APL treatment combined with radiotherapy. Here we describe the translation of perifosine treatment in combination with radiation, from *in vitro* and *in vivo* assays to a clinical study. *Chapter 1.2* provides the rationale and current status of this combined modality approach. In *Chapter 2.1* and *2.2*, the first step in tumor cell kill, drug uptake by tumor cells, and subsequent cytotoxic effects are discussed. We studied the role of lipid raft-mediated endocytosis of APLs in S49 lymphoma cells, and reported the involvement of this mode of uptake for all tested APL analogues. However, uptake of APLs is a tumor type-specific process, since in the squamous cell carcinoma KB, a tumor cell line that actively incorporates high amounts of APL, uptake was not related to such endocytic activity. A differential drug uptake by proliferating versus confluent endothelial cells underlies the hypothesis that APLs could inhibit angiogenesis *in vitro*. This hypothesis was confirmed using 2 assays,

in which APLs showed potent inhibition of the formation of bloodvessel-like structures *(Chapter 2.3)*. *Chapter 3* includes experimental animal studies on perifosine treatment, as single oral treatment and in combination with radiation. Perifosine showed slow pharmacokinetics, extensive body distribution and tumor accumulation in mice after oral administration. Of the 3 squamous cell carcinomas tested, the KB tumor was the most sensitive and displayed the highest degree of perifosine accumulation *in vitro* and as xenograft. In this tumor model we have found promising anti-tumor activity after treatment with perifosine in combination with radiotherapy, both *in vitro* and *in vivo*. This thesis is concluded with a phase I and pharmacokinetic study in which we report a good tolerability of daily intake of perifosine combined with radiotherapy *(Chapter 4)*. These results were the basis for a randomized phase II trial in patients with non-small cell lung cancer.

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Chapter 1.2

Rationale and clinical application of alkylphospholipid analogues in combination with radiotherapy

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Abstract

Concurrent treatment with radiotherapy and chemotherapy has emerged as an effective strategy to improve clinical outcome of cancer. In addition to combining radiation with classical anti-cancer agents, several new biological response modifiers are under investigation in pre-clinical and clinical studies. Synthetic alkylphospholipids (APLs) are anti-cancer agents that in contrast to most anti-cancer drugs do not target DNA, but insert in the plasma membrane and subsequently induce a broad range of biological effects, ultimately leading to cell death. APLs kill tumor cells directly by induction of both apoptotic and non-apoptotic cell death, and indirectly by interference with critical signal transduction pathways involved in phospholipid metabolism and cell survival. Due to their distinct mode of action, these drugs are considered as attractive candidates to combine with radiotherapy. In this review, we discuss several APLs that have reached clinical application. These include first-generation alkyl-lysophospholipids edelfosine and ilmofosine, second-generation alkylphosphocholine prototype miltefosine and the more recently developed analogues perifosine and erucylphosphocholine. We focus on mechanisms of action and the rationale to combine these agents with radiotherapy. The preclinical results on molecular targeting underlying this approach are reviewed, concluded with first clinical data on combined treatment of radiotherapy with perifosine.

Introduction

Approximately 50% of all cancer patients are treated with radiotherapy, either as primary treatment with curative intent or for palliation of cancer related symptoms. Likelihood of tumor response after radiotherapy is determined by the total radiation dose required for tumor cell kill and varies between tumor types, ranging from very radiosensitive low grade lymphomas (90% tumor control at 4 Gy [1]) to notoriously radioresistant malignant gliomas (not responsive at clinically achievable doses). During the last decades, many approaches have been applied to further improve treatment outcome after radiotherapy. One of these approaches involves the combination with agents that have the capability to increase the sensitivity of cells to radiation (radiosensitizers). Most conventional radiosensitizers are cytostatic agents themselves (platinum analogues, 5-fluorouracil, gemcitabine) and target primarily the structure and function of the DNA, integrity of which is essential for DNA synthesis and cell division [2]. Although chemoradiation has resulted in improved local tumor control in various solid tumors [3], its application is limited by an increase in normal tissue toxicity. Various molecular targeted agents, more specifically modulating signal transduction pathways in tumor cells, have the potential to further enhance tumor response to radiotherapy while limiting side effects [4,5]. In this review, we focus on the class of alkylphospholipids (APLs) and their use in anti-cancer therapy, with emphasis on clinically relevant analogues. In particular, we address cytotoxic effects of APL analogues on cellular targets, supporting the rationale that this class of membrane-targeted drugs could enhance the efficacy of radiotherapy. Pre-clinical results are discussed and finally first clinical data and implications are provided.

Alkylphospholipids as anti-cancer agents in historical perspective

APLs can be classified according to their molecular structure. First-generation APLs are distinguished by ether-linked aliphatic side-chains (a long and a very short one) to a glycerol backbone, and are referred to as alkyl-lysophospholipids (ALPs), or ether lipids. This class of compounds has been developed during the early 1970's, when these lysolecithin analogues were synthesized for immunomodulating applications [6]. During the process of finding biologically stable analogues, it was demonstrated that these ALPs possessed anti-proliferative properties in tumor cells [7]. To date, the most widely studied ALP is *rac*-1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine (Et-18-OCH$_3$; edelfosine, Fig. 1).
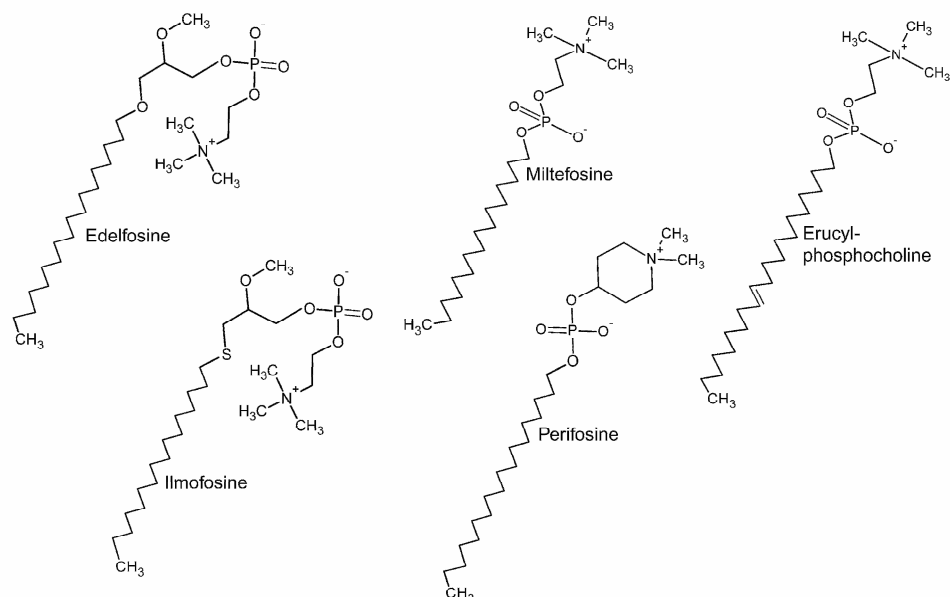
Figure 1. Chemical structures of clinically tested APLs. The alkyl-lysophospholipid (ALP)-prototype edelfosine is characterized by an ether-linked aliphatic side chain to a glycerol backbone. A decrease in alkyl chain length (C$_{18}$ → C$_{16}$) and substitution of the linker-oxygen by sulfur yields the thio-ether lipid ilmofosine. In the chemical structure of the alkylphosphocholine (APC)-prototype miltefosine, the glycerol moiety is lacking. Erucylphosphocholine differs from miltefosine only by a longer chain length (C$_{16}$ → C$_{22}$) and the introduction of a cis-double bond. Finally, an alkyl chain modification (C$_{16}$ → C$_{18}$) and substitution of the choline headgroup of miltefosine by a cyclic aliphatic piperidyl residue yields perifosine.

In addition to potent anti-tumor activity *in vitro*, edelfosine has shown *in vivo* efficacy in tumor models in both mouse [8,9] and rat [10,11]. Despite high anti-tumor activity of edelfosine in preclinical models, clinical activity is low, since an early clinical phase I trial with edelfosine in patients with advanced malignant disease showed only 2 partial remissions with non-small cell lung cancer (NSCLC) after intravenous treatment and none after oral treatment [12]. A multicenter phase II trial in patients with advanced NSCLC demonstrated stable disease in 87% of all cases. However, response rate and time to tumor progression were unfavorable when compared with an historical group of patients treated with ifosfamide and etoposide [13]. The only clinical application of edelfosine at this moment is for purging purposes of bone marrow in acute leukemia. The basis of this clinical use was the observation that edelfosine selectively kills leukemic cells, thus sparing normal bone marrow cells both *in vitro* and in laboratory animals [14]. Bone marrow purging with 75 µg/mL edelfosine for 4 h could be done safely in patients with

acute leukemia [15], and a subsequent phase II study showed low toxicity and results not dissimilar from purging trials with other agents [16].

A second ALP that has reached the clinic is the thioether variant of edelfosine, 1-hexadecyl-thio-2-methoxymethyl-*rac*-glycero-3-phosphocholine (BM 41.440; ilmofosine, Fig. 1). Ilmofosine has demonstrated both *in vitro* and *in vivo* anti-tumor activity in a variety of tumor cells [17-24]. Ilmofosine has been tested in phase I studies as a 2 h/weekly infusion in different treatment schedules [25,26]. Similar to edelfosine, gastrointestinal toxicity was dose limiting, and a dose of 450 mg/m^2 was recommended for phase II trials. However, uncertainty exists about clinical activity since these phase I trials and a phase II trial in patients with non-small cell bronchogenic carcinoma [27] did not report objective responses.

Structure-activity studies led to the generation of the group of alkylphosphocholines (APCs), in which the glycerol backbone is deleted [28]. Although the APC-prototype hexadecylphosphocholine (HePC; miltefosine, Fig. 1) is degraded by phospholipases yielding choline, phosphocholine and 1,2-diacylphosphatidylcholine after systemic treatment [29,30], potent anti-tumor activity *in vitro* against a variety of tumors has been described [30-33]. *In vivo* data are less clear-cut, since miltefosine reduced growth of the human squamous cell carcinoma KB xenograft in mice and both methylnitrosourea- and 7,12-dimethylbenzanthracene-induced mammary carcinomas in rats [34,35], but lacked activity in rats with serially-transplanted mammary tumors [11], benzo(a)pyrene-induced fibrosarcomas and acetoxymethylmethylnitrosamine-induced colorectal carcinomas [36]. Due to hemolytic effects when administered intravenously [37], miltefosine could only be developed as oral and topical formulation. A first dose escalation study reported nausea and vomiting as dose limiting, and recommended a 150 mg/day dose for further phase II testing. Unfortunately, lack of activity in patients with advanced soft tissue sarcoma [38], metastatic colorectal cancer [39] and squamous cell head and neck cancer [40] led to discontinuation of oral treatment development against cancer. First evidence of clinical activity was obtained when miltefosine was applied topically for treatment of skin metastases of breast cancer [41]. Subsequent phase II trials showed responses to topical treatment in patients with cutaneous lymphoma [42] and cutaneous breast cancer metastases [43,44]. It is worth noting that in addition to application in anti-cancer therapy, APLs have shown activity against protozoal disease [45], and miltefosine is now widely used as an effective oral treatment against leishmaniasis [46].

Replacing the choline moiety in miltefosine by a heterocyclic nitrogen group was

expected to yield derivates with an improved metabolic stability and therapeutic index [47]. One of these compounds is octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate, (D-21266; perifosine, Fig. 1), in which a piperidine has been attached to the alkyl-phosphate chain. This structural change apparently prevents degradation *in vivo*, since hardly any degradation products could be measured in plasma of mice treated with perifosine [48]. Although to date the available pre-clinical and clinical data remain limited, reports on anti-cancer activity are mounting. Perifosine showed toxicity similar to miltefosine against a variety of tumor cell lines *in vitro*, and the first *in vivo* results indicated equipotent anti-tumor properties in rats bearing dimethylbenzanthracene-induced mammary tumors, but with a far better tolerability [49]. Additional *in vivo* data are scarce, but a selective activity in some tumor types as is seen for the parental compound miltefosine is likely. Indeed, the relatively high drug levels measured in the miltefosine-responsive KB xenograft in nude mice after oral administration of perifosine suggest an *in vivo* selectivity for this tumor compared to A431 and HNXOE carcinomas [48]. The first phase I study in patients with solid tumors revealed a similar toxicological profile as miltefosine since gastrointestinal toxicity was dose limiting, and the maximum tolerable dose was established at 200 mg/day for 3 weeks [50]. A dose schedule involving a loading dose of 150 mg x 6, and a maintenance dose of 100 mg/day could be well tolerated and revealed modest activity in sarcoma and possibly, renal cell carcinoma [51]. At this moment, perifosine is tested in multiple clinical phase II trials. A detailed overview of clinical trials conducted with perifosine is shown in Table 1. Unfortunately, few clinical responses have been reported so far. No tumor response was reported in patients with metastatic melanoma [56], androgen independent prostate cancer [57], metastatic or locally advanced soft tissue sarcoma [58] or recurrent or metastatic head and neck cancer [59].

Currently, erucylphosphocholine (ErPC, Fig. 1) is being used in preclinical studies as a new potent alkylphosphocholine. Although erucylphosphocholine only differs from miltefosine in alkyl chain length and the presence of a double bond, striking differences were found in pharmacological behavior. This relatively minor structural modification increased hydrophobicity resulting in the formation of lamellar membranous erucylphosphocholine-structures, which prevents development of hemolytic toxicity [60]. This allows erucylphosphocholine to be applied intravenously, resulting in comparable tumor responses at 5x lower doses and with reduced gastrointestinal toxicity [61], suggesting a low bioavailability. Although

erucylphosphocholine displays dose limiting toxicity which is common for APLs, it differs from other analogues by distinct accumulation in brain tissue [62]. In view of the encouraging responses of brain tumors both *in vitro* [63,64] and *in vivo* [63], erucylphosphocholine is considered to be the most promising APL to date, with possible application in treatment of brain tumors. No clinical data on erucylphosphocholine are available yet.

Table 1. Clinical studies with perifosine

Phase	Tumor type (n)	Schedule	Toxicity	CTC	MTD (mg)	Response	Ref.
I	Solid, all types (22)	50-350 mg/d 3 wks	Nausea Vomiting Diarrhoea Fatigue	1-3	200	SD 2/16	[50]
I	Solid, all types (42)	L:400-900 mg M:50-150 mg/d	Nausea Vomiting Diarrhoea Fatigue	1-3	L:600 M:100	PR 1/36 SD 4/36	[51]
I	Solid, all types (21) 17/21 NSCLC	50-200 mg/d during radiation	Nausea Vomiting Anorexia Diarrhoea Fatigue	1-4	150	PR 5/21 CR 6/21 SD 10/21	[52]
II	Metastatic/advanced breast cancer (9)	L:300 mg d1 150 mg d2-21 q 4 wks	Vomiting Nausea Diarrhoea Anorexia	1-2	NR	SD 3/5	[53]
II	Advanced pancreatic cancer (19)	L:1050 mg d1-2 M:150 mg d3-21	Fatigue Anorexia Abdom. pain	NR	NR	SD 11%	[54]
II	Recurrent prostate cancer (25)	L:900 mg d1 M:100 mg d2-28 q 4 weeks	Arthritis Hyponatremia Hyperuricemia Vision change	3	NR	PSA 3/22	[55]
II	Metastatic/recurrent melanoma (18)	L:900 mg d1 M:150 mg d2-21	Fatigue Nausea	1-3	NR	SD 3/18	[56]
II	Metastatic prostate cancer (19)	L:900-600 mg d1 M:150 mg d2-21	Fatigue Nausea	1-2	NR	No clinical activity	[57]
II	Inoperable soft tissue sarcoma (16)	L:900-300 mg d1 M:150 d2-21	Fatigue Diarrhoea Nausea Vomiting	1-3	NR	SD 4/16	[58]
II	Recurrent/metastatic HNSCC (19)	L:900 mg d1-2 M:100 mg	Constipation Nausea Vomiting Anorexia	NR	NR	SD 1/19	[59]

Abbreviations: CTC = common toxicity criteria grade, MTD = maximum tolerated dose, NR = not reported, L = loading dose, M = maintenance dose, SD = stable disease, PR = partial response, CR = complete response, PSA = decline in prostate specific antigen > 50%, HNSCC = head and neck squamous cell carcinoma.

Mechanism of action

Interference with phospholipid turnover and lipid signaling. APLs differ from classical chemotherapeutic agents, since their primary target is the plasma membrane and not the DNA. Due to their chemical structure, they easily insert into the plasma membrane where they can act as detergent resulting in direct cell lysis. At more clinically relevant concentrations, they interfere with multiple cellular processes, including phospholipid turnover and signal transduction pathways. The biosynthesis and turnover of phospholipids are crucial for the maintenance of membrane integrity in the broadest sense, and thereby for cell survival. After internalization, edelfosine and miltefosine interfere with phosphatidylcholine (PC) synthesis by inhibition of CTP:phosphocholine cytidylyltransferase (CCT) [65], the key enzyme controlling *de novo* PC synthesis [66,67] (Fig. 2).
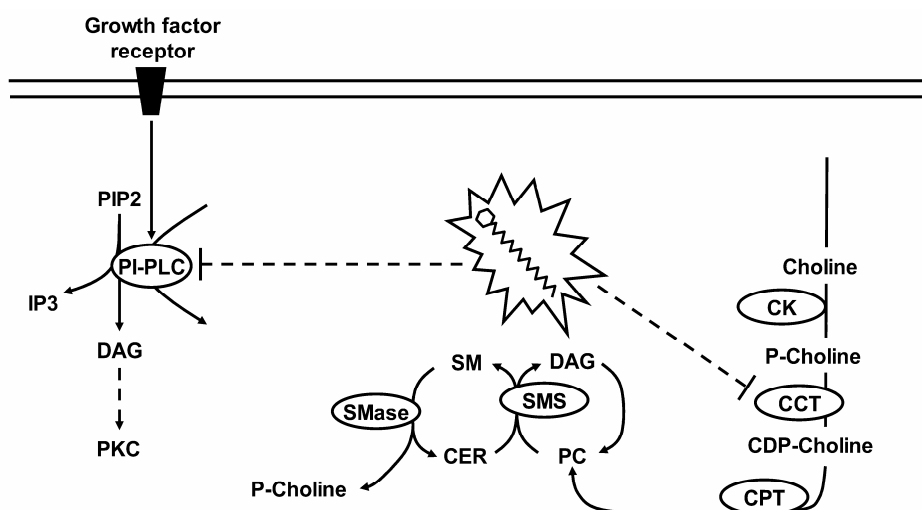
Figure 2. APLs interfere with phospholipid turnover. A key event in the cytotoxic action of APLs (in the figures represented by perifosine, but all these compounds are presumed to act by a similar fashion) is the inhibition of PC synthesis. Abbreviations not mentioned in the text: CDP-choline, cytidine 5'-diphosphocholine; CER, ceramide; CK, choline kinase; CPT, choline phosphotransferase; P-choline, phosphocholine; PKC, protein kinase C; PLC, phospholipase C; SM, sphingomyelin; SMS, sphingomyelin synthase; SMase, sphingomyelinase.

This directly triggers apoptosis, as is demonstrated by addition of lysoPC, an exogenous precursor for PC, which prevented edelfosine-induced cytotoxicity [68,69]. However, inhibition of PC synthesis has been suggested only to play a partial role in cell death induced by APCs [70]. It remains unclear whether this

discrepancy is dependent on the APL analogue used, or the cell type studied. Another cellular target described in the literature is the activation of phosphatidylinositol-specific phospholipase C (PI-PLC), subsequently blocking cleavage of phosphatidylinositol 4, 5 diphosphate (PIP2), formation of inositol 1,4,5-triphosphate (IP3), diacylglycerol (DAG) and activation of protein kinase C (PKC) [71] (Fig. 2). Contradictory results have been reported about the effect of hexadecylphosphocholine on another enzyme involved in proliferation, namely phospholipase D (PLD). Wieder and coworkers reported an acute, dose-dependent stimulation of PLD after treatment [72]. More recently however, it has been postulated that abrogation of PLD activation after chronic exposure to hexadecylphosphocholine may be underlying its anti-tumor activity [73].

Induction of stress signaling and apoptosis. It has become evident that the plasma membrane is more than a physical barrier that separates the intracellular compartment of the cell from the external environment. Numerous cellular processes are regulated at the level of the cell membrane, including phospholipid turnover, lipid second messenger formation and signal transduction pathways that are crucial for life and death decisions. Interference with these processes by APLs can result in cell death, predominantly via induction of apoptosis [74].

A family of aspartate-specific proteases known as caspases, plays a key role in the executioning of apoptosis [75-77]. Initiator caspases (like caspase 8/9/10) act upstream of effector caspases (caspase 3/6/7) that are involved in the cleavage of substrates which are essential for cell survival. In general, programmed cell death is initiated via 2 distinct pathways [78]. The *extrinsic* 'death receptor dependent' pathway is activated by binding of extracellular ligands to the receptors of the tumor necrosis factor-receptor superfamily, including TNFR, CD95/Fas and TRAIL-R. Upon triggering of the receptor, a complex consisting of an adaptor protein and initiator caspases is formed (death-inducing signaling complex – DISC), which is essential for death receptor-mediated apoptosis [79]. The *intrinsic* 'mitochondrial pathway' on the other hand, is regulated at the level of the mitochondria [80] by pro- and anti-apoptotic members of the Bcl-2 family [81]. The 2 pathways are interconnected by the Bcl-2 protein Bid. Bid is cleaved by caspase 8 as a consequence of death receptor ligation, and subsequently, cytochrome C is released by the mitochondria. This cascade results in the formation of a complex consisting of cytochrome C, Apaf 1 and caspase 9, also known as the 'apoptosome' (Fig. 3).
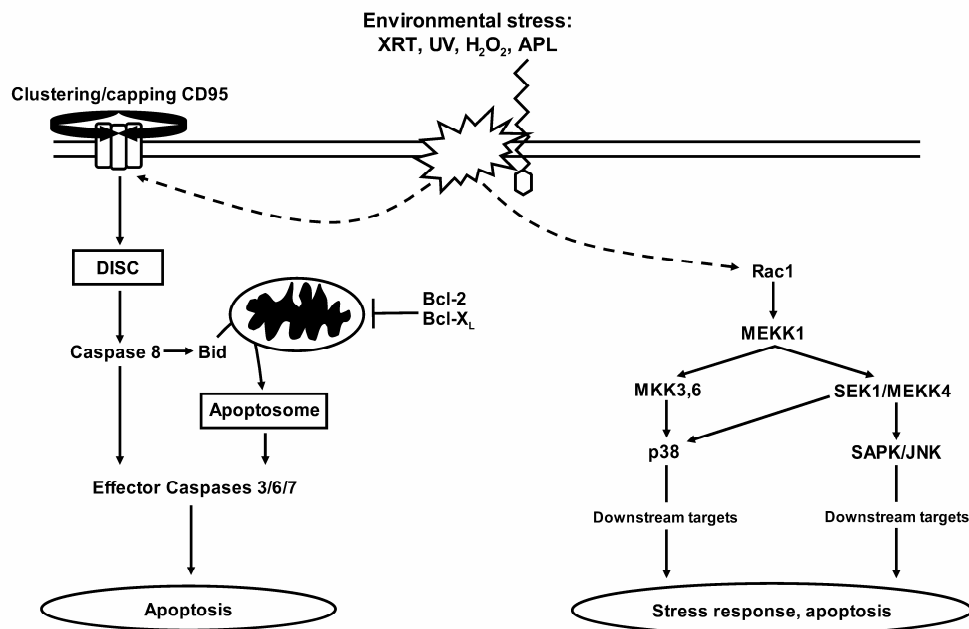
Figure 3. Apoptosis signaling and stress pathways induced by APLs. Treatment can result in both apoptotic and non-apoptotic cell death. Apoptosis induction can be direct, by activation of executioner caspases by caspase 8, or indirect by the amplification loop via the mitochondria.

Data on the mechanism by which ALPs and APCs induce apoptosis are controversial. Some investigators reported a Fas/CD95 death receptor-mediated induction of apoptosis, independent from its natural ligand FasL [74,82-84], whereas others excluded a role of the CD95 receptor in APL-induced apoptosis in general [85-87]. These investigators introduced a dominant negative FADD (receptor domain of CD95), thereby completely blocking CD95 receptor signaling. However, this modification did not rescue cells from APL-induced apoptosis. Involvement of the mitochondrial pathway in APL-induced apoptosis was demonstrated more recently [84,87-89].

I**nhibition of survival & proliferation pathways.** Cells are dependent on signals from the external environment for survival. Signaling pathways involved are often utilized by tumor cells to evade apoptosis and induce proliferation and ultimately survival after treatment. The G-protein RAS plays a central role in activation of these pathways (Fig. 4), and proteins acting at any level of these signal transduction cascades are therefore considered as potential pharmacological

targets. The serine/threonine kinase Akt is a component of the phosphatidylinositol 3'-kinase/Akt signal transduction pathway, which regulates a diversity of cellular processes, including apoptosis, proliferation, differentiation and metabolism. Three Akt-isoforms have been identified to date, Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ [90]. All isoforms are (co-) activated by components of the lipid kinase family of phosphatidylinositol-3 kinases (PI3Ks). Activation of the PI3K/Akt cascade is initiated by binding of a ligand to its growth factor receptor at the plasma membrane. After receptor activation, phosphatidylinositol-4,5-bisphosphate (PIP2) acts as substrate to generate the second messenger phosphatidylinositol-3,4,5-bisphosphate (PIP3). PIP3 recruits a subset of signaling proteins, including PDK and Akt, where Akt can be phosphorylated at Thr^{308} and Ser^{473}. PDK1 plays a central role in the phosphorylation of Akt at Thr^{308} [91], however the mechanism of Ser^{473} phosphorylation remains controversial. Dually-phosphorylated PKB/Akt is translocated to the nucleus, where it activates a number of downstream targets involved in cell proliferation, survival and differentiation [92]. The PI3K-Akt signaling pathway negatively regulates cell death by phosphorylation and inactivation of pro-apoptotic proteins Bad [93] and FKHRL-1 [94]. On the other hand, Akt promotes expression of anti-apoptotic molecules, for instance through activation of the NFκ-B pathway [95]. Other target proteins promote cell cycle progression and cell growth and include glycogen synthase kinase-3 (GSK-3), mammalian target of rapamycin (mTOR) and cyclin-dependent kinase inhibitors p21$^{CIP1/WAF1}$ and p27^{KIP1}.

Overexpression of the PI3K-PKB/Akt pathway is often associated with tumorigenesis [96,97] and subsequently with poor prognosis in cancer patients. The PKB/Akt pathway has extensively been implicated as a contributor to radioresistance [98-101]. In particular the epidermal growth factor receptor (EGFR) family has been targeted to overcome radiation resistance [102]. EGFR-activated PKB/Akt has been proposed to protect cells from radiation-induced apoptosis by multiple mechanisms, including phosphorylation of Bad and interference with apoptotic signaling at the level of the mitochondria [103]. These insights make PKB/Akt an attractive target for anti-cancer therapy and more specifically, for combined therapy. Several investigators have shown an enhanced tumor response to radiation after interference with the PKB/Akt signaling pathway [100,104]. In the last years, the PKB/Akt survival pathway has been identified as one of the targets of APLs [105,106]. Ruiter and coworkers showed a dose-dependent inhibition of insulin-induced PKB/Akt activation in tumor cells treated with edelfosine, miltefosine or perifosine. Interference with PKB/Akt by perifosine was studied in

more detail by Kondapaka *et al.*, who identified inhibition of recruitment of PKB/Akt to the membrane as mechanism of action [106]. This is likely due to inhibition of PI3K-mediated PIP3 formation [105].

A second mechanism by which APLs can interfere with survival of tumor cells is by targeting one of the mitogen-activated protein kinase (MAPK) pathways (Fig. 4).
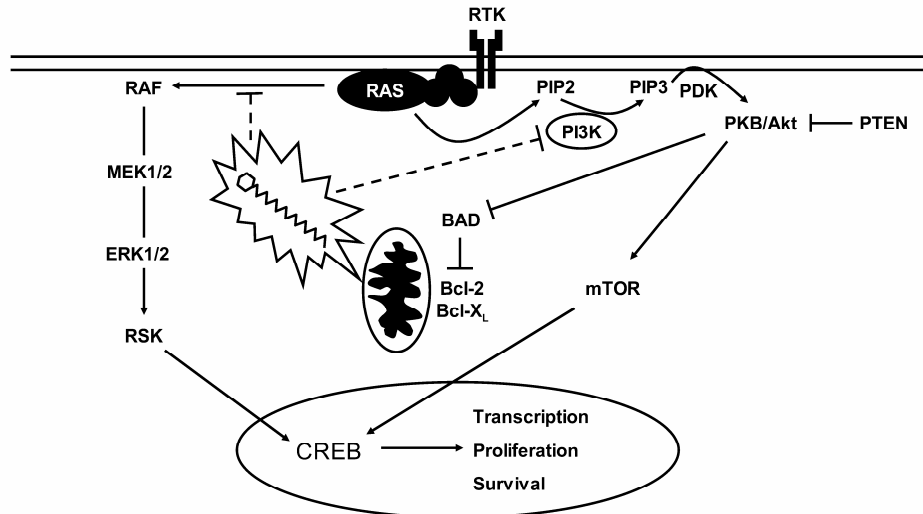
Figure 4. APLs block signaling of 2 prominent survival pathways downstream of RAS. The MAPK and PKB/Akt pathway can be activated by binding of growth factors to the receptor; activation of these pathways is associated with radioresistance of tumor cells.

The Ras/Raf/MEK/ERK signaling pathway is one of the best-characterized pathways downstream of RAS. ERK is frequently constitutively activated in tumor cells. RAS signaling is complex, and associated with counter regulatory processes, such as cell proliferation and cell cycle arrest. This review is limited to the role of MAPK signaling in proliferation and survival. After binding to a receptor tyrosine kinase, such as by EGF, PDGF or VEGF, RAS becomes activated and undergoes a conformational change, which results in the formation of a high affinity-binding site for RAF. RAF is consequently recruited to the membrane, where it can be phosphorylated at various sites and, in turn, can phosphorylate its substrate MEK. Downstream of this cascade can ERK, when activated, directly phosphorylate multiple transcription factors including c-Jun and c-Myc. Alternatively, ERK can activate transcription factor CREB via its downstream target RSK [107]. In addition to the promotion of proliferation, the MAPK pathway can negatively regulate the

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induction of apoptosis. Under certain conditions, RAF can translocate to the mitochondria and inactivate the Bad protein [108]. Bad indirectly promotes apoptosis by negative regulation of the anti-apoptotic Bcl-family. The ultimate outcome of MAPK signaling is diverse, and is dependent on several other factors like the cell type involved and the degree of phosphorylation at each level of this pathway. In recent years, APLs have been shown to be potent inhibitors of the MAPK-pathway [109,110]. One mechanism by which edelfosine inhibits MAPK signaling is by interference of the interaction of RAF with activated Ras, thereby reducing the levels of RAF that are translocated to the membrane [111].

Combination of alkylphospholipids and radiation

Evidently, the way by which APLs induce cytotoxicity is very complex, and involves numerous signal transduction pathways and a diversity of proteins in these pathways. Since some of these pathways have been implicated in influencing radiosensitivity and radioresistance of tumor cells, APLs have been considered attractive compounds to combine with radiotherapy. The first APL showing radiosensitizing properties was miltefosine [112]. However, this effect was limited to cell lines expressing an activated Ras oncogene. Berkovic *et al.* showed that miltefosine and edelfosine could reduce clonogenic survival after ionizing radiation, using the squamous cell carcinoma KB [113], a model cell system for APL cancer research. In addition, *in vitro* studies have identified perifosine as radiosensitizer in KB [5,114] and A431 [5] squamous cell carcinoma, and erucylphosphocholine in malignant glioma [115]. The inhibiting effects of APLs on survival and proliferation pathways, together with the induction of stress signaling led to the hypothesis that APLs would enhance radiosensitivity by enhanced induction of radiation-induced apoptosis [116]. This was tested in a lymphoma tumor model, and a prominent enhancement of radiation-induced apoptosis was found for edelfosine, miltefosine, and perifosine [110]. For edelfosine, this combination led to a synergistic apoptotic response. Crucial in this regard was activation of the SAPK/JNK signaling pathway (Fig. 3). This pathway is a component of the signal transduction pathway through mitogen-activated protein kinases (MAPK) that is mediated by the stress-activated protein kinase, also known as c-Jun N-terminal kinase (SAPK/JNK). This cascade is activated by inflammatory cytokines (TNFα, IL-1) and multiple environmental stress factors, like UV, heat shock, oxidation and ionizing radiation. SAPK, and its family member p38 are phosphorylated by SAPK/ERK kinase (SEK)1.

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Furthermore, p38 is a downstream target of MKK3 and MKK6. In turn, the p38 and SAPK/JNK-inducers are activated by MEKK1. An upstream regulator of MEKK1 is Rac1, and may be a primary target in the SAPK/JNK pathway. In addition, SAPK/JNK is able to directly induce mitochondrial-dependent apoptosis [117,118]. The SAPK/JNK pathway is involved in cell death induced by a variety of cellular stress factors, including CD95 stimulation, ionizing radiation and APL treatment [119-122]. Its role in enhanced radiation-induced apoptosis by APLs was demonstrated by the expression of a dominant-negative c-Jun, which led to a dramatic decrease in apoptosis induction by both single modalities as well as the combination [110]. Recently, enhanced radiation-induced apoptosis by erucylphosphocholine was linked to inhibition of PKB/Akt-mediated anti-apoptotic signaling [123].

The first translation of this combined strategy to an *in vivo* setting showed promising responses. Where escalating doses of radiation or perifosine induced dose-dependent tumor growth delay in mice, did a combination of both result in complete and sustained tumor remission at a clinically achievable perifosine plasma concentration [114]. Although the cytotoxic mechanism of action remains unclear, immunohistochemical analysis of tumor tissue after treatment indicated a prominent role of apoptosis, measured by caspase 3 activation. It should be noted that these proof-of-principle studies were done in a favorable experimental setting, using the highly responsive KB carcinoma. The issue of tumor specificity in this regard must be recognized, since this tumor displays a relatively high degree of drug internalization. A discrepancy between *in vitro* and *in vivo* radiosensitization was shown in a malignant glioma tumor model. Although De la Pena *et al.* showed clear radiosensitization by perifosine *in vitro*, subcutaneous gliomas did not show an enhanced response to radiation after treatment with perifosine [124]. Since only 1 dose schedule was tested, it is not clear whether an enhanced radiation response by perifosine could be obtained at optimal (clinically relevant) dose scheduling. Currently, only 1 clinical study on treatment with APLs combined with radiotherapy is available. A phase I trial was conducted in patients with advanced solid tumors, who were treated with fractionated radiotherapy concurrently with daily intake of perifosine [52]. Perifosine proved tolerable to a dose of 150 mg/day, combined with radiotherapy. Gastrointestinal toxicity was reported to be dose limiting.

Perspectives

APLs act distinct from DNA damaging agents, and are therefore attractive candidate drugs for combination therapy. Pre-clinical data on perifosine combined with radiation look all together promising. In addition, acceptable tolerability was reported in a phase I trial with patients with advanced solid tumors [52]. A randomized phase II study is underway to evaluate the activity of this combination in patients with locally advanced NSCLC.

Obviously, the generation of new APLs with a broader therapeutic window would increase their applicability in anti-cancer therapy. Optimal dose scheduling can further minimize normal tissue toxicity, which for all APLs tested to date is gastrointestinal. Due to their long elimination half-life, APLs are suitable for loading-maintenance dosing. This approach has been shown to result in stable mean plasma concentrations over time of study and a good tolerability in patients with advanced cancer treated with perifosine [51].

Data are lacking on *in vivo* tumor specificity of APLs. From *in vitro* studies, a clear difference in drug sensitivity is found among cell types. This difference is explained, at least partly, by selective drug accumulation. For instance, tumor cells that have gained resistance to APLs often display reduced drug accumulation [125-127]. In addition, selective cytotoxicity of edelfosine to leukemic cells compared to normal cells is correlated with uptake [74] and the highly sensitive KB carcinoma incorporates a relatively high amount of drug both *in vitro* and *in vivo* [48]. Studies are warranted to help to understand why some tumors incorporate lethal amounts of these lipids whereas other tumors do not. More insight in mechanisms of drug uptake might lead to the identification of markers for drug uptake and subsequently for clinical tumor response.

Although PKB is considered to be one of the main targets underlying the rationale for this combined strategy, the importance of PKB/Akt targeting in APL-induced radiosensitization remains to be confirmed. A more quantitative analysis of the effect of APL treatment *in vivo* on PKB/Akt and other candidate proteins would give more insight in the role for these proteins in the response to radiation. Effective biological targeting is an obvious requirement to be fulfilled both in monotherapy as well as in combined therapy. Whether this requirement can be met is determined by sufficient drug accumulation by tumor tissue on one side and (lack of) normal tissue toxicity on the other.

Due to its distinct mode of action, APLs have been tested in combination with

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regimens other than radiotherapy. Preliminary results about the cytotoxicity of perifosine combined with a diversity of compounds are encouraging: supra-additive cytotoxicity in combination with the protein kinase antagonist UCN-01 [128], enhanced activity when combined with anti-EGF receptor monoclonal antibody cetuximab (C225) in cancer cells with mutated PTEN [129], a synergistically enhanced apoptotic response in human leukemic cells when combined with either histone deacetylase inhibitors [130] or etoposide [131] and enhanced growth delay when combined with temozolomide in glioma [132]. However, most data are limited to *in vitro* assays. Therefore, more data on *in vivo* activity and tolerability might provide a basis for future clinical development of APLs in anti-cancer therapy. Results obtained to date indicate that application of this class of agents could have a role in the treatment of leukemic malignancies. For solid tumors accumulating evidence suggests that APLs, as part of a multimodality approach, could enhance the anti-tumor activity of existing anti-cancer regimens.

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In vitro studies

Chapter 2.1

A new class of anti-cancer alkylphospholipids uses lipid rafts as membrane gateways to induce apoptosis in lymphoma cells

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Abstract

Single-chain alkylphospholipids (APLs), unlike conventional chemotherapeutic drugs, act on cell membranes to induce apoptosis in tumor cells. We tested 4 different APLs, *i.e.* edelfosine, perifosine, erucylphosphocholine and compound D-21805, as inducers of apoptosis in the mouse lymphoma cell line S49. We compared their mechanism of cellular entry and their potency to induce apoptosis through inhibition of *de novo* biosynthesis of phosphatidylcholine (PC) at the endoplasmic reticulum. APL potency closely correlated with the degree of PC synthesis inhibition in the order edelfosine > D-21805 > erucylphosphocholine > perifosine. In all cases, exogenous lysoPC, an alternative source for cellular PC production, could partly rescue cells from APL-induced apoptosis, suggesting that PC biosynthesis is a direct target for apoptosis induction. Cellular uptake of each APL was dependent on lipid rafts, as pretreatment of cells with the raft-disrupting agents methyl-β-cyclodextrin, filipin, or bacterial sphingomyelinase, reduced APL uptake and/or apoptosis induction, and alleviated the inhibition PC synthesis. Uptake of all APLs was inhibited by siRNA-mediated blockage of sphingomyelin synthase (SMS1), which was previously shown to block raft-dependent endocytosis. Similar to edelfosine, perifosine accumulated in (isolated) lipid rafts independent on raft sphingomyelin content persé. However, perifosine was more susceptible than edelfosine to back-extraction by fatty acid-free serum albumin, suggesting a more peripheral location in the cell due to less effective internalization. Overall, our results suggest that lipid rafts are critical membrane portals for cellular entry of APLs depending on SMS1 activity, and therefore are potential targets for APL anti-cancer therapy.

Introduction

Synthetic lipase-resistant analogues of lysophosphadidylcholine, collectively named alkylphospholipids (APLs), exert cytotoxic effects against a wide variety of tumors [1-4]. The prototype of these compounds, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (Et-18-OCH$_3$; edelfosine), structurally resembles lysophosphatidylcholine (lysoPC) (Fig. 1) in that it has the same polar headgroup and a single long apolar hydrocarbon chain, which allows an easy insertion into the plasma membrane. Whereas membrane-inserted lysoPC undergoes rapid turnover, edelfosine with its stable ether bonds is not metabolized, leading to accumulation in cell membranes. This interferes with lipid-based signal transduction, often resulting in apoptosis of tumor cells. In later studies, the glycerol moiety in APLs was found not essential for the anti-tumor activity. A second generation of this class of compounds therefore comprised of phospho-ester-linked (single-chain) alkylphosphocholines and derivatives thereof. The first of these compounds, hexadecylphosphocholine (miltefosine), was clinically effective in patients with skin metastasis of breast cancers [2,5] and cutaneous lymphomas [6]. Erucylphosphocholine (ErPC) containing a much longer (22-carbon) chain with a *cis*-13,14 double bond (Fig. 1) could be applied intravenously and thereby showed improved anti-tumor activity with reduced hemolytic and gastrointestinal side effects [7,8]. Interestingly, it increased the cytotoxicity of ionizing radiation [9,10]. A structural analogue, octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate (perifosine; D-21266), in which the choline head group has been substituted by a piperidine moiety (Fig. 1) has received mounting attention as an anti-cancer agent, especially in combination with other pharmacological drugs [11-16] as well as with radiotherapy [4,17-19].

In contrast to classical chemotherapeutic drugs that target the DNA, APLs act at cell membranes by interfering with the turnover and synthesis of natural phospholipids and by disrupting membrane-signaling networks at multiple sites, leading to cell death [3,4,20]. For the APLs edelfosine and miltefosine, it has been shown that they induce apoptosis through inhibition of CTP:phosphocholine cytidylyltransferase (CCT), a key enzyme in phosphatidylcholine (PC) biosynthesis [21-23]. In order to inhibit this enzyme at the endoplasmic reticulum, APLs need to be internalized. We have demonstrated that edelfosine, after insertion in the plasma membrane, accumulates in lipid rafts and is then internalized by an endocytic pathway that depends on intact rafts and on the activity of sphingomyelin

synthase 1 (SMS1) [23-25]. How other APLs are taken up by cells, their relative potency and mechanism of apoptosis induction is currently unknown.

Here, we compared 4 anti-cancer APLs (molecular structures depicted in Fig. 1) with respect to their cellular uptake, cytotoxicity and mechanism of apoptosis induction in mouse S49 lymphoma cells. We found that all APLs utilize lipid rafts for internalization to inhibit PC synthesis to varying degrees, correlating with the efficiency and persistency of cellular uptake and potency to induce apoptosis.
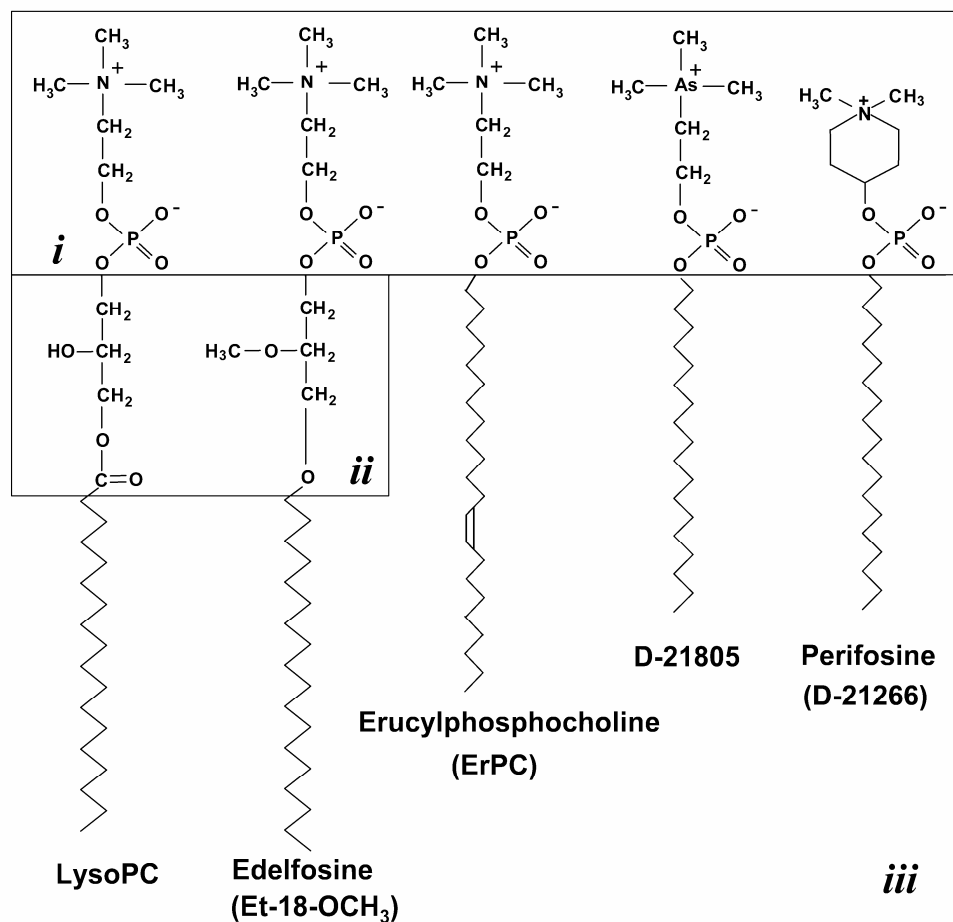

Figure 1. Chemical structures of synthetic APLs used in this study. Structures of edelfosine (also denoted as Et-18-OCH₃), erucylphosphocholine (ErPC), octadecyl-2-(trimethylarsonio)-ethyl-phosphate (D-21805), and perifosine (D-21266) in comparison to natural lysophosphatidylcholine (LysoPC). Note the 3 distinct submolecular structural moieties, *(i)* phosphocholine and related headgroup analogues, which are zwitterionic and represent the polar part of the molecule, *(ii)* glycerol backbone, present only in lysoPC and edelfosine, and *(iii)* the apolar alkyl-chain (acyl-chain in LysoPC).

Materials and Methods

Reagents. Edelfosine (1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine; Et-18-OCH$_3$) was purchased from BioMol (Plymouth Meeting, PA). [^{3}H]Et-18-OCH$_3$ (39 Ci/mmol) was synthesized by Moravek Biochemicals (Brea, CA). Perifosine (compound D-21266), octadecyl-2-(trimethylarsonio)-ethyl-phosphate (compound D-21805), erucylphosphocholine (ErPC), and [^{14}C]perifosine (31 mCi/mmol) were kindly provided by Zentaris GmbH (Frankfurt, Germany). [Methyl-^{14}C]choline chloride (58 mCi/mmol) was derived from Amersham Pharmacia Biotech. [^{3}H]Sphingosine was synthesized by Piet Weber at DSM (Delft, The Netherlands). Reagents for lipid extraction and subsequent analyses, as well as Silica 60 TLC plates (20 cm x 20 cm) were from Merck (Darmstadt, Germany). Sphingomyelinase (*Bacillus cereus*), filipin and MβCD were from Sigma Chemicals Co. (Zwijndrecht, The Netherlands).

Cell culture. Mouse S49.1 lymphoma cells (S49) were grown in Dulbecco's modified Eagle's medium, containing high glucose and pyruvate, supplemented with 8% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin at 37°C and 5% CO$_2$. Edelfosine-resistant variants (S49^{AR}) were isolated in 2 selection rounds of growth in 15 µM edelfosine for 72 h, followed by plating in semi-solid medium and isolation of colonies of surviving cells [26]. S49^{AR} cells could be grown continuously in 15 µM edelfosine with a doubling time of 12 h, similar to that of the parent S49 cells. S49^{mock} and S49^{siSMS1} cells were generated as described previously [25]. All experiments with S49^{AR} cells were performed with cells grown without the selection agent for at least 1 week.

Silencing of sphingomyelin synthase-1 by siRNA retroviral transduction. In order to suppress the expression of sphingomyelin synthase-1 (SMS1), S49 cells were retrovirally transduced by short interfering RNAs (siRNAs), yielding S49^{siSMS1} cells, as described previously [25]. Briefly, siRNAs directed to SMS1 were inserted into the retroviral vector pRETRO-SUPER. Retroviral supernatants were obtained from Phoenix cells and used to transduce S49 cells. Stable S49^{siSMS1} cells were selected with puromycin. The following siRNA primers were used: s*ense* GATCCCCGCATGGGAGTTGATTTAGATTCAAGAGATCTAAATCAACTCCCATG CTTTTTGGAAA and *antisense* AGCTTTTCCAAAAAGCATGGGAGTTGATTT-AGATCTCTTGAATCTAAATCAACTCCCATGCGGG. For mock transfection

(S49^{mock}), scrambled RNA was used.

Cellular uptake of APLs and apoptosis assay. Cells were grown to a density of 2.5x10^6/mL before [^{14}C]perifosine (0.2 μCi, 20 μM) or [^{3}H]edelfosine (0.2 μCi, 15 μM) was added. At given time points, samples were taken, incubated for 2 min on ice and washed with cold phosphate-buffered saline (PBS). Samples were lysed in 0.1 N NaOH for scintillation counting. For apoptosis, cells were seeded at 1.5x10^6 cells/mL, cultured overnight and incubated for indicated time periods with various concentrations of APLs. Cells were washed with PBS and lysed overnight at 4°C in 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100 and 50 μg/mL propidium iodide [27]. Fluorescence intensity of propidium iodide-stained DNA was determined on a FACScan (Becton Dickinson Advanced Cellular Biology), and data analyzed using Lysis software.

Lipid biosynthesis. To measure PC and sphingomyelin biosynthesis, cells at 2.5x10^6 cells/mL were incubated with [^{14}C]choline chloride (1 μCi/mL). At given time points, aliquots of cell suspension were taken, washed and resuspended in 200 μL PBS. Lipids were extracted with chloroform/methanol (1:2, v/v) and phase separation was induced using 1 M NaCl. The organic phase was washed in a solution of methanol/H$_2$O/chloroform (235:245:15, v/v/v), and separated by silica TLC, using choroform:methanol:acetic acid:water, 60:30:8:5, v/v/v/v). Alternatively, cells were radiolabeled for 24 h with 1 μCi/mL [^{3}H]sphingosine [28]. In this case, lipid extracts were separated by TLC using chloroform/methanol/0.2% CaCl$_2$ (60:40:9, v/v/v). Radioactive lipids were visualized and quantified using a Fuji BAS 2000 TR Phosphor Imager and identified using internal standards, which were visualized by iodine staining.

Isolation of lipid rafts. A detergent-resistant lipid raft fraction was prepared as described previously [23]. Briefly, cells (2.0x10^6/mL) were solubilized into 2 mL of ice-cold MBS buffer (25 mM MES, 150 mM NaCl), including 1% Triton X-100 and homogenized with a tight fitting Dounce homogenizer (10 strokes). The homogenate was adjusted to 40% sucrose and put on the bottom of an ultracentrifuge tube (4 mL). A discontinuous sucrose gradient was prepared by overlaying 5 mL of 30% sucrose and 3 mL of 5% sucrose (both in MBS), subsequently. The tubes were centrifuged at 39,000 rpm in a SW41 rotor for 16-18 h at 4°C and 12x 1.0 mL fractions were collected manually from the top of the

gradient. For incorporation of perifosine in lipid rafts, cells were incubated with [^{14}C]perifosine (0.2 µCi/mL; 20 µM) for 15 min to allow insertion into the outer leaflet of the plasma membrane lipid bilayer. Sphingomyelin levels in each fraction were determined after 24 h radiolabeling of cells with 1 µCi/mL [^{3}H]sphingosine, followed by Triton X-100 solubilization and sucrose gradient centrifugation and TLC separation (see above).

Results

Differential potency of 4 different APLs to induce apoptosis in S49 cells. We have previously described that the synthetic ether-lipid edelfosine (Et-18-OCH$_3$) can induce apoptosis in S49 lymphoma cells, in a dose- and time-dependent fashion [23]. The onset of apoptosis in these cells was relatively fast and already appeared after 3 h. As the structurally related analogues perifosine and erucylphosphocholine (ErPC) (Fig. 1) have more clinical potential, we examined the potency of these compounds, compared to edelfosine, to induce apoptosis in S49 lymphoma cells. We also tested a new APL derivative, Zentaris compound D-21805, in which the nitrogen in the choline moiety is substituted by arsenic (Fig. 1). It appeared that D-21805, ErPC and perifosine are less potent inducers of apoptosis than edelfosine, with IC$_{50}$ values of 15, 25, ≥ 50 and 12 µM, respectively (Fig. 2A).
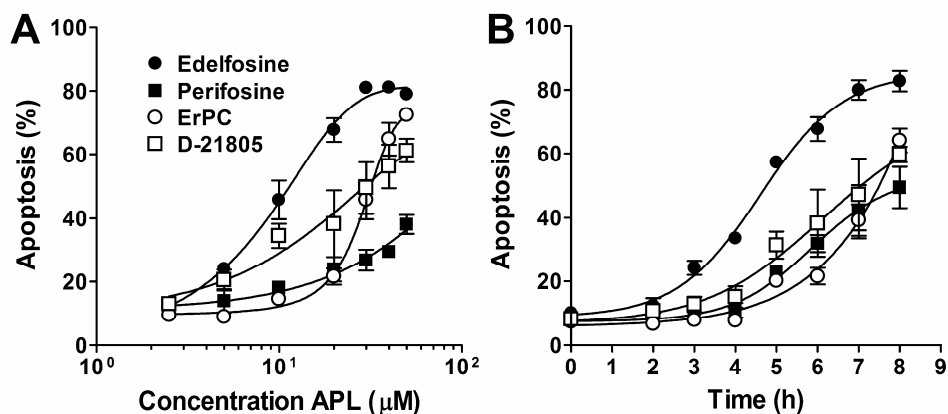
Figure 2. Dose-dependency and kinetics of apoptosis induction by various APLs. S49 cells were treated for 6 h with different concentrations of APL *(A)*, or for the times indicated with D-21805 (20 µM; open squares), perifosine (20 µM; closed squares), erucylphosphocholine (20 µM; open circles) or edelfosine (15 µM Et-18-OCH$_3$; closed circles) *(B)*. Apoptotic nuclear fragmentation was determined by FACS analysis (see Materials and Methods). Data are means of 4 experiments ± SD.

Contrary to edelfosine, D-21805 and perifosine did not reach plateau levels of apoptosis after 7 h incubation, even at a high concentration of 50 µM (Fig. 2A). In addition, the onset of apoptosis in cells treated with D-21805, ErPC, and perifosine was delayed, compared to edelfosine (Fig. 2B). Whereas maximal apoptosis by edelfosine was reached at 7 h, the other APLs required a prolonged (overnight) incubation time to reach maximum apoptosis.

Relative potency of individual APLs to inhibit PC synthesis correlates with their potency to induce apoptosis. We have previously demonstrated in S49 and HeLa cells, that continuous PC synthesis is crucial for cell survival and that edelfosine induces apoptosis by inhibiting *de novo* PC synthesis [23,24]. To test whether this holds true for the 3 other APLs, S49 cells were incubated with the PC precursor [^{14}C]choline in the absence or presence of edelfosine, D-21805, perifosine, or ErPC and the PC synthesized was measured after 2 h. The APLs were found to inhibit PC synthesis to various degrees, edelfosine being the most potent, followed by D-21805, ErPC and perifosine, amounting to 65, 60, 50 and 30% inhibition, respectively (Fig. 3A). These data correlate with the relative percentages of apoptosis induced by these compounds (Fig. 3B).
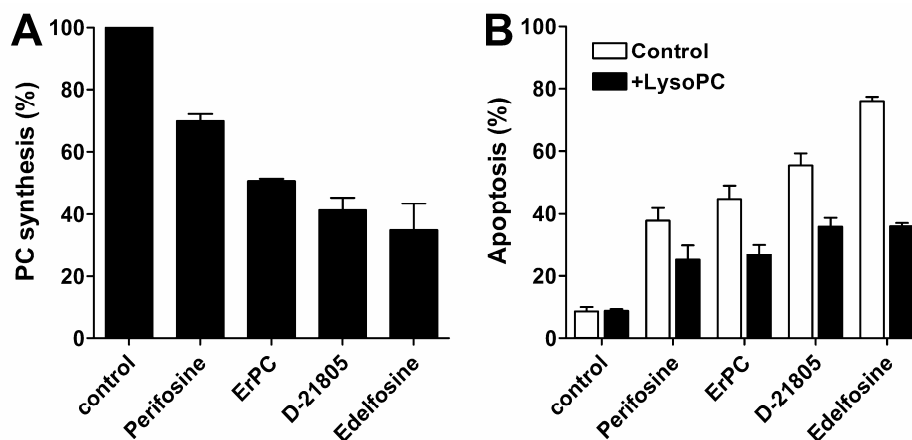
Figure 3. PC synthesis inhibition by APLs *(A)* and apoptosis suppression by exogenous LysoPC *(B)*. *(A)* S49 cells were pretreated for 30 min with perifosine (20 µM), erucylphosphocholine (ErPC, 20 µM), D-21805 (20 µM) or edelfosine (15 µM), and then incubated for 2 h with 0.2 µCi/mL [^{14}C]choline precursor to label cellular PC, which was visualized after lipid extraction and TLC analysis and was quantified using phosphor-imaging technology. *(B)* Apoptosis (nuclear fragmentation) induced in S49 cells by these concentrations of APLs after 5 h (open bars), and upon co-addition of 25 µM lysoPC (closed bars).

To demonstrate the importance of PC synthesis for cell survival, we administered exogenous lysoPC, an alternative precursor of PC [23], to the cells that were challenged with the various APLs. Figure 3B shows that, indeed, lysoPC rescued cells from apoptosis induction by perifosine, D-21805, ErPC and edelfosine, by 34, 36, 40 and 50% respectively.

From these results together, we conclude that all 4 APLs inhibit *de novo* PC synthesis in S49 cells to different degrees, which appeared to correlate with their potency to induce apoptosis. The observed protective effect of lysoPC indicates that this abrogation of PC synthesis is responsible (at least partly) for the onset of apoptosis induction by all tested APLs, as we concluded previously for edelfosine [23,24].
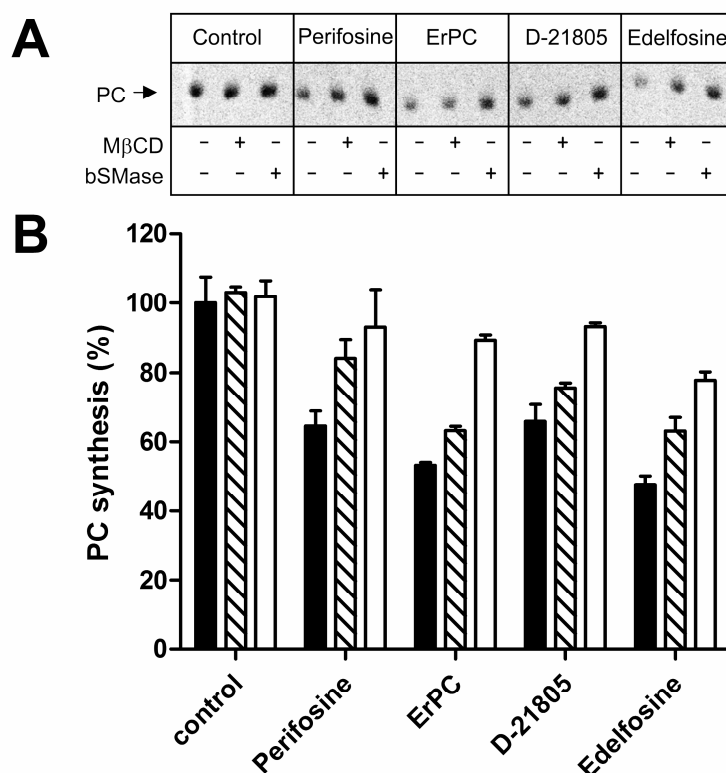
Figure 4. APL-induced inhibition of PC synthesis is alleviated by disruption of lipid rafts. S49 cells were preincubated (for 30 min) with MβCD (2 mg/mL; hatched bars), or with bSMase (150 milliunits/mL; open bars), or were not preincubated (black bars). Cells were then treated with buffer (control), or with the APLs perifosine (20 µM), ErPC (20 µM), D-21805 (20 µM) or edelfosine (15 µM), and were, 30 min later, incubated for 2 h with 0.2 µCi/mL [14C]choline precursor to label cellular PC, which was visualized after lipid extraction and TLC analysis; radioactive PC spots (A) were quantified using Phosphor Imaging (B).

Cellular uptake of APLs, PC synthesis inhibition and consequent apoptosis induction is mediated by lipid rafts. Inhibition of *de novo* PC synthesis by APLs occurs at the level of the rate-determining enzyme CTP:phosphocholine cytidylyltransferase located in the endoplasmic reticulum and the nucleus. Thus, APLs need to be internalized to inhibit this enzyme. Edelfosine was previously found to accumulate preferentially in detergent-resistant lipid raft fractions, and was internalized by raft-dependent endocytosis [23-25]. In order to investigate whether PC inhibition and apoptosis induction in S49 cells by perifosine, D-21805, and ErPC was likewise mediated by lipid rafts, we disrupted these membrane domains by extracting their cholesterol with methyl-β-cyclodextrin (MβCD), and by hydrolyzing their sphingomyelin (SM) with bacterial sphingomyelinase (bSMase). Fig. 4 shows that these treatments of cells alleviated the inhibition of PC synthesis for each APL.

In general, the PC synthesis rescuing effect of bSMase treatment was stronger than of MβCD. Fig. 5A shows that treatment of S49 cells with MβCD prevented apoptosis induction, for all tested APLs.
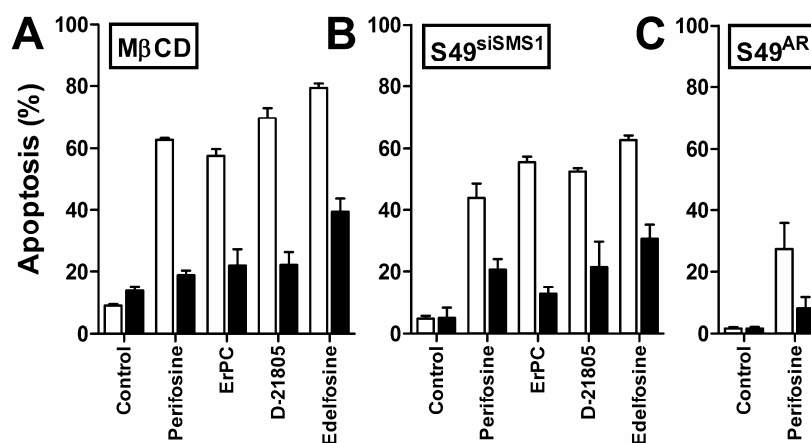
Figure 5. Apoptosis induction by APLs is prevented by cholesterol extraction and downregulation of SM synthesis. Apoptosis was induced at 7 h by the indicated APLs in S49 cells (open bars), in S49 cells preincubated (30 min) with methyl-β-cyclodextrin (MβCD, 2 mg/mL) (*A*, solid bars), and in the SMS1-deficient cell lines S49^siSMS1 (*B*, solid bars) and S49^AR (*C*, solid bars). Perifosine, erucylphosphocholine (ErPC) and D-21805 were used at 20 μM; edelfosine was used at 15 μM. Apoptosis was measured by nuclear fragmentation. Data are means of triplicates ± SD.

We recently described that edelfosine-resistant cells (S49^AR cells) lack the raft constituent SM, through a 50-fold downregulated SM synthase (SMS1) expression.

Genetically, SM deficiency can also be induced in S49 cells by RNAi-mediated SMS1 downregulation (Fig. 6F), yielding so-called S49[siSMS1] cells [25]. Fig. 5B and 5C show that both of these S49 cell variants are not only resistant to edelfosine but to the other APLs as well. Resistance in S49[siSMS1] cells was less stringent than in the S49[AR] cells because the RNAi-induced downregulation of SMS1 expression was not as pronounced as in S49[AR] cells [25]. The data presented in Fig. 5 confirm that apoptosis sensitivities to all APLs are dependent on lipid raft integrity (including appropriate cholesterol content) and SMS1-mediated SM production in the lipid rafts.

Perifosine accumulates in lipid rafts, independent of their sphingomyelin levels, but internalization depends on sphingomyelin synthesis. As edelfosine was previously shown to accumulate in lipid rafts prior to its raft-mediated internalization [23-25], we investigated possible raft accumulation of perifosine, the only APL available in ^{14}C-labeled form. Fig. 6A-C show that, similar to edelfosine [23], [^{14}C]perifosine accumulated in lipid rafts to similar levels for S49 cells and S49[AR] and S49[siSMS1] cells, that are deficient in SM synthesis (Fig. 6D and F). In addition, when S49 cells were pretreated with bacterial SMase to decrease SM levels (Fig. 6E), there was no effect on the preference of perifosine to accumulate in rafts (Fig. 6B). Therefore, we conclude that the levels of SM in lipid rafts are irrelevant for the accumulation of perifosine into these membrane microdomains. While the content of SM was irrelevant, its synthesis (which has functions beyond simply contributing to raft structure; see Discussion) was important for the active cellular uptake of perifosine, as it was for edelfosine [25].
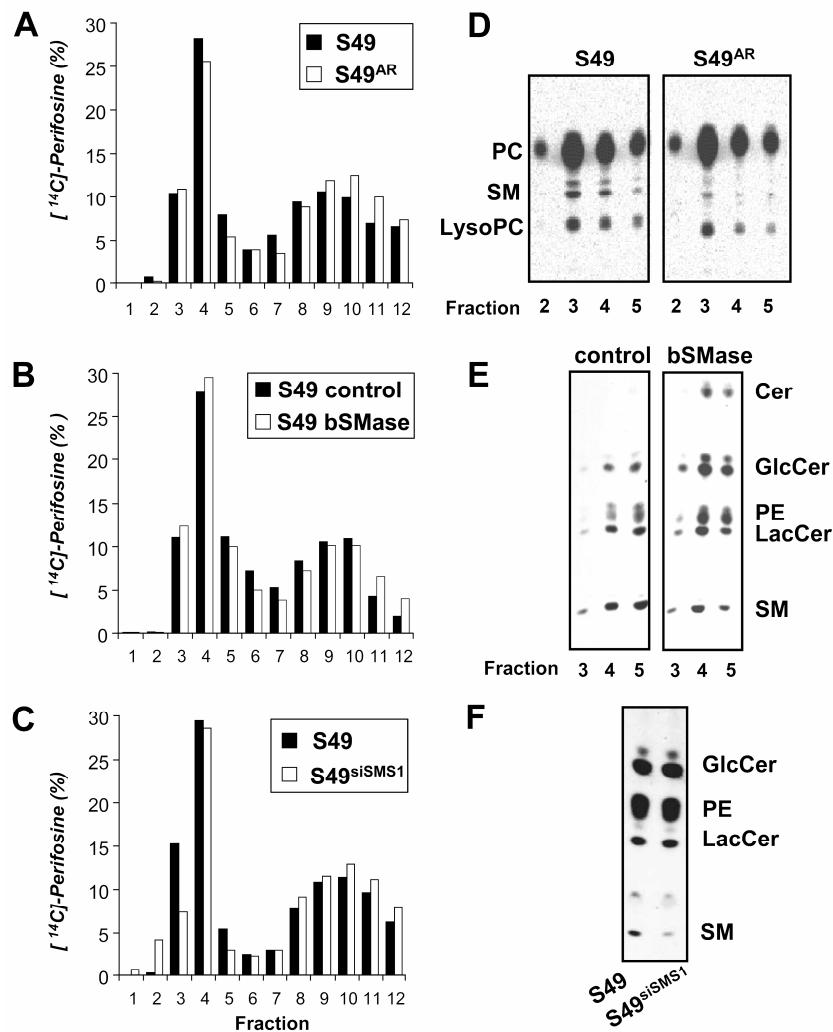
Figure 6. Perifosine accumulates in lipid rafts independent of the sphingomyelin content. *(A-C)* S49 control cells (solid bars) or sphingomyelin-deficient cells (S49[siSMS1], S49[AR], and bSMase-treated S49 cells (open bars) were incubated for 15 min with [^{14}C]perifosine (20 µM, 0.02 µCi/mL), washed and detergent-insoluble lipid rafts were then isolated on sucrose density gradients (typically fractions 3-5). Radioactivity in the gradient fractions was counted and expressed as a percentage of total activity. *(D)* S49 and S49[AR] cells (indicated) were labeled with [methyl-^{14}C]choline (1 µCi/mL, overnight). Detergent-insoluble lipid rafts were isolated on sucrose gradients, extracted and separated by TLC. Positions of phospholipids are indicated. SM appears typically as 2 spots [25]. *(E)* S49 cells were labeled with [^{3}H]sphingosine (1 µCi/mL, 4 h) and then treated with bacterial sphingomyelinase (bSMase, 150 milliunits/mL, 30 min). (Sphingo)lipids were extracted and separated by TLC. The location of SM and other sphingolipids, ceramide (Cer), glucosylceramide (GlcCer) and lactosylceramide (LacCer) is indicated. Phosphatidylethanolamine (PE) is a catabolic end-product of sphingosine-1-phosphate degradation [25]. *(F)* S49 cells and sphingomyelin-downregulated S49[siSMS1] cells were labeled, extracted and analyzed on TLC as in *(E)*.

Similar to [³H]edelfosine (Fig. 7A), the time-dependent uptake of [¹⁴C]perifosine at 37°C was decreased in SMS1-downregulated cells (S49^{AR} and S49^{siSMS1}) as compared with the parental S49 cells (Fig. 7B).
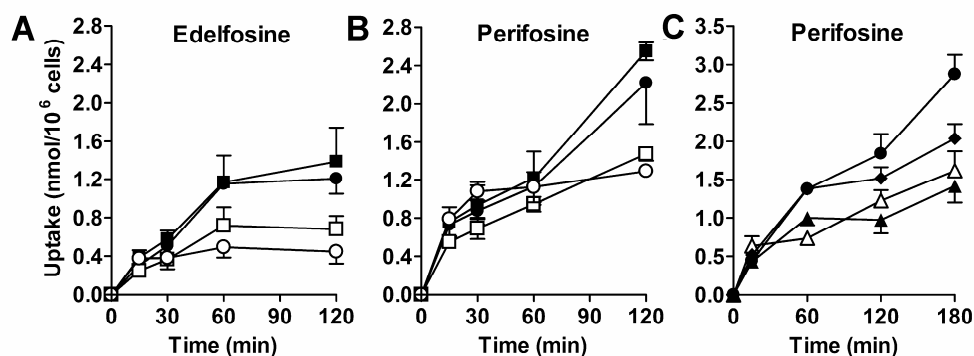
Figure 7. Time-dependent cellular uptake of edelfosine and perifosine; dependence on SMS1 expression *(A,B)* **and lipid raft integrity** *(C)***. Cells were incubated at 37°C with [³H]edelfosine (15 µM, 0.02 µCi/mL)** *(A)* **or [¹⁴C]perifosine (20 µM, 0.02 µCi/mL)** *(B, C)* **for the times indicated, and then washed with cold PBS, solubilized and the radioactivity was counted. From this the amount of uptake in nmol (± SD; n=4) was calculated. Symbols for cells in panels** *(A,B)***: S49, closed circles; S49^{AR}, open circles; S49^{mock}, closed squares; S49^{siSMS1}, open squares.** *(C)* **S49 cells remained untreated (closed circles), were pretreated (30 min) with filipin (1 µg/mL; closed diamonds), methyl-β-cyclodextrin (2 mg/mL; open triangles), or bacterial sphingomyelinase (150 milliunits/mL; closed triangles).**

Also similar to edelfosine [23], internalization of [¹⁴C]perifosine was dependent on lipid raft integrity, since raft disruption of S49 cells with the cholesterol sequestrating agents filipin and methyl-β-cyclodextrin or with bSMase, decreased the uptake of this APL (Fig. 7C).

Different levels of cellular uptake of perifosine compared to edelfosine. When comparing the uptake at 37°C of edelfosine with perifosine in more detail, we found that the perifosine taken up by S49 cells remained less persistently associated with (bound to) the cells than edelfosine (Fig. 8). This persistence of APL accumulation in the cells was demonstrated by back-extraction with fatty acid-free bovine serum albumin (BSA), a technique that we used previously to analyze internalization of exogenous lysoPC in cells [24]. BSA will only back-extract the APL fraction that remains in the outer leaflet of the plasma membrane lipid bilayer, not the APL that has been internalized by spontaneous and/or protein-mediated transbilayer 'flipping' or endocytosis. Fig. 8A and B show the relative amount of radiolabeled

edelfosine that, after 10 min or 1 h incubation at 37°C remains associated to cells after BSA back-extraction, which was a about 2-fold higher than for perifosine. These levels of internalized APL were higher in S49 cells than in S49^{AR} cells, which are explained by the lack of raft-mediated endocytosis in the latter cells [23,25].
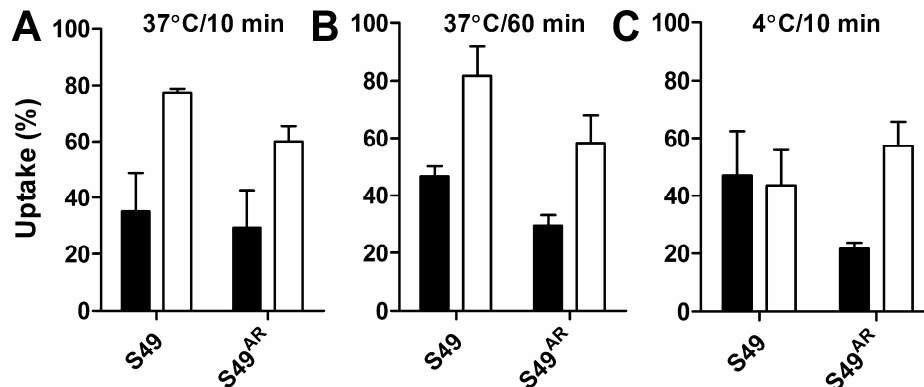

Figure 8. Fraction of the cellular uptake of edelfosine and perifosine that is resistant to BSA back-extraction. S49 and S49^{AR} cells were incubated at 37°C *(A, B)* or at 4°C *(C)* with [^{14}C]perifosine (20 μM, 0.02 μCi/mL; closed bars) or [^{3}H]edelfosine (15 μM, 0.02 μCi/mL; open bars) for the times indicated (10 min, 1 h), and then washed 3 times with cold PBS or with fatty acid-free bovine serum albumin (BSA) (1%). Data are expressed as the percentage of radioactivity left in the cells after BSA washing relative to PBS washing. Data are means of 3 experiments ± SD.

We next determined the persistent uptake at 4°C, a temperature which still allows spontaneous transbilayer flipping [24] but no 'active' endocytic uptake. Fig. 8C shows that the same percentage (about 45%) of edelfosine and perifosine remained associated to S49 cells after BSA back-extraction, suggesting that the internalization by spontaneous flipping is the same for the 2 APLs. Intriguingly, however, from S49^{AR} cells much more perifosine (80%) than edelfosine (45%) can be back-extracted under these low-temperature conditions, suggesting that in these APL-resistant cells, perifosine is less subject to trans-bilayer flipping than edelfosine, for reasons that are not clear.

The most important conclusion from these data is that the cellular uptake of perifosine is less efficient than of edelfosine, so that more perifosine than edelfosine remains located at the plasma membrane outer leaflet, available to BSA back-extraction. While spontaneous membrane traversal (flipping) in S49 cells appears the same for the 2 compounds, temperature dependent 'active' uptake is higher for edelfosine than for perifosine.

Discussion

In this paper, we have shown that S49 lymphoma cells are sensitive to a class of structurally related synthetic APLs, which comprises edelfosine, D-21805, erucylphosphocholine and perifosine. The relative potency of these 4 anti-cancer agents to induce apoptosis in S49 cells differed and was correlated with their capacity to inhibit PC synthesis in the cell. We demonstrated for each of these compounds that this inhibition of PC synthesis was a direct trigger for apoptosis induction because exogenous lysoPC, an alternative source for PC production (through acylation inside the cell), rescued the cells from APL-induced apoptosis. For 2 of these compounds, (radiolabeled) edelfosine and perifosine, we demonstrated that they accumulated in the detergent-resistant lipid raft fractions and that cellular uptake was impaired when the rafts were disrupted by cholesterol extraction (using MβCD) or when the synthesis of a major raft phospholipid, SM, was downregulated. These latter interventions protected the cells against apoptosis induction by all APLs. It is therefore likely that each of these APLs, after initial insertion in the outer leaflet of the plasma membrane lipid bilayer, accumulates in lipid rafts and from there, is taken up by raft-mediated endocytosis, as we demonstrated previously for edelfosine in more detail [23-25].

The differential efficacy of apoptosis induction by the 4 APLs (Fig. 2 and 3B) is likely related to their different chemical structure (Fig. 1). Edelfosine, the most effective one, contains a glycerol backbone with 2 ether-linked substituents: a long alkyl chain and a short *O*-methyl group, properties that allow its easy partitioning into lipid rafts, as argued before [23,28,29]. In model membranes, hexadecylphosphocholine (miltefosine; lacking the glycerol-backbone) was less miscible with SM than edelfosine, yet stabilized SM-cholesterol-rich ordered domains, similar to edelfosine [29]. This is in line with our finding that perifosine, like edelfosine, accumulates in raft fractions, but possibly in weaker association with SM. If true, it remains to be seen if this relates to the degree of cellular uptake, the route by which this occurs and the apoptotic potency. The lower potency of perifosine to induce apoptosis cannot be ascribed to metabolic breakdown, since the molecule remained essentially intact within the cell [30].

When comparing the most potent APL, edelfosine, with the least effective one, perifosine, we find some similarities, but also differences in their behavior in cells. Both compounds accumulate in rafts, but they differ in the capacity to inhibit PC synthesis. It is possible that different APLs have differential inhibitory effects on the

CTP:phosphocholine cytidylyltransferase persé. However, is also possible that not every APL can reach this enzyme in the endoplasmic reticulum by the same route and to the same extent. In this regard, it is of interest that perifosine, after cellular uptake, can be much more easily back-extracted by BSA than edelfosine. This would suggest a less pronounced cellular internalization, possibly by a different route and/or by localization more in the cell periphery than edelfosine. The mechanism of cellular internalization and distribution of APL is probably also dependent on the cell type, as we previously observed that KB cells showed a remarkably high uptake and sensitivity for perifosine compared with the other squamous carcinoma cell lines A431 and HNXOE [30].

It is a remarkable finding that perifosine incorporation in lipid rafts is independent of SM content. Neither inhibition of SM synthesis by RNAi-mediated SMS1 downregulation, nor SM breakdown by bacterial SMase affected perifosine incorporation into the lipid raft fractions. Similar results were recently published for edelfosine [25]. Yet, SM synthesis was important for cellular uptake of perifosine and the subsequent induction of apoptosis, since downregulation of SMS1 by RNAi prevented these events, again, similar as we found for edelfosine [25]. In this regard, it should be noted that SMS1 activity in the trans-Golgi [31] has important cell biological implications that goes beyond the mere production of SM for nascent lipid rafts [25]. SMS1 also produces diacylglycerol that activates protein kinase D, which is essential for anterograde vesicular trafficking towards the plasma membrane [32]. We have argued that APL internalization by raft-dependent endocytosis may represent the retrograde route of constitutive lipid raft-vesicular cycling that may exist between the trans-Golgi, the plasma membrane and the endosomal compartments [25].

Our results strengthen the idea that inhibition of PC synthesis is a major insult to cells, which is apparently sufficient to initiate their apoptotic machinery, at least in S49 lymphoma cells. Not only edelfosine but also other APLs can induce apoptosis in this way. How exactly PC synthesis inhibition by APLs results in apoptosis is unknown. Rescue of cells from cell death by exogenous lysoPC, which is rapidly converted to PC [24], suggests that a continuous PC synthesis is crucial for cell survival. We have argued that a continuous phospholipid supply by vesicular trafficking might support a survival mechanism [33]. In line with this speculative idea is that proper PC synthesis may regulate PKB/Akt kinase activity, as it was shown that an inhibition in PC synthesis precedes PKB/Akt inactivation [34]. This might be a reason why perifosine and edelfosine inhibited PKB/Akt activation in

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various cell types [16,35,36]. However, the precise mechanism by which this PC synthesis inhibition connects in molecular terms to the initiation of the apoptotic machinery remains to be investigated. Also, future studies should reveal to what extent such a raft- and SMS1-dependent APL uptake mechanism applies to other types of (human) cancer cells.

Acknowledgement

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Chapter 2.2

Lipid rafts and metabolic energy differentially determine uptake of anti-cancer alkylphospholipids in lymphoma versus carcinoma cells

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Abstract

Perifosine is a member of the class of synthetic alkylphospholipids (APLs), and is being evaluated as anti-cancer agent in several clinical trials. These single-chain APLs accumulate in cellular membranes and disturb lipid-dependent signal transduction, ultimately causing apoptosis in a variety of tumor cells. The APL prototype edelfosine was previously found to be endocytosed by S49 mouse lymphoma cells via lipid rafts. An edelfosine-resistant cell variant, S49^{AR}, was found to be cross-resistant to other APLs, including perifosine. This resistance was due to defective synthesis of the raft constituent sphingomyelin, which abrogated APL cellular uptake. Sensitivity of S49 cells to edelfosine was higher than to perifosine, which correlated with a relatively higher uptake. Human KB epidermal carcinoma cells were much more sensitive to APLs than S49 cells. Their much higher APL uptake was highly dependent on intracellular ATP and ambient temperature, and was blocked by chlorpromazine, independent of canonical endocytic pathways. We found no prominent role of lipid rafts for APL uptake in these KB cells: Contrary to S49^{AR} cells, perifosine-resistant KBr cells display normal sphingomyelin synthesis, whereas APL uptake by the responsive KB cells was insensitive to treatment with methyl-β-cyclodextrin, a cholesterol-sequestrator and inhibitor of raft-mediated endocytosis. In conclusion, different mechanisms determine APL uptake and consequent apoptotic toxicity in lymphoma versus carcinoma cells. In the latter cells, APL uptake is mainly determined by a raft- and endocytosis-independent, but metabolic energy-dependent process, possibly by a lipid transporter.

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Introduction

The group of synthetic single-chain alkylphospholipids (APLs) is a heterogeneous class of anti-cancer agents, which exerts cytotoxic effects against a wide variety of tumors [1-5]. In contrast to classical chemotherapeutic drugs, APLs do not target DNA but primarily act at the level of cell membranes. This distinct mode of action, which includes inhibition of phospholipid turnover and interference with signal transduction pathways, makes APLs attractive candidates for combination therapy with classical anti-cancer agents and ionizing radiation [6,7]. Several APLs are currently under investigation as radiosensitizers, since they have been shown to enhance radiation-induced cell death, both *in vitro* [6,8-13] and *in vivo* [11].

The prototype of these APL compounds is 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (Et-18-OCH$_3$; edelfosine), an ether-linked phospholipid with a glycerol backbone. Other members of the APL family lack this glycerol backbone, their alkyl chain being directly esterified to a phosphocholine moiety or analogue thereof, for example hexadecylphosphocholine (miltefosine), erucylphosphocholine (ErPC, which is intravenously applicable) and octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate (compound D-21266; perifosine [14]). This latter compound has been tested in phase I trials in patients with solid tumors as single agent [15,16] and in combination with radiotherapy [17], and is currently being evaluated in phase II studies.

In general, tumor cells can gain resistance to cytotoxic agents via multiple mechanisms, such as increased drug metabolism, enhanced drug efflux and reduced drug accumulation. There is no evidence for increased APL degradation. Similar to edelfosine [18], we found that perifosine is almost completely metabolically stable, both *in vitro* and *in vivo* [19]. Reduced APL uptake, however, is often observed in tumor cells that have gained resistance to these synthetic lipids [18,20-22]. Conversely, the high sensitivity of KB squamous cell carcinoma to APLs is correlated with a high degree of drug accumulation [19]. The precise mechanism by which APLs are internalized has long been a matter of debate. Whereas several investigators have reported a reduced uptake by tumor cells after treatment with pharmacological inhibitors of endocytosis [23-25], others have failed to show a correlation between resistance to APLs and endocytosis [26,27].

Our group has recently demonstrated that in S49 lymphoma cells, edelfosine accumulates in sphingolipid- and cholesterol-enriched plasma membrane microdomains, known as lipid rafts [28,29]. The drug is then rapidly internalized via

these domains in S49 cells, whereas this process is disturbed in the edelfosine-resistant cell variant S49[AR] [22,29]. This abrogated uptake of edelfosine was linked to reduced levels of sphingomyelin (SM), an essential raft constituent. Recently, we showed this defect to occur at the level of SM synthase 1 (SMS1) expression [30]. Although lipid rafts are clearly involved in APL uptake by lymphoma cells, their role in the uptake by solid tumors remains unknown. The human squamous cell carcinoma KB has previously been studied as an APL-responsive tumor model, both *in vitro* [19,31] and *in vivo* [11,32]. Here, we compared the APL uptake in the S49/S49[AR] lymphoma with the KB carcinoma model and an APL-resistant cell variant, KBr. APL resistance was induced differently in these cells. S49[AR] cells were selected after continuous edelfosine treatment [33] whereas KBr was generated after mutagenesis and subsequent continuous culturing in the presence of perifosine. In both cell models, we find a correlation between cellular APL uptake and sensitivity, as well as cross-resistance to other APLs. However, contrary to S49 cells, APL uptake by KB cells is predominantly raft- and endocytosis-independent, but dependent on metabolic energy, possibly an ATP-dependent lipid transporter.

Materials and methods

Materials. [Methyl-^{14}C]choline chloride (56 mCi/mmol) and L-lyso-3-phosphatidylcholine, 1-[1-^{14}C]palmitoyl ([^{14}C]LysoPC, 56 mCi/mmol; Code CFA633) were purchased from Amersham Pharmacia Biotech. Erucylphosphocholine (ErPC), compound D-21805 (with arsenic substituting the choline nitrogen atom), perifosine (octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate; compound D-21266), and [^{14}C]perifosine (31 mCi/mmol) were kindly provided by Zentaris GmbH (Frankfurt, Germany). Edelfosine (Et-18-OCH$_3$) was from Kamiya Biomedical Company (Seattle, WA). [^{3}H]Edelfosine (39 Ci/mmol) was synthesized by Moravek Biochemicals (Brea, CA). Miltefosine (hexadecylphosphocholine) was from Cayman Chemical (Ann Arbor, MI). Reagents for lipid extraction and subsequent analyses, as well as Silica 60 TLC plates (20 cm x 20 cm) were from Merck (Darmstadt, Germany). BODIPY-lactosylceramide, Alexa-488-labeled transferrin and FITC-dextran were from Molecular Probes (Leiden, The Netherlands). All other chemicals were from Sigma (Zwijndrecht, The Netherlands).

Cell culture. The human head and neck squamous cell carcinoma KB and resistant KBr were kindly provided by dr. F. Gamarro (Granada, Spain). The KBr

cells (also named KB PER® clone 10) were obtained by mutagenesis with ethyl-methanesulfonate, followed by intervals of incubation with increasing concentrations of perifosine, as described in detail elsewhere (F. Munoz-Martinez, C. Torres, S. Castanys and F. Gamarro, manuscript in preparation). KB and KBr were maintained in Dulbecco's Modified Eagles Medium (Gibco, Rockville), supplemented with 10% (v/v) fetal bovine serum (Life Technologies) and antibiotics. Mouse S49.1 lymphoma cells (S49) were grown in Dulbecco's modified Eagle's medium, containing high glucose and pyruvate, supplemented with 8% fetal calf serum, 2 mM L-glutamine and antibiotics. Edelfosine-resistant variants (S49^{AR}) were isolated in 2 selection rounds of growth in 15 µM edelfosine (Et-18-OCH$_3$) for 72 h, followed by plating in semi-solid medium and isolation of colonies of surviving cells, as described by Smets *et al.* [33]. The selected S49^{AR} clone could be grown continuously in 15 µM edelfosine with a doubling time of 12 h, similar to the parental S49 cells. Experiments with S49^{AR} cells were performed with cells grown without the selection agent for at least 1 week. All cell lines were tested negative for mycoplasma.

Phospholipid synthesis. KB/KBr cells (2.5x10^5/well in 6-well plates) and S49/S49^{AR} cells (1x10^6/well in 6-well plates) were incubated overnight, and labeled for 8 h with 1 µCi/mL [methyl-^{14}C]choline chloride to measure SM synthesis. For PC synthesis from exogenous LysoPC, cells were incubated for the indicated times with 0.025 µCi [^{14}C]LysoPC. Cells were harvested, washed and fixed in methanol. Lipids were extracted with chloroform/methanol (1:2, v/v) and phase separation was induced using 1 M NaCl. The organic phase was washed in a solution of methanol/H$_2$O/chloroform (235:245:15, v/v/v), and separated by silica TLC, using chloroform/methanol/acetic acid/H$_2$O, 60:30:8:5, v/v/v/v). Radioactive phospholipids, among which [^{14}C]PC and [^{14}C]SM, were identified using internal standards and quantified using a Fuji BAS 2000 TR Phosphor Imager.

Apoptosis assay. KB/KBr cells were plated in 6-well plates (1x10^5 cells/well) and allowed to attach overnight, whereas S49/S49^{AR} cells were plated in 96-well plates (1x10^5 cells/well) before incubation for indicated time periods with APLs. Cells were washed in phosphate-buffered saline (PBS) and incubated at 4°C in 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100 and 50 µg/mL propidium iodide [33]. Fluorescence intensity of propidium iodide-stained sub-nuclear DNA fragments was determined by FACScan analysis (Becton Dickinson, San Jose, CA). The data

were fitted to a sigmoidal concentration-response curve and IC_{50} calculation was done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

APL uptake and ATP depletion. Cells were plated in 12-well dishes ($S49/S49^{AR}$ – $1x10^6$ cells/mL, KB/KBr – $5x10^4$ cells/well) and incubated overnight. Cells were pretreated for 30 min with 2.5 mg/mL methyl-β-cyclodextrin or 20 μM chlorpromazine, where indicated. Hereafter, cells were incubated with [^{14}C]perifosine (0.03 μCi/mL, 1 μM) or [^{3}H]edelfosine (0.15 μCi/mL, 1 μM). At appropriate time points, cells were washed with PBS, subsequently lysed in 1 M NaOH and diluted in Ultima Gold scintillation liquid (PerkinElmer, Wellesley, MA) for scintillation counting in a TRI-CARB liquid scintillation analyzer. ATP depletion was obtained by pre-incubation for 30 min with DMEM lacking D-glucose and sodium pyruvate, and containing 20 mM 2-deoxy-D-glucose and 10 mM sodium azide. Statistical analysis of the data was performed using Student's *t*-test.

K^+ depletion and ^{125}I-EGF internalization. KB cells were incubated for 5 min in DMEM/H_2O (1:1 (v/v)) at 37°C, followed by a 60-min incubation at 37°C in minimal medium without K^+ (20 mM Hepes at pH 7.5, 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose and 0.5% BSA). Control cells were incubated in minimal medium supplemented with 10 mM KCl. Cells were incubated for 5 min with 1 ng/mL [^{125}I]EGF (epidermal growth factor) (684 mCi/mmol; from Amersham) or for 1 h with radiolabeled APL (see above). Membrane-bound EGF was stripped off by washing with acidified PBS (HCl, pH 2.1) for 2 min at 4°C. Radioactivity in cells was measured by scintillation counting.

Isolation of lipid rafts. A lipid raft fraction was prepared by detergent extraction of cells and sucrose gradient centrifugation for the determination incorporation of radiolabeled APL in lipid rafts [22]. S49 cells were grown to a density of $2.0x10^6$/mL, incubated with [^{14}C]perifosine (0.03 μCi/mL, 20 μM) or [^{3}H]edelfosine (0.15 μCi/mL, 15 μM). KB cells were plated in 8 cm diameter dishes ($5x10^6$/dish – 2 dishes for each raft isolation), allowed to attach overnight and incubated with [^{14}C]perifosine or [^{3}H]edelfosine (0.02 μCi/mL, 1 μM) for 30 min. Cells were washed twice with ice cold PBS, solubilized into 2 mL of ice-cold MBST buffer (25 mM MES, 150 mM NaCl, 1% Triton X-100) and homogenized with a tight fitting Dounce homogenizer (10 strokes). The extract was adjusted to 40% sucrose by the

addition of 2 mL of 80% sucrose in MBS (lacking Triton X-100) and put on the bottom of an ultracentrifuge tube. A discontinuous sucrose gradient was prepared by overlaying 5 mL of 30% sucrose and 3 mL of 5% sucrose (both in MBS), respectively. The tubes were centrifuged at 39,000 rpm in a SW41 rotor for 16-18 h at 4°C and 12x 1.0 mL fractions were collected manually from the top of the gradient.

Confocal microscopy. $1x10^5$ cells KB/KBr cells were grown on glass coverslips overnight. Cells were incubated in serum-free DMEM for 30 min, and subsequently incubated for 30 min with BODIPY-lactosylceramide (5 µM), Alexa-488-labeled transferrin (10 µg/mL) or FITC-dextran (2 µg/mL). The cells were subsequently washed twice with ice-cold PBS and fixed for 15 min in 4% formaldehyde/PBS. Thereafter, cells were washed with PBS and mounted in Vectashield (Vector Laboratories, Ltd., Peterborough, England). Microscopy was done using a TCS SP2 confocal microscope (Leica Microsystems B.V., Rijswijk, The Netherlands).

Results

Drug resistance induced by either edelfosine or perifosine causes cross-resistance to other APLs. We used 2 tumor cell models, the mouse lymphoma S49 with its edelfosine-resistant variant S49[AR] [33] and the human squamous cell carcinoma KB with its perifosine-resistant variant KBr to study APL uptake and consequent cellular sensitivity or resistance towards apoptosis induction by these drugs. The dose-response curves reveal that S49 cells are approximately 5-fold more sensitive to edelfosine than to perifosine. The data furthermore indicate that the edelfosine-resistant S49[AR] are cross-resistant to perifosine. However, the difference in sensitivity towards perifosine compared to edelfosine between S49 and S49[AR] is only moderate, yet statistically significant (1.2-fold, $P < 0.05$) (compare Fig. 1A and B). Parental KB cells were equally sensitive to perifosine and edelfosine, with EC_{50}'s of about 2 µM for both compounds. APL resistance was pronounced (> 40-fold) in KBr and comparable for both perifosine and edelfosine (Fig. 1C and D).
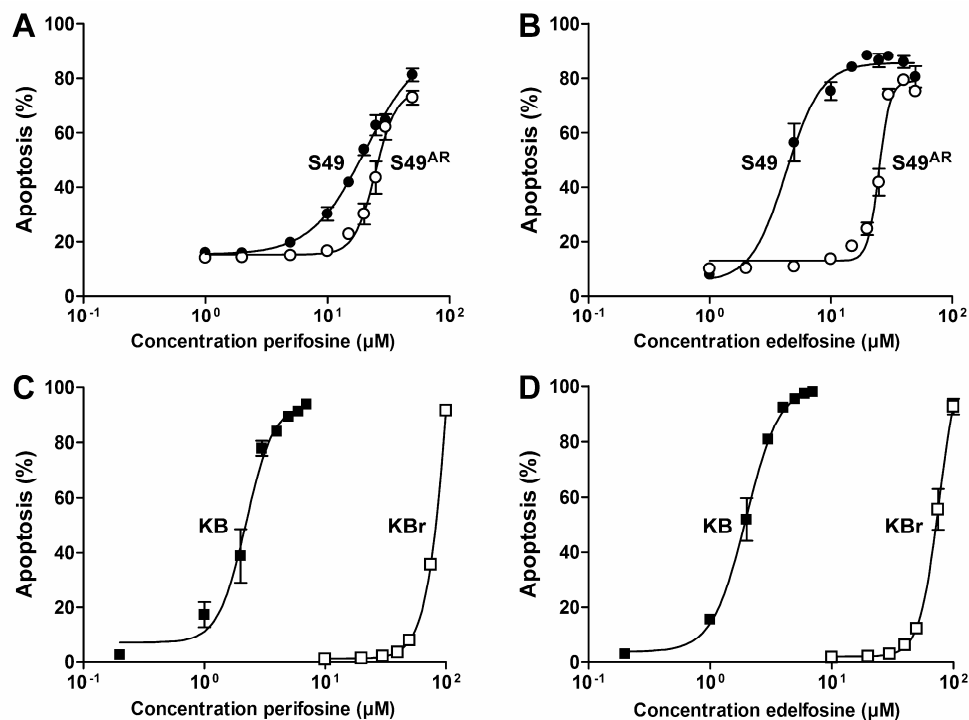
Figure 1. Differential sensitivity of S49 and KB cells and their APL-resistant cell variants S49^AR and KBr, respectively, to perifosine or edelfosine. Dose-dependent induction of apoptosis, measured by nuclear fragmentation, in S49 (solid circles) and S49^AR cells (open circles) 24 h after treatment with either perifosine *(A)* or edelfosine *(B)*. Apoptosis in KB (solid squares) and KBr cells (open squares) was determined after exposure for 48 h to perifosine *(C)* or edelfosine *(D)*. Values are means of quadruplicates ± SD.

Cells were treated with a panel of APLs to test whether resistance induced by a single APL analogue resulted in cross-resistance to other APL members. In addition to perifosine and edelfosine, cells were treated with the APL analogues miltefosine, erucylphosphocholine (ErPC) and Zentaris compound D-21805, in which the choline nitrogen atom has been replaced by arsenic. APL-induced apoptosis in S49 and KB cells was measured at different time points, 24 h and 48 h respectively, because of different apoptosis kinetics in these cells [11,29]. All tested compounds induced apoptosis in S49 at a concentration of 20 µM, edelfosine being most potent and miltefosine being only moderately effective (Fig. 2A). Interestingly, the edelfosine-resistant S49^AR cells showed cross-resistance to all other APLs, although the degree of resistance varied between the APLs tested

(Fig. 2A). KB cells were overall highly affected by APLs, applied at 5 µM (deduced from Fig. 1), whereas the perifosine-resistant KBr cells were fully cross-resistant to apoptosis induction by the other APLs (Fig. 2B). The different degree of cross-resistance for other APLs observed for S49[AR] and KBr suggests distinct underlying mechanisms determining their resistance.
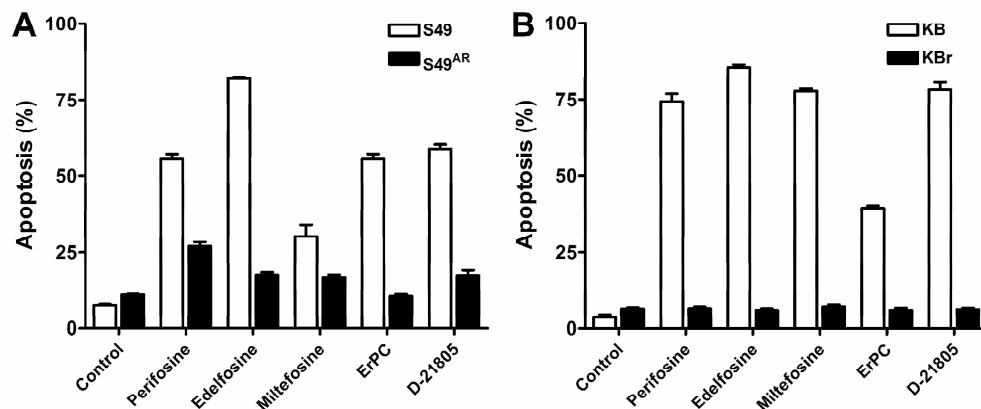

Figure 2. Differential sensitivity of S49 and KB cells and their APL-resistant cell variants S49[AR] and KBr, respectively, to apoptosis induction by a panel of APL analogues. Apoptosis was determined by propidium iodide staining and FACS analysis of subdiploid nuclear fragments. *(A)* Apoptosis in S49 and S49[AR] cells, 24 h after treatment with 20 µM APL. *(B)* Apoptosis in KB and KBr cells, 48 h after treatment with 5 µM APL. Values are means of triplicates ± SD.

APL uptake depends on metabolic energy, especially in KB cells. We next assessed the relationship between APL sensitivity and cellular uptake in S49 and KB cells. We also compared the mechanism of uptake of 2 APLs, perifosine and edelfosine, in these 2 cell types. Evidence exists that after initial insertion into the outer leaflet of the plasma membrane, APLs accumulate in lipid rafts [22,35] (see below) and undergo raft-dependent endocytosis [22,36]. To assess the possible role of endocytosis, APL uptake at 37°C was compared with 4°C, a temperature at which endocytosis is blocked. In S49 cells, edelfosine uptake (at 30 min) was more pronounced, and more temperature-dependent than the uptake of perifosine (Fig. 3A). Remarkably, KB carcinoma cells showed a much higher APL uptake (more than 10-fold for perifosine) than S49 cells (at the same concentration, 1 µM), and the KB cellular uptake of perifosine was twice as high as edelfosine. Furthermore, APL uptake by KB cells was almost completely (94%) blocked at 4°C (Fig. 3B). For perifosine, the temperature effect and hence the mode of cellular uptake was quite

contrasting for KB and S49 cells. Perifosine uptake by S49 was only moderately (35%) sensitive to low temperature, compared to edelfosine (64%). To confirm that KB cells display a more active, temperature and energy-dependent uptake of APL, we subjected the 2 cell types to ATP depletion prior to APL uptake. It appeared that the APL uptake by KB cells was much more compromised (55%) by such energy depletion than the S49 cells (15% for perifosine, 36% for edelfosine) (Fig. 3C).
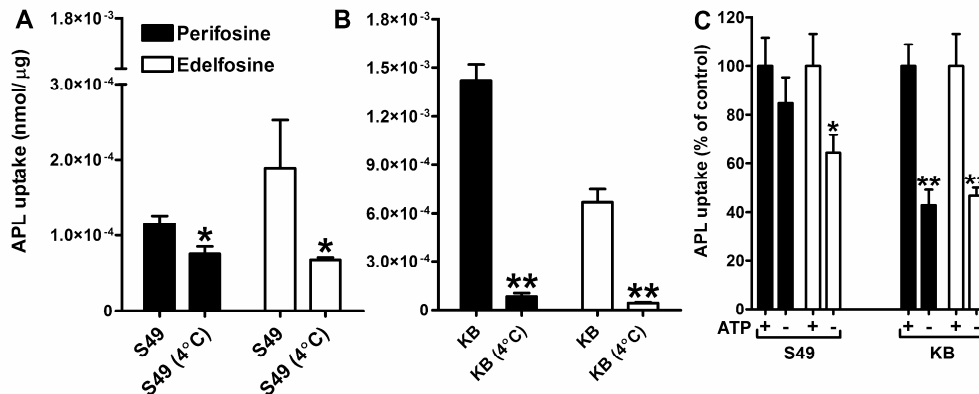
Figure 3. Differential uptake of perifosine and edelfosine by S49 and KB cells, depending on temperature and cellular ATP. Uptake of [^{14}C]perifosine (0.03 µCi/mL, 1 µM) and [^{3}H]edelfosine (0.15 µCi/mL, 1 µM) was determined in S49 cells *(A)* and KB cells *(B)* after 30 min incubation at 37ºC or 4ºC (indicated). *(C)* Effect of ATP depletion on the uptake of APL, 20 µM or 1 µM by S49 and KB cells, respectively. Values are means of triplicates ± SD. *P < 0.05; **P < 0.001.

APLs accumulate in lipid rafts in KB and S49 cells independent of sphingomyelin content. We have previously shown that edelfosine accumulates in lipid rafts of S49 cells [22]. We pre-incubated S49 and KB cells with radiolabeled edelfosine and perifosine, and isolated detergent-resistant lipid raft fractions from these cells. We found a similar APL distribution among the sucrose gradient fractions of both cell types. APLs accumulated in the lipid raft fractions 2 to 4, perifosine even more prominently (about 2-fold) than edelfosine (Fig. 4A and B). Furthermore, APL accumulation in lipid raft fractions was comparable between parental and resistant cells (data not shown). The bulk APLs distributing at the higher density (non-raft) fractions 7-11 is derived from all other membranous parts (plasma- and endoplasmic membranes) of the cell. We calculated that, based on protein content, perifosine and edelfosine in the combined raft fractions 2-4 were 46- and 34-fold enriched, respectively, relative to the non-raft fractions 7-11, for

S49 cells. For KB cells, these enrichments were 43- and 16-fold, respectively. Our previous data [22,29] (see also Fig. 5) have revealed that in S49 cells, mainly the raft-dependent routes of APL internalization are relevant for apoptosis sensitivity in these cells.
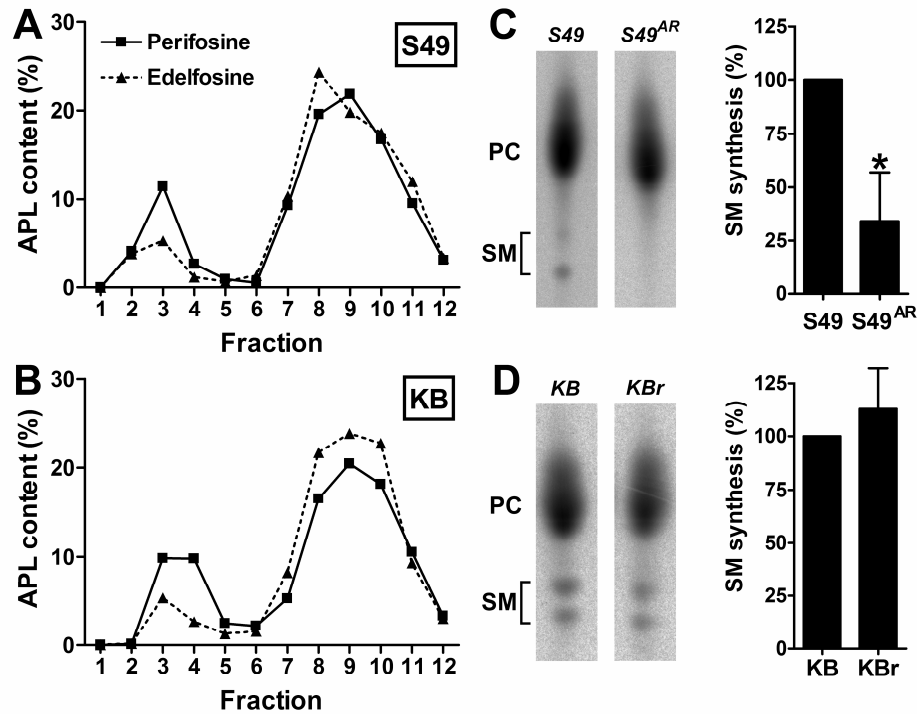
Figure 4. Edelfosine and perifosine accumulate in lipid rafts of S49 and KB cells, independent of sphingomyelin (SM) synthesis. Cells were treated with perifosine or edelfosine for 30 min, and rafts were subsequently isolated. Fractions 2-4 represent detergent-resistant rafts. Representative distributions are shown after incubation with either 20 μM or 1 μM APL for S49 *(A)* and KB *(B)*, respectively. SM synthesis is abrogated in the APL-resistant S49[AR] cells *(C)*, but not in KBr cells *(D)*. Cells were labeled for 8 h with [14C]choline. Lipids were extracted, separated by TLC, visualized and quantified by PhosphorImaging (right panels; n=3 ± SD; *P < 0.05). PC, phosphatidylcholine; SM, sphingomyelin.

Reduced APL uptake in S49[AR] has previously been shown to be the result of impaired raft-mediated endocytosis, due to downregulated SMS1 expression and consequently reduced SM synthesis [22,30]. Indeed, S49[AR] cells were deficient in SM production, as shown by the lack of [14C]choline incorporation (Fig. 4C). In contrast to S49[AR] cells, APL resistance in KBr cells was not accompanied by reduced SM synthesis. The TLC separation of radiolabeled lipid extracts from KB and KBr cells showed two comparable SM spots (Fig. 4D), typically corresponding

to a SM pool with relatively short acyl chains (16 C-atoms) and a SM pool with long acyl chains (predominantly C24:1) and containing a C16-dihydro- (sphinganine) species, as described previously [30]. We conclude that, unlike S49^{AR} cells, downregulation of SM synthesis is not the mechanism of APL resistance induction in KBr.

APL internalization is raft-dependent in S49, but raft-independent in KB cells. To get more insight in the mode of uptake of APLs in the 2 cell types, we used 2 inhibitors of endocytosis, methyl-β-cyclodextrin (MβCD) and chlorpromazine, that act in different ways: MβCD inhibits raft-mediated endocytosis by extracting cholesterol from the membrane, whereas chlorpromazine is commonly used as an inhibitor of clathrin-mediated endocytosis, as it inhibits clathrin-coated pit formation at the plasma membrane [37]. Cells were pretreated with either of these inhibitors, followed by treatment (for 1 h) with equi-effective concentrations of APLs (20 μM for S49, 1 μM for KB). The effect of these inhibitors on the relative APL uptake by the 2 cell types was very different (Fig. 5). MβCD reduced the uptake of respectively edelfosine and perifosine in S49 cells by as much as 64% and 77%, but only by 15% and 8% in KB cells. Chlorpromazine on the other hand, more effectively reduced the edelfosine and perifosine uptake by KB cells (56% and 68%, respectively) than by S49 cells (34% and 24%, respectively).
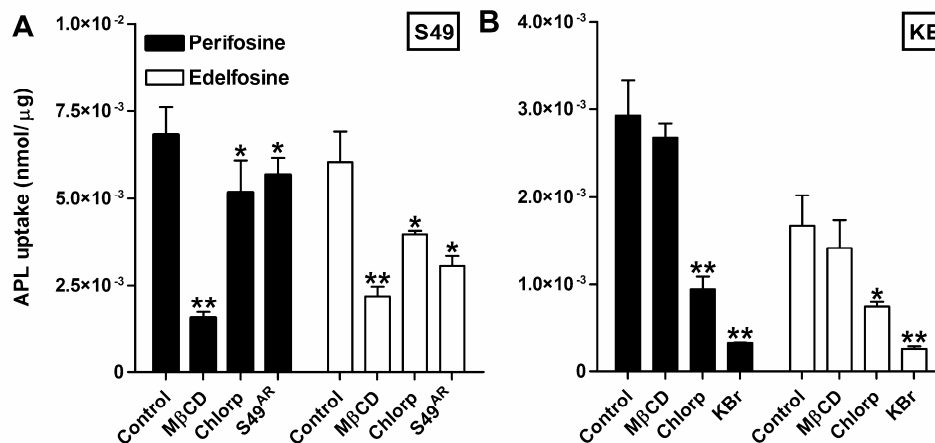
Figure 5. Cell type-dependent effect of pharmacological inhibitors of endocytosis on the uptake of APLs. Cells were pretreated for 30 min with methyl-β-cyclodextrin (MβCD; 2.5 mg/mL) (blocks raft-dependent endocytosis) or chlorpromazine (20 μM) (blocks raft-independent endocytosis), and subsequently incubated for 1 h with 0.03 μCi [^{14}C]perifosine or 0.15 μCi [^{3}H]edelfosine at 20 μM for S49/S49^{AR} cells *(A)* and at 1 μM for KB/KBr cells *(B)*. Uptake values are expressed in nmol APL per μg cellular protein, as means of triplicates ± SD. *$P < 0.05$; **$P < 0.001$.

When we compare cellular uptake of edelfosine with perifosine, we find relatively little difference in perifosine uptake between S49 and S49^{AR} cells, in agreement with Fig. 1A. Although absolute edelfosine uptake by KB cells was lower than perifosine uptake, the effects of MβCD and chlorpromazine on cellular uptake were similar for both APLs (Fig. 5B). Neither of the 2 endocytosis inhibitors reduced the APL uptake to the level of the APL-resistant KBr clone, which displayed a 9-fold reduced perifosine uptake.

Although the chlorpromazine effect on APL uptake would suggest the involvement of clathrin-mediated endocytosis [37], we did not find a defect in the clathrin-dependent internalization of fluorescently labeled transferrin by its cognate receptor in KBr cells (Fig. 6B). Moreover, we found that another established way to block clathrin-mediated endocytosis, by K$^+$ depletion of cells, failed to reduce the uptake of both edelfosine and perifosine by KB cells, whereas as a positive control, the uptake of [^{125}I]EGF via the epidermal growth factor receptor (EGFR) was fully blocked (Fig. 6A).
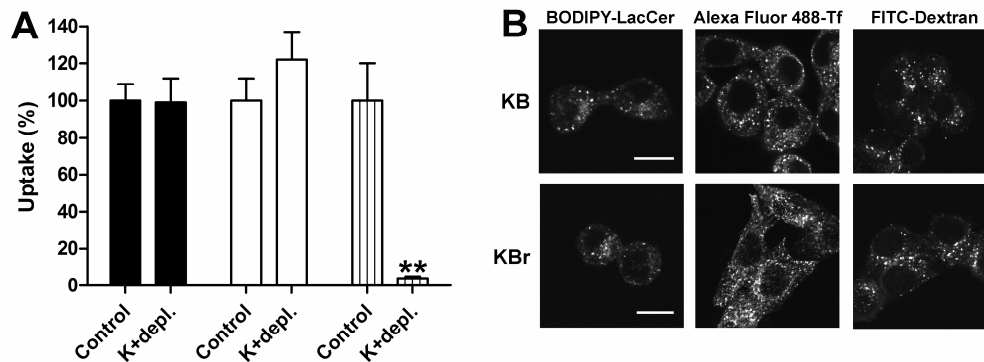
Figure 6. No defects in canonical endocytosis routes can explain abrogated APL uptake in KBr cells. *(A)* **APL uptake in KB cells is not affected by K$^+$ depletion (which blocks clathrin-mediated endocytosis). Cells were left untreated or were K$^+$ depleted, as described in Materials and Methods. Uptake of [^{14}C]perifosine (0.03 μCi, 1 μM) (closed bars), or [^{3}H]edelfosine (0.15 μCi, 1 μM) (open bars), or 1 ng [^{125}I]EGF (684 mCi/mmol) (positive control; hatched bars) was measured at 30 min. Values represent percentage uptake relative to controls (mean of triplicates ± SD of a representative experiment. *****P* < 0.001). *(B)* Confocal microscopy of fluorescently labeled markers of endocytosis. KB and KBr cells, grown on cover slips, were incubated with BODIPY-lactosylceramide (5 μM) (marker for raft-mediated endocytosis), Alexa-488-labeled transferrin (10 μg/mL) (marker for clathrin-mediated endocytosis) or FITC-Dextran (2 μg/mL) (marker for fluid-phase endocytosis) for 30 min at 37ºC. Uptake of these fluorescent markers by KB cells (upper panels) and KBr cells (lower panels) was visualized by confocal microscopy. The bar in the figure represents 20 μm.**

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Therefore, we do not believe that the clathrin-dependent pathway is a major route of APL endocytosis in KB cells. We also found no differences between KB and KBr cells in the raft-mediated endocytosis of a fluorescent raft marker, BODIPY-lactosylceramide [38] and the uptake of fluorescent dextran via fluid-phase endocytosis (Fig. 6B). In conclusion, we found no clear defect in 3 different routes of endocytosis that would explain the resistance of KBr cells to APLs.

To address the mechanism of internalization of APL-like compounds further, we studied the uptake of exogenous lysoPC. From our previous work in HeLa cells [36], we know that exogenous lysoPC does not accumulate in lipid rafts and is internalized independently from these microdomains, by transbilayer flipping, at least to a significant extent. Immediately after membrane traversal, lysoPC is fully acylated to PC [36]. We therefore incubated KB and KBr cells with [^{14}C]lysoPC and followed in time the production of [^{14}C]PC. From a comparison of the absolute (arbitrary) units of [^{14}C]PC synthesized in time (Fig. 7A) with the [^{14}C]lysoPC left in the cells (Fig. 7B), it follows that indeed, most of the LPC is rapidly (within a few min) converted into PC. This is even more clear when expressed in percentages conversion of LysoPC into PC (Fig. 7C). Furthermore, similar to edelfosine and perifosine, the LysoPC internalization and subsequent conversion to PC was much decreased in the KBr cells (Fig. 7A).
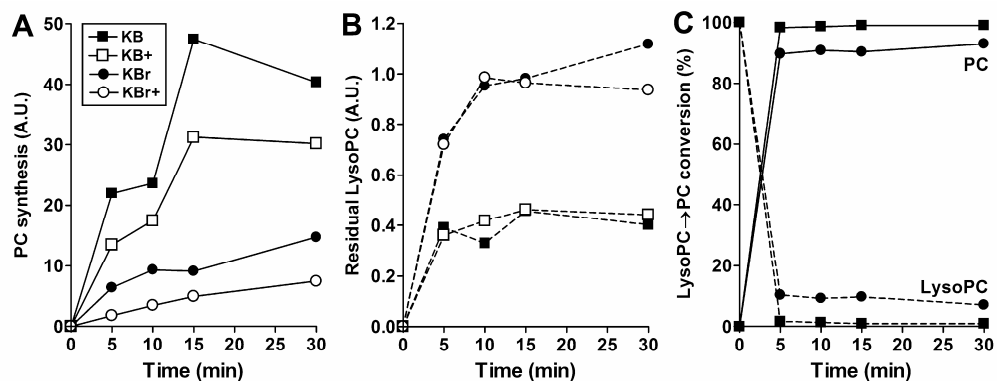
Figure 7. Internalization of exogenous LysoPC and subsequent acylation to PC in KB and KBr cells. KB cells (squares) and KBr cells (circles) were incubated for indicated time periods with 0.025 µCi [¹⁴C]LysoPC, in the absence (closed symbols) or presence of chlorpromazine (20 µM; 30 min preincubation) (KB+, KBr+; open symbols). Cells were then washed 3 times with PBS. Lipids were extracted and separated by TLC. [¹⁴C]PC (panel *A,* solid lines) and residual [¹⁴C]LysoPC (panel *B,* broken lines) were quantified by Phosphor Imaging. Values represent arbitrary units (A.U.) of a representative experiment. *(C)* Percentage of conversion of [¹⁴C]LysoPC (broken lines) into [¹⁴C]PC (solid lines) as a function of time.

The residual LysoPC in KBr cells was higher than in KB cells (Fig. 7B), indicative of impaired internalization in the KBr cells. As a control, LysoPC to PC conversion in KB and KBr cell lysates was not different (data not shown), indicating that the intracellular acyl-transferase activities were similar in these cells, and that the reduced LysoPC to PC conversion in intact KBr cells was indeed the consequence of a blockade in transbilayer movement of the lysoPC. Similar to APL uptake, this lysoPC internalization was sensitive to chlorpromazine treatment, but the relative reduction of PC formation by chlorpromazine was not as dramatic as in the KBr cells (Fig. 7A).

Collectively, these results suggest that, contrary to S49 cells, APL uptake in KB cells is mainly raft-independent, but dependent on a yet undefined energy-dependent (active) uptake mechanism that can be inhibited by chlorpromazine. We find no clear evidence for a defective endocytosis in APL-resistant KBr cells. Since these cells are also resistant to uptake of natural LysoPC, we rather think of a defect in transmembrane flipping, possibly at the level of an unknown transporter.

Discussion

In this study, we show that the cellular uptake and consequent toxicity of anti-cancer APLs in the KB squamous cell carcinoma differs significantly from S49 lymphoma cells. For this latter cell type, we previously described in detail how the APL edelfosine is internalized by clathrin-independent, raft-mediated endocytosis [22,30]. The present study suggests that, in S49 cells, the other APLs are also taken up by this route. We showed for example that the uptake of edelfosine and perifosine in these cells was inhibited by the cholesterol-chelating agent MβCD, which disrupts lipid rafts. KB cells on the other hand, were much more sensitive to APLs than S49 cells due to a higher cellular uptake. Contrary to S49 cells, this high APL uptake in KB cells was mainly raft-independent but, instead, more dependent on ambient temperature and metabolic energy. In addition, APL uptake in KB cells was inhibited by chlorpromazine, which is usually suggestive of a clathrin-dependent uptake [37] but, relevant to our present study, may have relatively non-specific phospholipid 'translocase' effects as well [39,40]. However, K^+ depletion, another way to block clathrin-dependent endocytosis [37], had little effect on APL uptake in KB cells, yet prevented internalization of [^{125}I]EGF by its cognate receptor via the clathrin pathway. Moreover, APL-resistant KBr cells show dramatically impaired uptake of (radiolabeled) edelfosine, perifosine and even natural lysoPC,

while their uptake of transferrin, presumably via clathrin-dependent receptor internalization, was unaffected. So, there is much evidence against clathrin-dependent endocytosis of APL as a major route to induce toxicity in KB cells. In this regard, there is a remarkable resemblance of the KB/KBr system with the macrophage-like RAW cells and their APL-resistant counterparts, described previously [18].

The crucial role of lipid rafts in the uptake of edelfosine by S49 lymphoma cells [22] was recently further explained. We found that the edelfosine-resistant cell variant S49[AR] was deficient in the raft constituent sphingomyelin (SM) due to downregulated expression of SMS1. This inhibition of SM synthesis in the trans-Golgi network abrogated lipid raft vesicular trafficking/recycling and raft-dependent endocytosis of edelfosine and apoptosis induction in these cells [30]. We now demonstrate that these SM-deficient S49[AR] cells are not only resistant to edelfosine but also to the other APLs. Interestingly, we found a similar APL cross-resistance in the KBr cells. While parental KB carcinoma cells take up high amounts of APLs, such as miltefosine [31] and perifosine [19] (also shown in this study), the KBr variant cells, originally made resistant to perifosine, were even more cross-resistant to APLs than S49[AR] cells. Importantly however, this resistance is based on a different mechanism: contrary to S49[AR] cells, the KBr cells displayed normal SM synthesis, suggesting that APL resistance was independent on lipid rafts. This notion is supported by the different effects in these cells of MβCD, which sequesters cholesterol and inhibits raft-dependent endocytosis: we found that this lipid raft-disrupting agent dramatically reduced APL uptake in S49 cells, but not in KB cells.

Targeting of lipid rafts was recently suggested to underlie the selective induction of apoptosis in multiple myeloma cells by both edelfosine and perifosine [41]. Following this concept, Mollinedo and coworkers reported that edelfosine was localized in rafts in leukemic cells, in agreement with our data, but not in solid tumor cells [35]. We found some APL accumulation in isolated lipid rafts of the solid tumor cell KB, but without an apparent consequence. Thus it seems that APL incorporation in lipid rafts and the role of these rafts in APL-induced cytotoxicity is very much dependent on the cell type.

The group of Berkovic and Fleer has made KB cells resistant to miltefosine (hexadecylphosphocholine) (yielding a distinct KBr cell variant) by prolonged culturing in the presence of this drug [21], in a similar way as the S49[AR] cells were made resistant to edelfosine. Their KBr cells contained even more SM than the

parental KB cells, while the uptake of miltefosine was reduced to 20-40%. These cells were cross-resistant to edelfosine and ilmofosine (another APL) and showed reduced uptake of inositol, for reasons that remain unclear. The KBr cells used in our studies were obtained via a process that involved mutagenesis. This method possibly induced genetic defects related to energy-mediated uptake processes, since active, energy-dependent APL uptake was completely blocked in these KBr cells, whereas SM synthesis and uptake of the raft marker lactosylceramide was not impaired. Taken together, our results and those of the German group [21,31] strongly argue against a role for SM synthesis and lipid rafts, but suggest a defect in energy-dependent active uptake to explain the APL resistance in KBr carcinoma cells.

While no apparent defects in SM synthesis, lipid raft- or clathrin-dependent, or fluid-phase endocytosis are detectable in KBr cells to explain their resistance to APLs, what other energy-dependent uptake mechanism could be involved in these cells? We found that APL uptake in KB cells was inhibited by chlorpromazine and by cytochalasin B (data not shown), which disrupts actin filaments. Both of these drugs can inhibit endocytosis, and although the mentioned canonical routes of endocytosis seem not to be affected in the KBr cells, it remains possible that APLs are taken up by an as yet poorly defined endocytic pathway in KB cells. Alternatively, the active APL uptake by KB cells could be mediated by an unknown ATP-driven transporter (see below) that is somehow compromised by chlorpromazine and cytochalasin B-induced cytoskeleton disruption.

In 2 non-mammalian cell systems, there is evidence for the involvement of a P-type transmembrane ATPase that actively translocates APL molecules like miltefosine and perifosine over the plasma membrane. First, in the parasite Leishmaniana, resistance to miltefosine has been attributed to inactivation of the transporter protein LdMT and its beta-subunit LdRos3 [42,43]. Secondly, in yeast there are similar P-type ATPases, Dnf1p and Dnf2p that, in association with their non-catalytic beta-subunit Lem3p, act as "flippases" for inward transbilayer movement of aminophospholipids, lysophospholipids and APLs [44-46]. It is possible that mammalian counterparts of these ATPases exist that can act as a flippase for APL uptake. A recent study in Caco-2 intestinal epithelial cells indeed suggests the existence of a yet undefined carrier-mediated uptake of miltefosine [47]. Whether such a flippase exists in KB cells and is inactivated in KBr cells, remains unknown.

In conclusion, we report a critical role of raft-mediated endocytosis in the uptake of APLs by S49 lymphoma cells, whereas uptake by KB carcinoma cells occurs

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mainly by a raft-independent, energy-dependent route that is sensitive to chlorpromazine and does not involve the canonical endocytosis pathways. Individual APLs are taken up to different extents, which correlates with cell sensitivity towards the drug. The precise mode of APL internalization by KB cells and the mechanism of resistance in KBr cells need to be further defined with the help of additional specific markers for endocytic activity or the possible identification of an APL transmembrane transporter.

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Chapter 2.3

Alkylphospholipids inhibit capillary-like endothelial tube formation *in vitro:* anti-angiogenic properties of a new class of anti-tumor agents

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Abstract

Synthetic alkylphospholipids (APLs), such as edelfosine, miltefosine and perifosine constitute a new class of anti-neoplastic compounds with various clinical applications. Here we have evaluated the anti-angiogenic properties of APLs. The sensitivity of 3 types of vascular endothelial cells (EC) (bovine aortic EC, human umbilical vein EC, human microvascular EC) to APL-induced apoptosis was dependent on the proliferative status of these cells and correlated with the cellular drug incorporation. Whereas confluent, non-dividing endothelial cells failed to undergo apoptosis, proliferating endothelial cells showed 3-4 fold higher uptake and significant levels of apoptosis after APL treatment. These findings raised the question whether APLs interfere with new blood vessel formation. To test the anti-angiogenic properties *in vitro*, we studied the effect of APLs using 2 different experimental models. In the first one we tested the ability of human microvascular EC to invade a 3-dimensional human fibrin matrix and form capillary-like tubular networks. In the second model bovine aortic EC were grown in a collagen gel sandwich to allow tube formation. We found that all 3 APLs interfered with endothelial tube formation in a dose-dependent manner with a more than 50% reduction at 25 μM. Interference with the angiogenic process represents a novel mode of action of APLs and may significantly contribute to the anti-tumor effect of these compounds.

Introduction

Synthetic alkylphospholipids (APLs) represent a group of membrane-permeable compounds with anti-neoplastic properties and a broad range of clinical applications. For example, edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) has been used as a purging agent in autologous bone marrow transplantation [1]. Topical application of miltefosine (hexadecylphosphocholine) was shown to be an effective therapy for skin metastases of breast cancer [2] and cutaneous lymphomas [3]. Oral administration of miltefosine is successfully used in the treatment of visceral leishmaniasis, a systemic protozoal infection [4]. The most recent derivative, perifosine (octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate), has been evaluated as an oral anti-cancer drug in clinical phase I [5,6] and II [7,8] studies, and as a potential radiosensitizer in a clinical phase I study we recently concluded [9].

APLs differ from most currently used cytotoxic drugs with respect to their cellular targets. APLs primarily act on cell membranes where they accumulate in sphingolipid- and cholesterol-enriched microdomains, known as lipid rafts [10]. Following raft-dependent internalization, these compounds interfere with the rapid and continuous phospholipid turnover that is essential for cell survival [11,12]. This interference occurs at different levels: edelfosine and miltefosine inhibit phosphoinositide-specific phospholipase C and consequent formation of the second messengers diacylglycerol and inositol 1,4,5-trisphosphate [13,14]. In addition, both APLs inhibit phosphatidylcholine (PC) turnover at the level of PC degradation as well as PC resynthesis [11,15,16]. The latter inhibition occurs at the level of CTP:phosphocholine cytidylyltransferase [17,18], the rate-determining enzymatic step in PC biosynthesis. Signaling events downstream of these disturbing effects of APLs on lipid metabolism and signaling, include inhibition of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway [19,20], activation of pro-apoptotic stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signaling [21,22] and, as we and others reported more recently, inhibition of the Akt/protein kinase B (PKB) survival pathway [23,24]. These effects most likely contribute to a change in the balance between pro- and anti-apoptotic signaling. Indeed, APLs are potent inducers of apoptosis in a variety of tumor cell lines [25-27]. In addition, APLs enhance radiation- and chemotherapy-induced cytotoxicity, both *in vitro* [22,28-32] and *in vivo* [32].

Angiogenesis is the outgrowth of new blood vessels from pre-existing ones and occurs during development, but normally stops at maturity. In the healthy adult it is only found in the endometrium and ovaries during the menstrual cycle, and in conditions associated with tissue repair and inflammation. Angiogenesis is increased in a number of diseases including rheumatoid arthritis, diabetic retinopathy and cancer. This increase is accompanied by changes in the behavior of endothelial cells, which are reflected in a large increase in their proliferation rate, increased migration and invasion into the extracellular matrix, and the formation of new tubular structures. The increased vascular bed nourishes the malignant tissue and accelerates the growth of many tumors. In the last 2 decades not only have the mechanisms and factors that underlie the angiogenic process become better known, but insight has also grown into the possibilities that inhibition of the angiogenic process may contribute to the treatment of solid tumors [33-38]. In this context, the recombinant humanized anti-VEGF monoclonal antibody bevacizumab represents an apposite example of an anti-angiogenic approach that increases the effectiveness of chemotherapy and radiotherapy [33].

In the present studies we used 3 well-characterized and clinically relevant APLs (edelfosine, miltefosine and perifosine) to evaluate their effect on endothelial integrity. We found that each compound induced apoptosis in endothelial cells from both human and bovine origin depending on the proliferative status of the cells. Confluent, quiescent endothelial cells were relatively resistant, whereas proliferating endothelial cells were highly sensitive to APL-induced apoptosis. In addition, we investigated whether APLs were capable to interfere with angiogenesis *in vitro*. For these studies 2 experimental models were selected. In the first, human microvascular endothelial cells were cultured on top of a 3-dimensional fibrin matrix and allowed to migrate and form an invasive capillary-like tubular network [39,40]. In the second model, bovine aortic endothelial cells were grown in a collagen gel sandwich to re-organize and form sustained tubular structures [41]. In both models, APLs inhibited the formation of endothelial tube-like structures. We therefore conclude that besides the preferential apoptotic effect on malignant cells, interference with angiogenesis may contribute to the anti-tumor effect of these compounds.

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Materials and Methods

Reagents. Miltefosine was purchased from Sigma Chemical Co. (Zwijndrecht, The Netherlands). Edelfosine was from Biomol (Plymouth Meeting, PA) and PAF-18 from ICN Biomedical Inc. (Aurora, OH). [^{3}H]Edelfosine (specific activity 58 mCi/mmol) was synthesized by Moravek Biochemicals (Brea, CA). [^{14}C]Miltefosine (specific activity 42 mCi/mmol), perifosine and [^{14}C]perifosine (specific activity 31 mCi/mmol) were kindly provided by Zentaris GmbH (Frankfurt, Germany). These compounds were diluted in serum-free culture medium. Thrombin was purchased from Leo Pharmaceutic Products (Weesp, The Netherlands) and human fibrinogen from Chromogenix AB (Mölndal, Sweden). Factor XIII was generously provided by dr. H. Metzner and dr. G. Seemann (Aventis Behring, Marburg, Germany), bFGF was obtained from PeproTech Inc. (London, UK), human recombinant TNFα from Biogent (Gent, Belgium) and human recombinant VEGF-A$_{165}$ from ReliaTech (Braunschweig, Germany). A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine hypothalamus as described [42]. Collagen was purchased from Vitrogen 100 (Cohesion Palo Alto, CA).

Cell culture. The human squamous carcinoma cell lines A431 and HeLa and human fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (50 units/mL), and streptomycin (50 μg/mL). Human monoblastic leukemia U937 cells and human T lymphoid leukemic Jurkat cells (J16; kindly provided by prof. J. Borst, The Netherlands Cancer Institute, Amsterdam, The Netherlands) were grown at a density between 0.1×10^6 and 1×10^6 cells/mL in Iscove's modified Dulbecco's medium (GIBCO-BRL, Paisley, Scotland), supplemented with 10% heat-inactivated FCS, penicillin (50 units/mL) and streptomycin (50 μg/mL). Prior to APL treatment, cells were resuspended in serum-free medium (RPMI-1640 or DMEM) and kept overnight. Endothelial cells from human umbilical veins (HUVEC; kindly provided by dr. J.A. van Mourik, Sanquin, Amsterdam, The Netherlands) were cultured in plastic 6-well plates, precoated with human fibronectin (2 mg/mL). The medium consisted of an equal mixture of RPMI-1640 and M199 (GIBCO-BRL), 20% (v/v) heat-inactivated pooled human serum, 2 mM glutamine (Merck, Darmstadt, Germany), penicillin (100 units/mL), streptomycin (100 units/mL) and fungizone (2.5 μg/mL) (GIBCO-BRL). When human serum had to be omitted from the medium, 0.5% human serum albumin

(Sanquin, Amsterdam, The Netherlands) and human transferrin (20 µg/mL, Sigma) were added. Confluent monolayers were harvested by trypsinization, resuspended in medium and subcultured. Subcultured cells from passages 1 and 2 were used. The medium was replaced every 3 days. Endothelial cells from bovine aortic origin (BAEC; kindly provided by dr. Haimovitz-Friedman, Memorial Sloan-Kettering Cancer Center, New York, USA) were grown to confluence in DMEM low glucose (1 g/L) (GIBCO-BRL), supplemented with 10% bovine calf serum (BCS), penicillin (50 units/mL) and streptomycin (50 µg/mL). For serum-free conditions medium containing 0.5% BCS was used. Confluent monolayers were either used for experiments or further subcultured at a plating density of $0.75x10^5$ cells/cm^2. Confluence (cell density $6x10^5$/cm^2 and > 90% of cells in G_0-G_1) was reached at 4-5 days after plating. Human foreskin microvascular endothelial cells (HMVEC) were isolated, cultured and characterized as previously described [43]. HMVEC were cultured on gelatin-coated dishes in M199 supplemented with 20 mmol/L HEPES (pH 7.3), 10% heat-inactivated pooled human serum, 10% heat-inactivated newborn bovine calf serum (NBCS), 150 µg/mL crude ECGF, 2 mM glutamine, 5 units/mL heparin, 100 units/mL penicillin and 100 units/mL streptomycin. Cells were used after they had reached confluence and had been cultured without growth factor for at least 24 h. In some experiments proliferating endothelial cells were used. For these studies, cultures were harvested at 1-2 days after plating, *i.e.* during the exponential phase of cell growth (cell density $1.5x10^5$/cm^2).

Apoptosis assay. Apoptosis was determined by either staining with the DNA-binding fluorochome bisbenzimide (Hoechst 33258, Sigma) [44] to detect morphological nuclear changes or by propidium iodide staining and fluorescence-activated cell sorting (FACS) analysis [45] to determine the percentage of subdiploid apoptotic nuclei. For the bisbenzimide staining, APL-treated cells were harvested at the indicated time points, washed once with phosphate-buffered saline (PBS) and resuspended in 3.7% (v/v) paraformaldehyde/PBS solution. After 10 min at room temperature, the fixative was removed and the cells were resuspended in 15 µL of PBS containing 16 µg/mL bisbenzimide. Following a 15-min incubation at room temperature, a 10 µL aliquot was placed on a glass slide, and 400 cells/slide were scored in duplicate for the incidence of apoptotic nuclear changes under an Olympus AH2-RFL fluorescence microscope using a BH2-DMU2UV exciter filter.

For the propidium iodide staining, cells were seeded at $2x10^5$ cells/mL, 100 µL/well

in round-bottomed, 96-well microtiter plates in serum-free RPMI-medium. Cells were lysed overnight in 200 μL Nicoletti Buffer (0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/mL propidium iodide) and the percentage of apoptotic nuclei, recognized by their subdiploid DNA content, was determined on a FACScan (Beckton Dickinson, San Jose, CA) using Lysis II software.

Incorporation of APLs. Cultures of confluent or proliferating BAEC were incubated in low serum (0.5%) culture medium containing 15 μM APL, traced with 0.05 μCi/mL radiolabeled compound. At various time intervals up to 2 h the medium was removed and cells were washed 3 times with ice-cold PBS and subsequently lysed in 0.1 N NaOH. The incorporated radioactivity was quantified by liquid scintillation counting and normalized for total cell number.

***In vitro* angiogenesis models.** Two *in vitro* angiogenesis models were used to study the formation of tubular structures as previously described [39-41]. For the first model, human fibrin matrices were prepared by addition of 0.1 units/mL thrombin to a mixture of 2.5 units/mL factor XIII (final concentrations), 2 mg/mL fibrinogen, 2 mg/mL sodium citrate, 0.8 mg/mL NaCl, and 3 μg/mL plasminogen in M199 without indicator, and 300 μL aliquots of this mixture were added to 48-well plates. After clotting at room temperature, the fibrin matrices were soaked with 0.5 mL M199 supplemented with 10% heat-inactivated pooled human serum and 10% heat-inactivated NBCS for 2 h at 37°C to inactivate the thrombin. Highly confluent HMVEC (0.7x10^5 cells/cm^2) were seeded in a 1.25:1 split ratio on the fibrin matrices and cultured for 24 h in M199 without indicator supplemented with 10% heat-inactivated pooled human serum, 10% heat-inactivated NBCS and penicillin/streptomycin. Confluent monolayers of HMVEC were then stimulated with the indicated mediators (2.5 ng/mL TNFα and 10 ng/mL bFGF or 25 ng/mL VEGF) for 8 to 10 days in the absence or presence of APL. Every second day the culture medium was removed and fresh medium containing appropriate mediators and test compounds was added. An important feature of this model is that it does not allow endothelial cells to proliferate. Instead, cells migrate and invade the underlying matrix. The formation of capillary-like tubular structures of endothelial cells in the 3-dimensional fibrin matrix was analyzed by phase contrast and dark-field microscopy. The total length of capillary-like tubular structures of 6 randomly chosen microscopic fields (7.3 mm^2/field) was measured using a Nikon FXA microscope equipped with a monochrome CCD camera (MX5) connected to a

computer with Optimas image analysis software (Tokyo, Japan), and expressed as mm/cm^2. For the second model, 0.25 mL of collagen solution (5 mL collagen, 1 mL 10x DMEM, 1 mL 0.1 M NaOH, 1 mL 0.1 M Na$_2$CO$_3$, 2 mL 1x DMEM) was placed in a 24-well plate. After polymerization, 3.5x10^5 cells/0.5 mL DMEM medium supplemented with 0.5% BCS were evenly distributed in the wells. After the cells were attached, the medium was aspirated and the top layer of collagen was added. After polymerization medium with APL was added and incubated for 24–48 h at 37°C. After incubation the cells were fixed with 3% paraformaldehyde. In this model, endothelial cells re-organize, mimicking the resolution phase of angiogenesis; the read-out used is the total additive sprout length [41].

Statistical analyses. Statistical analyses of the data were performed by standard procedures, using Student's *t*-test. Differences were considered significant when *P* values were smaller than 0.05.

Results

APL-induced apoptosis in malignant versus normal cells. Three clinically relevant APLs (edelfosine, miltefosine and perifosine) were assayed for their capacity to induce apoptosis in a panel of cancer cell lines and a variety of normal cell types. As illustrated in Fig. 1, edelfosine induced apoptosis in all human tumor cell lines tested, both from solid (A431 and HeLa) and leukemic origin (U937 and Jurkat T). In contrast, 3 types of confluent normal vascular endothelial cells (BAEC, HUVEC and HMVEC) failed to undergo apoptosis after APL treatment. This resistance was observed for all 3 compounds even after doses as high as 30 µM (not shown). Table 1 shows the ED$_{50}$ values in the different tumor cell lines for the 3 APLs used. The most potent APL was edelfosine, which is considered as the prototype of this group of compounds.

Table 1. Alkylphospholipid-induced apoptosis in a panel of human carcinoma and leukemic cell lines.

Cell type	Edelfosine	Miltefosine	Perifosine
A431	15.4 ± 2.9	17.2 ± 3.0	23.1 ± 2.7
HeLa	5.1 ± 1.6	8.1 ± 0.4	9.2 ± 1.8
U937	6.2 ± 0.3	7.9 ± 2.3	10.3 ± 1.2
Jurkat T	5.0 ± 1.3	8.0 ± 1.9	8.2 ± 0.6

ED$_{50}$ values (µM) were calculated from full dose-response curves at t=16h. Apoptosis was quantified by bisbenzimide staining. Data are expressed as mean ± SD from 3 independent experiments.

Figure 1. APL-induced apoptosis in tumor cells (A431, HeLa, U937, Jurkat T) and normal confluent endothelial cells (BAEC, HUVEC, HMVEC). Apoptosis was determined at 16 h after treatment with 10 µM edelfosine by FACScan analysis after propidium iodide staining. Data are expressed as mean ± SD from 3 independent experiments.

APL-induced apoptosis in confluent versus proliferating endothelial cells. We observed a striking difference in the propensity to undergo APL-induced apoptosis between confluent and proliferating endothelial cells (Fig. 2). As discussed above and consistent with our previous observations [22], APLs did not induce significant levels of apoptosis in confluent cultures of BAEC, HUVEC and HMVEC. Up to concentrations of 25 µM for 48 h, APLs exerted no significant effect on endothelial cell viability in confluent culture (not shown).
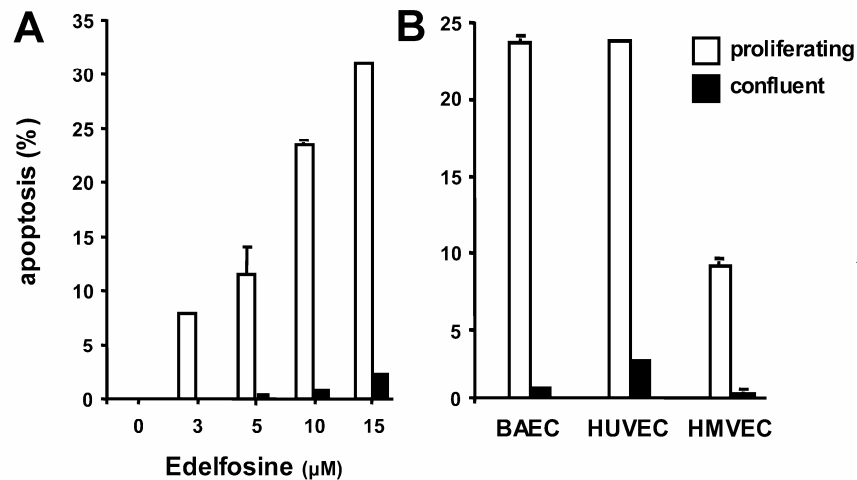
Figure 2. APLs induce apoptosis in proliferating but not in confluent endothelial cells. *(A)* Dose-effect relationship of edelfosine-induced apoptosis at 24 h in proliferating and confluent BAEC. *(B)* Edelfosine (10 µM)-induced apoptosis in proliferating and confluent BAEC, HUVEC and HMVEC at 24 h. Apoptosis was determined by FACS analysis after propidium iodide staining. Data are expressed as mean ± range from 2 independent experiments.

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However, as shown in Fig. 2A, exponentially proliferating endothelial cells showed a dose-dependent increase in apoptosis after edelfosine treatment. Similar differences in apoptosis sensitivity between confluent and proliferating cells were observed in HUVEC and HMVEC (Fig. 2B) and after treatment with the 2 other APLs (not shown).

Incorporation of APLs in confluent versus proliferating endothelial cells. Because it has been shown that the cytotoxic effect of APLs correlates with its cellular uptake [25,46,47], we measured the incorporation of [³H]edelfosine, [¹⁴C]miltefosine and [¹⁴C]perifosine in cultures of confluent and proliferating endothelial cells. We found that proliferating BAEC incorporated much larger amounts of APLs than confluent BAEC, namely by a factor of approximately 3-4 at 2 h (Fig. 3A). The kinetics of APL uptake was also different with a more rapid and prolonged uptake in proliferating BAEC. Fig. 3B shows the uptake of edelfosine over a period of 2 h. Similar kinetics were observed for miltefosine and perifosine (not shown). In confluent BAEC the uptake reached its maximum at about 30 min after addition. It should be noted that the incorporation of APL in proliferating BAEC preceded the appearance of apoptotic morphology that was detected after 4-6 h (not shown).
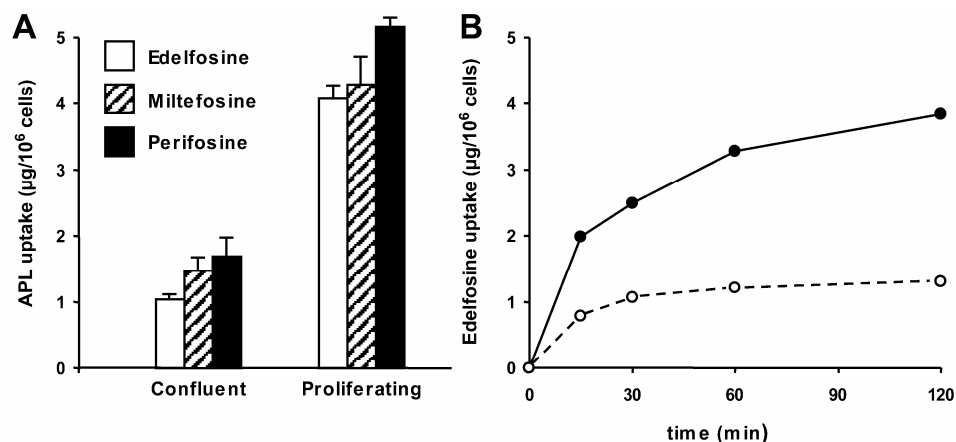
Figure 3. Incorporation of radiolabeled APL in proliferating (closed circles) and confluent (open circles) BAEC. At the indicated time points after addition of 0.05 µCi/mL [³H]edelfosine, [¹⁴C]miltefosine or [¹⁴C]perifosine (final APL concentration 15 µM), the incorporation of the compound was measured by liquid scintillation and normalized for total cell number. *(A)* APL uptake at 2 h. Data are expressed as mean ± range from 2 independent experiments. *(B)* Time course of [³H]edelfosine uptake. Data shown are representative of 3 experiments performed.

Effect of APLs on endothelial tube formation *in vitro*. Because endothelial proliferation and apoptosis are both major determining factors in angiogenesis, we reasoned that APLs might interfere with new vessel formation. To test this hypothesis, we employed 2 *in vitro* angiogenesis models. It is important to note that both models do not allow endothelial proliferation, thereby excluding an anti-proliferative effect of APLs as the main cause of their potential anti-angiogenic properties. In the first model, described by Koolwijk *et al.* [39,40], HMVEC are seeded on a 3-dimensional human fibrin matrix to form a confluent monolayer. In the continuous presence of the combination of an angiogenic factor (VEGF or bFGF) and TNFα, outgrowth of capillary-like tubular structures in the fibrin matrix is observed over a period of 8-10 days. The total length of these tubular networks is quantified by computer-assisted image analysis [39]. Fig. 4 shows a set of phase contrast microscopy images of a representative experiment. In the unstimulated cultures, the confluent monolayer of HMVEC remained on top of the 3-dimensional fibrin matrix. Invading endothelial cells and tubular structures could not be observed (Fig. 4A).
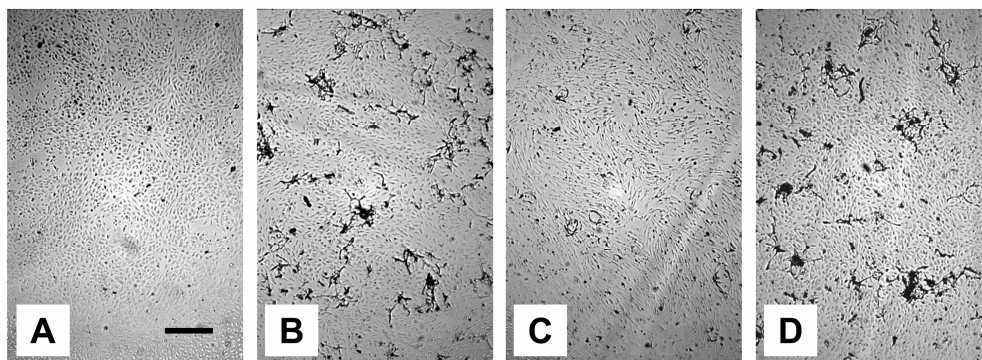
Figure 4. Capillary-like tube formation is inhibited by APLs. HMVEC cultured on top of a 3-dimensional fibrin matrix were not stimulated *(A)*, or stimulated with 10 ng/mL bFGF and 2.5 ng/mL TNFα *(B)*, or with bFGF and TNFα in the presence of 100 μM edelfosine *(C)*, or with bFGF and TNFα in the presence of 100 μM PAF-18 *(D)*. After 8 days of culture representative phase contrast photographs were taken (bar: 300 μm). Similar results were obtained in 3 independent experiments.

The addition of bFGF or TNFα alone was not sufficient to induce tube formation (not shown). However, the simultaneous addition of bFGF and TNFα resulted in the outgrowth of tubular structures invading the fibrin matrix and forming a capillary network (Fig. 4B). The number of endothelial cells on top of the fibrin matrix was not significantly changed compared with unstimulated cultures (95% of control; not

shown). In the presence of APL, a significant inhibition in the formation of tubular structures was observed. Fig. 4C shows the effect of 100 μM edelfosine. The morphology of the endothelial monolayer covering the fibrin matrix was slightly altered, but no significant detachment of cells was observed. The specificity of APL-induced interference with tube formation was demonstrated by the use of platelet-activating factor-18 (PAF-18), a structurally related, but ineffective counterpart of edelfosine [25]. The addition of PAF-18 up to 100 μM did not significantly affect the outgrowth of tubular structures (Fig. 4D and 5).

The inhibitory effect of edelfosine on bFGF/TNFα-induced tube formation was dose-dependent, as shown in Fig. 5. At 25 μM, this inhibition was 54% of controls and reached statistical significance. At 100 μM, the inhibition was complete. We note that the final concentration of APL in the angiogenesis studies was kept higher than in the apoptosis assays, because the higher serum concentration in the former type of experiments sequesters APLs and thus diminishes the effective concentration by a factor of 2-3. To assure that the cell membrane integrity was not impaired under these conditions, we performed a separate set of standard culture experiment, in which endothelial lactate dehydrogenase (LDH) release and trypan blue exclusion were measured after APL treatment. No significant changes in both parameters were found in APL-treated endothelial cell cultures as compared with controls, confirming the viability of the cells (data not shown).

Figure 5. Dose-dependent inhibition of capillary-like tube formation by APLs. HMVEC seeded on top of a 3-dimensional fibrin matrix were not stimulated (control) or stimulated with 10 ng/mL bFGF and 2.5 ng/mL TNFα in the presence of increasing amounts of edelfosine, or 100 μM PAF-18. After 8 days of culturing total tube length/cm^2 ± SD of triplicate wells was measured (*P < 0.005 compared with 0 μM edelfosine). Similar results were obtained in 3 independent experiments.

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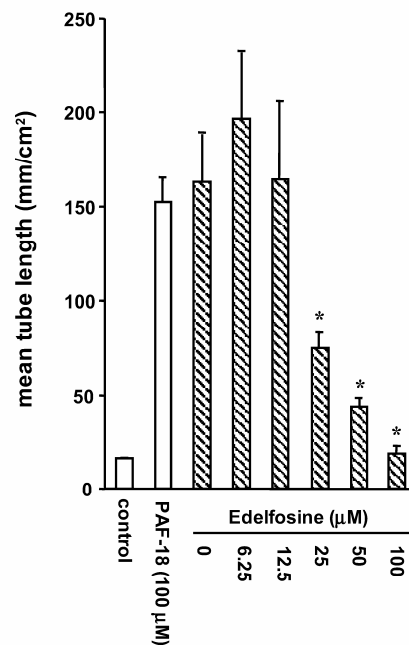
Next, we introduced another angiogenic factor in this system and investigated the effect of other APLs on tube formation. Like we found for bFGF (Fig. 6A), VEGF added in combination with TNFα to the endothelial monolayers induced the formation of tubular structures (Fig. 6D). Furthermore, like edelfosine, miltefosine (Fig. 6B,E) and perifosine (Fig. 6C,F) interfered with the outgrowth of endothelial tubes by both bFGF/TNFα and VEGF/TNFα.
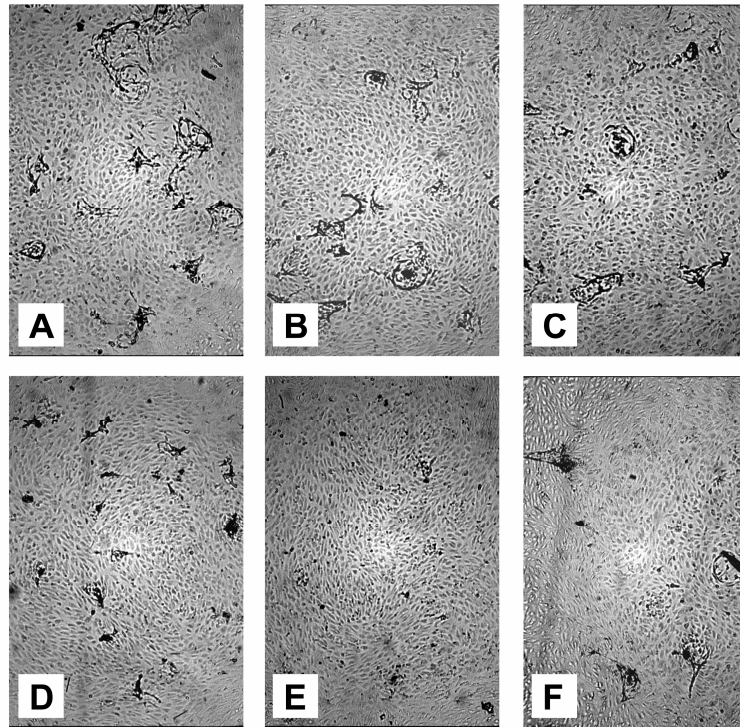
Figure 6. Different APLs inhibit bFGF/TNFα- and VEGF/TNFα-mediated tube formation. HMVEC cultured on top of a 3-dimensional fibrin matrix were stimulated with 10 ng/mL bFGF and 2.5 ng/mL TNFα *(A,B,C)* or with 25 ng/mL VEGF and 2.5 ng/mL TNFα *(D,E,F)*. No APL added *(A,D)*; 50 μM miltefosine *(B,E)* and 50 μM perifosine *(C,F)*. After 8 days of culture representative phase contrast photographs were taken. Similar results were obtained in 3 independent experiments.

To confirm these inhibitory effects of APLs on tube formation, we employed a second model [41], using endothelial cells from bovine origin. In this model BAEC are seeded between a collagen sandwich and allowed to re-organize and rapidly form tubular structures within 48 h (Fig. 7A). Similar to HMVEC grown on a fibrin matrix, APL inhibited tube formation by BAEC in a collagen sandwich as well. Fig.

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7D-F shows the dose-dependent interference with tube formation by edelfosine. At 20 μM and higher, no tubular structures could be discerned; quantification of the total sprout length was therefore not possible. Miltefosine (Fig. 7B) and perifosine (Fig. 7C) inhibited this process in a comparable fashion.
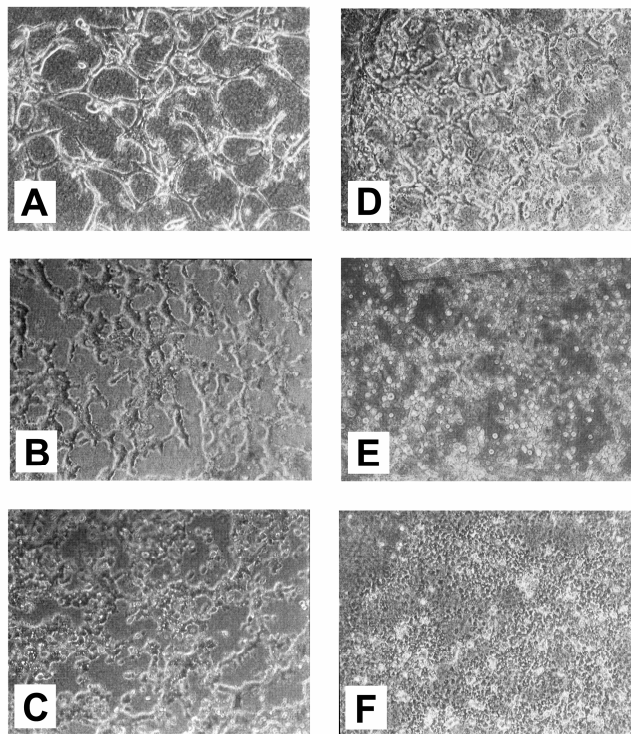
Figure 7. APL-induced interference with endothelial tube formation. BAEC were seeded between a collagen sandwich and allowed to rapidly re-organize and form capillary-like tubular structures within 48 h. No APL added (A); 10 μM miltefosine (B); 10 μM perifosine (C) and 10, 20, 50 μM edelfosine (D,E,F). Similar results were obtained in 3 independent experiments. Magnification 100x.

Discussion

The present study was undertaken to investigate the effect of 3 clinically relevant APLs on normal vascular endothelial cells in comparison with a panel of tumor cell lines, and to study the anti-angiogenic properties of these compounds *in vitro*. Edelfosine, miltefosine and perifosine induced a time- and dose-dependent increase in apoptosis in a variety of human leukemic and solid tumor cell lines. Importantly, the ED_{50}-values we found here fall within the same micromolar range

as obtained in plasma from APL-treated patients [5,6]. In our phase I study, we measured a dose-dependent steady state plasma concentration of perifosine between 4 and 23 µM, which was maintained throughout the 4-week treatment period [9]. In contrast to the effect on tumor cells, 3 types of normal quiescent endothelial cells were insensitive to APL-induced apoptosis. This differential cytotoxic effect of APLs is consistent with data obtained in other cell systems [25,27,48] and offers a solid basis for further clinical evaluation of these compounds as selective anti-cancer drugs. Another attractive biological property of APLs is their capacity to strongly enhance radiation-induced apoptosis of tumor cells *in vitro* and *in vivo*, as we described recently [22,28,32]. In the present studies we observed a remarkable difference in APL-induced apoptosis between confluent, resting versus actively proliferating endothelial cells. These findings are consistent with Araki *et al.* [49], who reported on apoptosis induced by the structurally related compound Et-16-OCH$_3$ (edelfosine = Et-18-OCH$_3$) in subconfluent cultures of HUVEC. We found that APL-induced endothelial apoptosis correlated with the cellular uptake of the compound. Proliferating endothelial cells incorporated large amounts of APL, resulting in significant levels of apoptosis. In quiescent endothelial cells, on the other hand, the uptake of APL was only one-third of proliferating cells and insufficient to induce significant apoptotic cell death. The relationship between APL uptake and apoptosis sensitivity is emerging as a more general phenomenon. Mollinedo *et al.* [25] demonstrated that upon transformation with SV40, 3T3 fibroblasts became sensitive to edelfosine and incorporated high amounts of the lipid. Similarly, apoptosis sensitivity was restored in human T lymphocytes after activation with mitogens [46]. Thus, the amount of APL incorporated by the endothelial cell, most likely in combination with the enhanced metabolic activity of the cell, apparently dictates the biological effect. Because endothelial apoptosis has been identified as an important determinant in tumor angiogenesis [50-52], these observations prompted us to study anti-angiogenic properties of APLs *in vitro*.

Angiogenesis is a complex and tightly regulated process of new blood vessel formation from pre-existing vasculature. Its role in tumor growth and metastases has now clearly been established and several strategies of anti-angiogenic therapy have been developed and tested clinically [33-35]. During angiogenesis several phases can be distinguished: (a) degradation of the basement membrane, (b) endothelial migration and invasion in the extracellular matrix, (c) endothelial proliferation, and (d) the formation of capillary-like tubes [36]. A large number of

angiogenic factors have been identified in recent years, including VEGF and bFGF [37,38]. The formation of capillary-like structures can be studied *in vitro* using different model systems. For our experiments we employed 2 well-characterized models. The first consists of a 3-dimensional human fibrin matrix covered by human microvascular endothelial cells [39,40]. This model mimics the *in vivo* situation where fibrin is a common component of the matrix present at sites of chronic inflammation and tumor stroma [53]. Both an angiogenic factor (bFGF or VEGF) and a factor to induce urokinase-type plasminogen activator (*e.g.* TNFα) are required in this *in vitro* model to induce endothelial migration and the formation of capillary-like tubular structures without endothelial proliferation [34,35]. The present studies demonstrate that APLs are efficient inhibitors of both VEGF/TNFα- and bFGF/TNFα-induced tube formation from pre-existing monolayers of confluent HMVEC. Moreover, the structurally related, but ineffective compound PAF-18 failed to interfere with this process. Also in a second re-organization model using a collagen sandwich and endothelial cells from bovine origin, APLs interfered with tube formation (Fig. 7). The anti-angiogenic action of APLs cannot be explained by an anti-proliferative effect, because both models do not allow endothelial proliferation. Instead, these models study endothelial migration and re-organization. It is also unlikely that extensive cytotoxic effects account for the inhibition of angiogenesis, as the endothelial monolayer remains intact throughout the observation period (Fig. 4 and 5).

It remains to be established which signal transduction pathways are important for the apoptotic and anti-angiogenic effects of APLs. In this context, we have previously shown that APLs activate the pro-apoptotic SAPK/JNK pathway. In addition, APLs efficiently prevent serum- and growth factor-induced MAPK/ERK signaling both in tumor and endothelial cells [19,20,22,54]. More recently, we found that APLs also inhibit the Akt/PKB survival pathway [28]. These signaling systems are not only important for cell death and survival, but have been implicated in angiogenesis as well [55-58]. In different *in vitro* and *in vivo* angiogenesis models it has been shown that blockade of the MAPK/ERK or Akt/PKB pathway by pharmacological or molecular approaches induces apoptosis and inhibits angiogenesis [55,56,59]. Our current line of research is focused on the identification of additional, critical (intra-)cellular targets of APLs [10].

In conclusion, our data show that not only tumor cells, but also normal endothelial cells can be a target for APLs. The cytotoxic effect, however, depends on the proliferative status with actively dividing cells incorporating more APL and thus

being apoptosis-sensitive. Furthermore, we demonstrated that APLs are effective inhibitors of endothelial capillary-like tube formation *in vitro*. Taken together, these results support the concept that APLs exert their anti-tumor effect both directly through apoptosis and indirectly, through interference with the angiogenic process.

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In vivo studies

Chapter 3.1

Tumor and normal tissue pharmacokinetics of perifosine, an oral anti-cancer alkylphospholipid

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Abstract

Clinical use of anti-cancer alkylphospholipids (APLs) is limited by gastrointestinal toxicity. However, new interest has emerged since it was shown that these drugs enhance the cytotoxic effect of conventional chemotherapy and radiotherapy in preclinical models. The aim of this study was to characterize the pharmacokinetic profile of perifosine, an oral analogue of alkylphosphocholine (APC), and to compare *in vitro* drug uptake with *in vivo* drug accumulation in 3 human-derived squamous cell carcinomas (A431, HNXOE and KB). *In vitro*, KB cells showed a remarkably high uptake and sensitivity for perifosine compared with A431 and HNXOE cells. *In vivo*, perifosine reached a clinically relevant plasma concentration in mice after a single oral dose of 40 mg/kg. Perifosine was not metabolized and displayed slow elimination, with a terminal half-life of 137 (± 20) h and an apparent volume of distribution of 11.3 L/kg. Comparable tumor accumulation was observed for A431 and HNXOE tumors, whereas perifosine uptake by KB xenografts was substantially higher. Tissue distribution occurred throughout the whole body reaching high perifosine levels in the gastrointestinal tract, while heart and brain tissue contained relatively low levels. Based on its stability and relatively high tumor uptake *in vivo*, perifosine is an attractive candidate for further evaluation, *e.g.* as radiosensitizer.
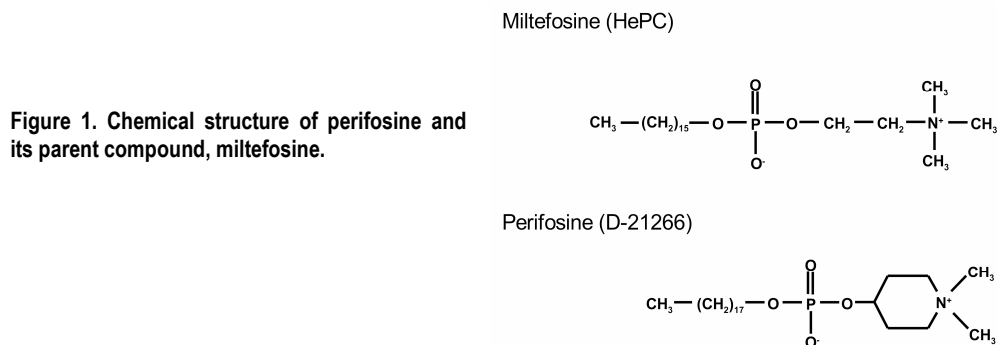
Introduction

Perifosine (D-21266 - octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate) is a heterocyclic member of membrane-permeable single-chain anti-tumor alkylphosphocholines (APCs). This class of synthetic anti-cancer agents primarily acts at the level of the cell membrane, in contrast to most conventional chemotherapeutic drugs that target the DNA. APCs are selectively toxic for malignant cells, both *in vitro* [1] and *in vivo* [2-5]. Although the precise mechanism of action is not yet fully elucidated, APCs have been shown to interfere with phospholipid metabolism and survival signaling, to induce apoptosis, inhibit neovascularization, prevent invasion and induce tumor cell differentiation (reviewed in [6-11]).

APC(-like compounds) that are currently applied clinically are edelfosine, miltefosine and its derivative, perifosine. Miltefosine is effective as an oral drug against leishmaniasis [12] and as a topical treatment of breast cancer skin metastases [13]. Edelfosine has been applied as a purging agent in autologous bone marrow transplantation [14] and perifosine has entered several phase I and II trials. The clinical use of APCs is limited due to major side effects. The APC prototype miltefosine (hexadecylphosphocholine) for instance, causes hemolysis when administered parenterally [15]. Further, its oral application was ceased in a phase II trial due to cumulative gastrointestinal toxicity, especially vomiting and diarrhea [16].

Perifosine, in which the choline moiety of miltefosine is replaced by a cyclic aliphatic piperidyl residue (Fig. 1), was designed for an improved systemic therapeutic index. Indeed, perifosine was shown to be better tolerated than miltefosine in rats bearing DMBA-induced tumors [17]. Recently, perifosine has been evaluated in phase I trials using different dose schedules in patients with solid tumors [18,19]. Renewed interest in APCs has emerged since they were shown to enhance cell death induced by conventional chemotherapeutics [20] and more recently, by radiation [21,22]. The enhanced radiation-induced cell kill is thought to depend on interference with survival signal transduction pathways by APCs [23]. We and others have shown that APCs induce stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signaling and inhibit mitogen-activated protein kinase (MAPK) and protein kinase B (Akt/PKB) signaling, resulting in an enhanced apoptotic response [22,24]. In addition, APCs act as radiosensitizers in different solid tumor cell lines [21,25].

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Despite several *in vitro* and *in vivo* studies, the pharmacokinetic behavior and biodistribution of perifosine in an animal tumor model have not yet been described. The pharmacokinetic profile of perifosine and its selective uptake by malignant cells are important determinants of the anti-tumor response after perifosine treatment, both as a single agent treatment and in combined modality strategies. The aim of this study was to determine *in vitro* and *in vivo* perifosine uptake by tumor cells. Furthermore, we aimed to quantitate plasma pharmacokinetics, biodistribution and metabolic fate after oral perifosine treatment.

Miltefosine (HePC)

Figure 1. Chemical structure of perifosine and its parent compound, miltefosine.

Perifosine (D-21266)

Material and Methods

Chemicals. Perifosine and [2,6-^{14}C]perifosine (66.8 µCi/mg) were kindly provided by Zentaris GmbH (Frankfurt, Germany).

Cell culture. The A431 epidermoid vulva carcinoma cell line and KB head and neck carcinoma cell line were obtained from the ATCC. The HNXOE is a head and neck squamous cell carcinoma cell line derived from a metastasis of the oral cavity [26]. A431, KB and HNXOE cells, routinely tested for absence of mycoplasma, were cultured in Dulbecco's Modified Eagles Medium (Gibco, Rockville, supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin and 10% (v/v) fetal bovine serum (Life Technologies).

***In vitro* drug uptake and cytotoxicity.** *Drug uptake*: 5x10^4 cells in 2 mL DMEM/well were plated in 6-well plates and incubated overnight. [^{14}C]perifosine was added to a final concentration of 1 µM. After 1, 2, 4, 8 and 24 h of incubation, medium was removed and the cells were washed twice with PBS. The cells were

dissolved in 1 mL Solvable (Packard Instrument Co., Groningen, The Netherlands), diluted in Ultima Gold scintillation liquid and measured using a TRI-CARB liquid scintillation analyzer (Canberra Packard Benelux B.V., Groningen, The Netherlands). *Cytotoxicity assay:* The sulforhodamine B (SRB) assay was used to determine the cytotoxicity of perifosine in the A431, HNXOE and KB cell lines. Cells (3,000 in 200 µL DMEM/well) were allowed to attach overnight, followed by incubation with perifosine. After 48 h, the cells were fixed in 10% TCA/HBSS, washed with H_2O and stained with 0.4% SRB/1% acetic acid. Plates were washed with 1% acetic acid, and protein bound SRB was dissolved in 10 mM Tris-HCl (pH 7.5). The extinction was measured at 540 nm with a microplate reader (Bio-Tek Instruments, Winooski, VT). The surviving fraction was calculated by dividing the mean extinction for each concentration by the mean extinction of the control wells (medium).

Animals and tumors. Female BALB/c nude mice (18-28 g), were obtained from the animal department of the Netherlands Cancer Institute. Animals, kept and handled according to institutional guidelines complying with Dutch legislation under a 12/12 h light/dark cycle at a temperature of $22^{o}C$, received a standard diet and acidified water *ad libitum*. Mice were injected subcutaneously on the lower dorsum with $1x10^6$ A431 cells, $1x10^6$ HNXOE cells or $3x10^6$ KB cells in 50 µL PBS.

Pharmacokinetic analysis. Tumor bearing animals were administered a single oral dose of 40 mg/kg (A431, HNXOE and KB xenografts) or 4 daily administrations of 10 mg/kg (A431 xenografts) perifosine, using a stomach tube. At various time points after administration (0-168 h), animals were anesthetized and blood was collected by way of heart puncture (for each tumor 3-5 animals/time point). Blood was centrifuged at 14,000 rpm for 5 min ($4^{o}C$), and 10-20 µL plasma was diluted in Ultima Gold scintillation liquid (Packard Bioscience B.V., Groningen, The Netherlands) and [^{14}C]perifosine was measured using a TRI-CARB liquid scintillation analyzer (Canberra Packard Benelux B.V., Groningen, The Netherlands). Pharmacokinetic analysis was done for a single dose of 40 mg/kg, with plasma samples obtained from experiments with all 3 tumors. The area under the curve (AUC) of perifosine in plasma after a single administration of 40 mg/kg was calculated with the linear trapezoidal rule. The elimination half-life ($t_{1/2}$) was calculated using linear regression analysis of the log plasma concentration-time curve. The maximum plasma concentration (C_{max}) was calculated using the

software program MW/Pharm (Mediware, Groningen, The Netherlands) [27]. Remaining plasma after heart puncture was stored at -20°C until thin layer chromatography (TLC).

Thin Layer Chromatography. Plasma samples obtained at various time points after oral administration, containing approximately 3,000 dpm [^{14}C]perifosine, were adjusted to 200 µL with PBS. Lipids were extracted with methanol/chloroform (1:2, v/v) and phase separation was induced with 1 M NaCl. The organic phase was dried, dissolved in chloroform and applied on a silica TLC plate. The plate was developed in chloroform/methanol/25% ammonia (7:4:1, v/v/v). [^{14}C]Perifosine was visualized and quantified using a Fuji BAS 2000 TR Phosphor Imager (Fuji Photo Film Co., Tokyo, Japan).

Tumor and normal tissue distribution studies. Mice were allowed to grow tumors with an initial mean diameter of at least 5 mm (measured in 3 orthogonal directions). The mice received, by way of a stomach tube, a single dose of 40 mg/kg perifosine/[^{14}C]perifosine (0.1 µCi/g) or 4 daily dosages of 10 mg/kg perifosine/[^{14}C]perifosine (0.05 µCi/g) dissolved in PBS, in a volume of 5 µL/g body weight. At various time points (0-168 h) after the first administration, animals were anesthetized, sacrificed by cervical dislocation and tumors and organs were collected. The gastrointestinal tract was cleared from content and all tissues were dissolved in 1-6 mL Solvable (Packard Instrument Co., Groningen, The Netherlands) at 60°C overnight, bleached with 30% H_2O_2, diluted in Ultima Gold scintillation liquid and measured using a TRI-CARB liquid scintillation analyzer.

Results

In vitro studies
Perifosine uptake and sensitivity. To determine *in vitro* uptake of perifosine, A431, HNXOE and KB cells were incubated with 1 µM [^{14}C]perifosine. Uptake by A431 and HNXOE cells was in the same range, reaching a plateau of around 1 µg/10^6 cells after approximately 4 h of incubation. KB cells however, showed significantly higher uptake reaching 8 µg/10^6 cells at 24 h (Fig. 2A).
Perifosine cytotoxicity is shown in Fig. 2B. The remarkably high level of perifosine uptake by KB cells was accompanied by a relatively high sensitivity for this drug (IC$_{50}$=0.84 ± 0.08 µM). A431 and HNXOE cells showed a comparable perifosine

uptake, with A431 cells being slightly more sensitive than HNXOE cells (IC_{50} values 9.95 ± 0.82 μM and 24.55 ± 0.64 μM, respectively).
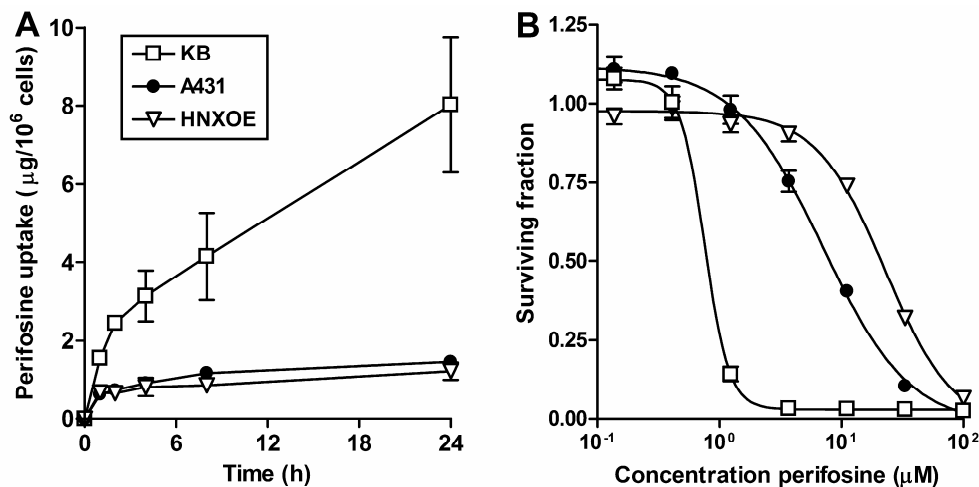
Figure 2. *In vitro* **determination of perifosine uptake and sensitivity of A431, HNXOE and KB cells.** *(A)* **Drug uptake after incubation with 1 μM [**[14]**C]perifosine (mean values of 3 independent experiments, ± SD).** *(B)* **Cytotoxicity after 48 h of incubation with perifosine. Shown are representative dose response curves.**

In vivo studies

Tumor and normal tissue pharmacokinetics after oral perifosine treatment were studied in female BALB/C nude mice, bearing subcutaneous tumors.

Plasma pharmacokinetics. Pharmacokinetic analysis of [^{14}C]perifosine in blood plasma after a single oral dose of 40 mg/kg showed a C_{max} of 5.7 μg/mL, 22 h after administration and a $t_{1/2}$ of 137 ± 20 h (Fig. 3; Table 1). Four daily administrations of 10 mg/kg perifosine resulted in a C_{max} of about 6 μg/mL (measured 24 h after the last administration), similar as obtained with a single dose of 40 mg/kg (Fig. 3).

Table 1. Pharmacokinetic parameters of a single dose of perifosine of 40 mg/kg					
C_{max}	T_{max}	$T_{1/2} \pm$ SEM	AUC $\pm$ SEM	Cl	V_d
(μg/mL)	(h)	(h)	(μg/mL.h)	(L/h/kg)	(L/kg)
5.7	22	137 ± 20	698 ± 19	0.057	11.3

C_{max} = peak plasma drug concentration; T_{max} = time to maximum plasma concentration; $T_{1/2}$= half life; $AUC_{(0-168 h)}$ = area under the concentration-time curve; Cl = clearance; V_d = apparent volume of distribution.

Figure 3. Plasma levels of perifosine after oral administration of a single dose of 40 mg/kg and 4 daily dosages of 10 mg/kg (values are means ± SEM, n=4-13).
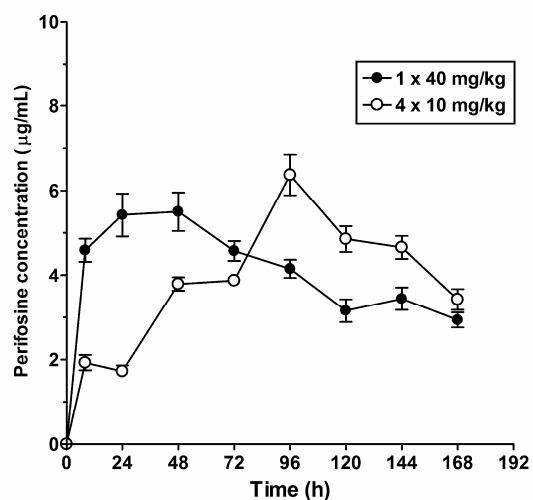
Metabolic fate. Plasma levels of [^{14}C]perifosine were used to determine the metabolic fate of perifosine after oral administration. Plasma was collected from 8-168 h after administration, and plasma lipids were extracted and separated by TLC. Autoradiography of [^{14}C]perifosine in the plasma extract compared to the stock solution revealed a high *in vivo* stability. Up to 168 h after administration, only a very small fraction of radiolabeled by-product was present in the plasma, and this fraction seemed to be constant over time (Fig. 4). From the total amount of [^{14}C] that was present in plasma 168 h after administration, 96% had the same Rf value as authentic perifosine and is therefore assumed to be the original non-degraded compound.
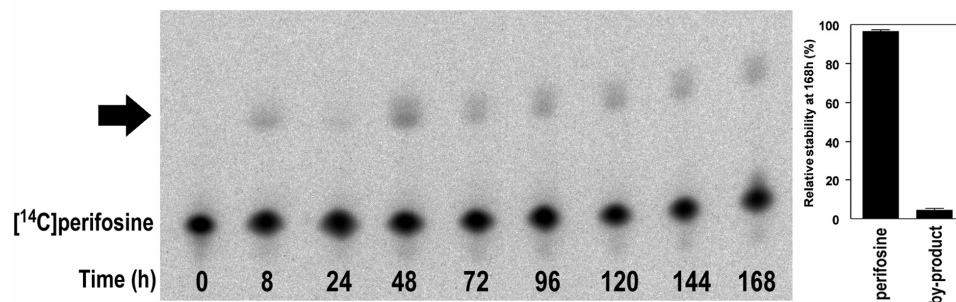
Figure 4. Visualization of [^{14}C]perifosine and by-product (arrow), extracted from plasma up to 168 h after administration and separated using thin layer chromatography. Perifosine/[^{14}C]perifosine before administration (t=0) is used as marker. Graph: Quantification of perifosine and by-product, 168 h after administration.

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Tumor pharmacokinetics after single and repeated oral administration. *In vivo* tumor uptake was studied using A431, HNXOE and KB xenografts. Tumor bearing mice were administered a single dose of 40 mg/kg perifosine, and drug levels were measured up to 7 days after administration. For the A431 and HNXOE tumors, maximum drug levels of approximately 70 and 60 μg/g were reached, respectively, at 48 h after administration (Fig. 5A).
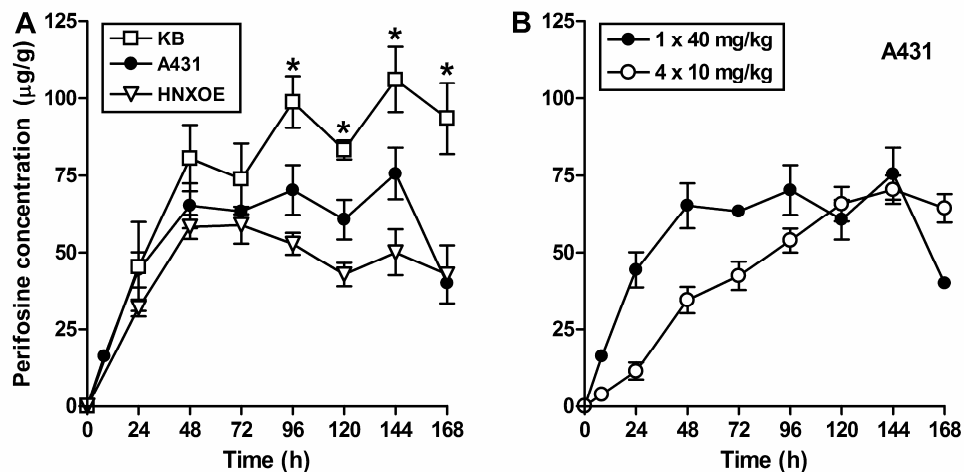
Figure 5. *(A)* Perifosine uptake by A431, HNXOE and KB tumors after a single dose of 40 mg/kg. *(B)* Perifosine uptake by A431 tumors after a single dose of 40 mg/kg and 4 daily dosages of 10 mg/kg. All values are means ± SEM (n=3-5; *P < 0.05, Student's *t*-test).

No significant decrease in perifosine concentration was observed in the A431 tumor until 144 h after administration, whereas the HNXOE tumor only showed a moderate decrease from 96 h onwards after administration. KB tumors displayed a more pronounced drug uptake, with increasing levels measured at least up to 144 h, which resulted in substantially higher perifosine levels (approximately 100 μg/g) as compared to A431 and HNXOE tumors.

To compare perifosine uptake after single and repeated administration, A431 tumor bearing animals received 4 daily administrations of 10 mg/kg perifosine. Similar A431 tumor drug levels were reached for both single and repeated administration. While peak perifosine levels were reached at 48 h after a single administration, maximum levels were measured after 120 h for the repeated administration. Drug levels declined from 144 h onwards for the repeated dose schedule (Fig. 5B).

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Biodistribution after single and repeated oral administration. The biodistribution of perifosine after single and repeated oral administration is shown in Fig. 6. The major organs were analyzed for the presence of [^{14}C]perifosine from 8-168 h after oral administration. Radiolabel was encountered in all analyzed organs, from relatively low levels in the heart and brain, to relatively high levels in the gastrointestinal tract. Single administration of a dose of 40 mg/kg resulted in maximum levels measured in stomach, small intestine, colon, liver and kidneys at 8 h after administration. Delayed distribution was observed in lungs, spleen, heart and brain. Four daily administrations of 10 mg/kg resulted in increasing perifosine levels in all organs up to 96 h after the first administration.
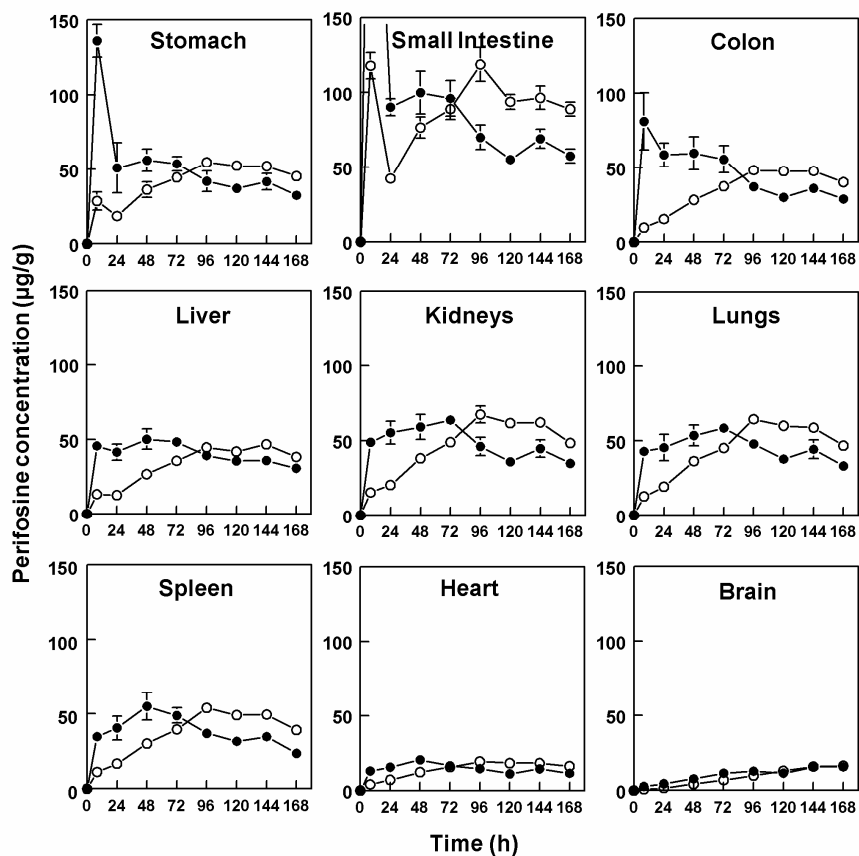
Figure 6. Tissue distribution of perifosine after administration of a single dose 40 mg/kg (closed circles) and 4 daily dosages of 10 mg/kg (open circles). Shown is the perifosine concentration (µg/g wet weight) in different organs until 168 h after administration. The first data point in small intestine at high perifosine dose amounted to 370 µg/g. Values are means ± SEM (n=4).

Toxicity. As an index of systemic toxicity, changes in body weight after oral perifosine administration were measured. The results are shown in Fig. 7. A single dose of 40 mg/kg resulted in a slight, reversible weight loss, and 4 daily dosages of 10 mg/kg each did not have a significant effect on body weight. No significant differences in mean body weight were observed between treated and control animals beyond 9 days after administration (not shown).
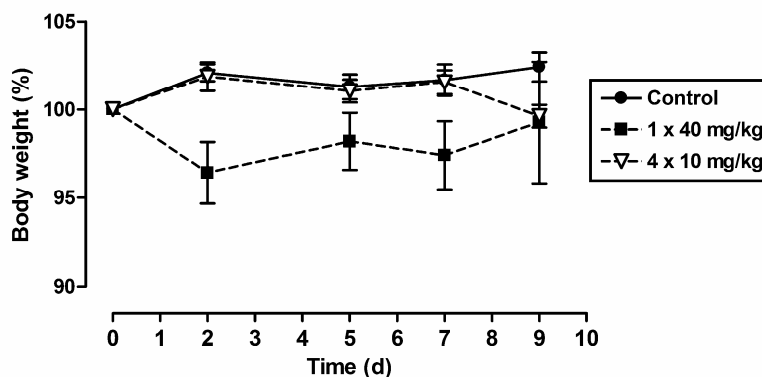
Figure 7. Perifosine toxicity after administration of PBS (control), a single dose of 40 mg/kg or 4 daily doses of 10 mg/kg. Perifosine toxicity is represented as changes in mean body weight. Values are means ± SEM (n=3-8).

Discussion

In this study we show the *in vitro* and *in vivo* uptake of perifosine in 3 human squamous cell carcinomas (vulvar, head and neck). *In vitro*, the KB cell line shows the highest level of drug accumulation and a high sensitivity compared with A431 and HNXOE cell lines. *In vivo*, perifosine accumulation reached levels of 60-70 µg/g in A431 and HNXOE xenografts and 100 µg/g in KB tumors. Further, orally administered perifosine displayed a slow elimination and extensive distribution in normal tissues.

APCs have been studied as anti-cancer agents for more than 2 decades. Although these agents showed promising properties *in vitro*, clinical use is limited, mainly due to gastrointestinal side effects. Based on animal studies, the APC analogue perifosine was expected to have a higher tolerability than its parent compound miltefosine. However, a phase I trial of perifosine revealed a similar toxicological profile as miltefosine, with a MTD of 200 mg/day [18]. More interest has arisen to combine APCs with conventional chemotherapy and, more recently, radiotherapy,

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since it was shown that APCs can enhance cell death induced by these regimens [20,22,25]. Currently, perifosine combined with radiotherapy is evaluated in a phase I study [28]. To support clinical data and to optimize drug scheduling, additional *in vitro* and *in vivo* data are needed.

Here we show a good correlation between *in vitro* perifosine uptake and cytotoxicity, and the *in vivo* uptake of three human squamous cell carcinomas. Several animal tumor models have been used to study the effect of APCs, the KB tumor being one of these models. Indeed, the APC-sensitive KB tumor shows a remarkable perifosine uptake compared with the other tested tumors. A431 and HNXOE cells incorporate comparable amounts of perifosine, with an uptake plateau at 4 h of incubation with 1 µM perifosine. For both cell lines, no cytotoxicity at this concentration is observed after 48 h of incubation.

The plasma levels reached after oral administration of a single sub-toxic dose of 40 mg/kg (C_{max} = 5.7 µg/mL, or 12.3 µM) are in agreement with plasma concentrations measured in phase I trials using a daily dose schedule [18] or a loading dose/maintenance dose schedule [19]. In addition, these concentrations have been shown to be effective *in vitro*. Not only is this concentration cytotoxic for the sensitive KB carcinoma, it also exceeds the IC_{50} of the A431 cell line. At these concentrations, APCs have not only been shown to induce apoptosis and to inhibit cell proliferation, but also to act as a radiosensitizer in these cell lines [25].

In vivo, similar perifosine uptake by A431, HNXOE and KB cells is observed. A repeated schedule was slightly better tolerated than a single dose, and both schedules resulted in similar tumor levels. This is in line with the linear dose-response relationship that has been described for miltefosine treatment of KB tumors [29]. A plateau is reached at roughly 48 h after a single dose for the 3 tumor types tested. Further, maximum perifosine levels in A431 tumors are measured 48 h after the last administration when a repeated dose schedule is applied. A 48 h interval between perifosine administration and local tumor irradiation might therefore improve the *in vivo* efficacy of this combined modality.

Perifosine treatment is limited, like other APCs, by gastrointestinal toxicity. In several *in vitro* studies, perifosine cytotoxicity is comparable to other APCs, like miltefosine and edelfosine. However, we show that perifosine is not a substrate for phospholipases, or any other catabolic enzymes. Whereas miltefosine has been shown to be metabolized, yielding choline, phosphocholine and 1,2-diacylphosphocholine [30], we show a high degree of stability of [^{14}C]perifosine after oral administration. Up to 168 h after administration, the great majority of the

plasma-extracted drug is still present as parent compound. This relates to a slightly longer $t_{1/2}$ for perifosine compared to miltefosine (137 and 96 h, respectively). This could be beneficial, since APC-induced cytotoxicity has been shown to be partially reversible, depending on drug concentration and duration of treatment [31].

Perifosine tissue distribution after oral administration appeared to involve the whole body, similar as previously shown for the APCs hexadecylphosphocholine (miltefosine), octadecylphosphocholine and erucylphosphocholine [2]. In general, oral administration of perifosine resulted in similar drug profiles in most organs. However, relatively high drug levels were found in the small intestine, in contrast to heart and brain in which relatively low levels were measured. Indeed, gastrointestinal toxicity has been reported to be dose limiting in phase I studies [18,19]. This toxicity may be associated with the presence of the drug in the upper part of the gastrointestinal tract after oral intake. In this context, accumulation in the small intestine after intravenous administration was not observed for the structurally related miltefosine [30]. The low perifosine levels of perifosine that we found in brain tissue would imply that erucylphosphocholine might be a better candidate for treatment of brain tumors, since this APC specifically accumulates in brain tissue [32]. In contrast to miltefosine [2,30,33], perifosine does not accumulate specifically in the kidney. Therefore, renal dysfunction, as described after miltefosine treatment [34], is not to be expected after perifosine treatment.

In summary, the APC-sensitive KB carcinoma accumulates significantly higher amounts of perifosine than the more resistant A431 and HNXOE cells, both *in vitro* and *in vivo*. Perifosine is absorbed from the gastrointestinal tract within 24 h, is not metabolized and distributes over the body. Maximum tumor levels after a sub-toxic total dose of 40 mg/kg are reached at 48 h after administration. These findings are a sound basis for further investigation of oral application of perifosine, possibly combined with other regimens, for instance radiotherapy.

Acknowledgements

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Chapter 3.2

Radiosensitization of squamous cell carcinoma by the alkyl-phospholipid perifosine in cell culture and xenografts

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Abstract

Purpose: Combined modality treatment has improved outcome in various solid tumors. Besides classical anti-cancer drugs, a new generation of biological response modifiers has emerged that increases the efficacy of radiation. Here, we have investigated whether perifosine, an orally applicable, membrane-targeted alkylphospholipid (APL), enhances the anti-tumor effect of radiation *in vitro* and *in vivo*. **Experimental design:** Several long- and short-term *in vitro* assays (clonogenic survival, sulforhodamine B cytotoxicity, apoptosis and cell cycle analysis) were used to assess the cytotoxic effect of perifosine in combination with radiation. *In vivo*, the response of human KB squamous cell carcinoma xenografts was measured after treatment with perifosine, irradiation and the combination. Radiolabeled perifosine was used to determine drug disposition in tumor and normal tissues. At various intervals after treatment, tumor specimens were collected to document histopathological changes. **Results:** *In vitro*, perifosine reduced clonogenic survival, enhanced apoptosis and increased cell cycle arrest after radiation. *In vivo*, radiation and perifosine alone induced a dose-dependent tumor growth delay. When combining multiple perifosine administrations with single or split doses of radiation, complete and sustained tumor regression was observed. Histopathological analysis of tumor specimens revealed a prominent apoptotic response after combined treatment with radiation and perifosine. Radiation-enhanced tumor response was observed at clinically relevant plasma perifosine concentrations and accumulating drug disposition of >100 µg/g in tumor tissue. **Conclusions:** Perifosine enhances radiation-induced cytotoxicity, as evidenced by reduced clonogenic survival and increased apoptosis induction *in vitro*, and by complete tumor regression *in vivo*. These data provide strong support for further development of this combination in clinical studies.

Introduction

Alkylphospholipids (APLs), such as alkylphosphocholines and alkyl-lysophospholipids, have been identified as synthetic anti-tumor agents that, in contrast to most classical chemotherapeutic drugs, primarily accumulate in the cell membrane [1]. There, they interfere with signal transduction pathways and subsequently affect multiple cellular processes, including apoptosis, proliferation and survival. Despite encouraging preclinical results, clinical use of APLs has been limited due to severe gastrointestinal [2,3] and hemolytic toxicity [4]. The alkylphosphocholine prototype miltefosine (hexadecylphosphocholine) is currently used as a topical formulation against cutaneous lymphomas and breast cancer metastases [5] and as an oral treatment against leishmaniasis [6]. Perifosine (D-21266; octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate), a heterocyclic analogue of miltefosine, has been evaluated as an oral anti-cancer drug. Initial studies showed an improved therapeutic index in preclinical models [7]. In several phase I and pharmacokinetic studies, gastrointestinal adverse effects were reported as dose limiting toxicity [8,9]. Recently, phase II studies in patients with metastatic or recurrent melanoma [10] and androgen-independent prostate cancer [11] revealed no objective responses after treatment with perifosine as single agent.

Combined modality treatment has led to improved treatment results in patients with advanced solid tumors, as has been demonstrated in several clinical studies during the last decade. In particular, the concurrent use of radiotherapy and chemotherapy resulted in reduced recurrence rates and improved survival and has become standard therapy in advanced head and neck, lung, cervical and anal cancer [12]. The combination of these classical anti-cancer regimens with novel biological response modifiers, has emerged as an attractive strategy to further increase tumor response and limit normal tissue toxicity [13,14]. Based on their potential to modulate signal transduction pathways involved in apoptosis, proliferation and survival, APLs are attractive candidates for such a combined modality approach. Indeed, perifosine demonstrates synergistic cytotoxicity *in vitro* when combined with other cytotoxic drugs, *e.g.* the cyclin-dependent kinase antagonist UCN-01 (7-hydrostaurosporine) [15] and histone deacetylase inhibitors (HDACIs) [16]. In addition, several APLs have been shown to enhance radiation-induced cell death in a variety of tumor types *in vitro*. Erucylphosphocholine enhanced radiation-induced apoptosis in glioblastoma cells [17], whereas

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edelfosine (Et-18-OCH$_3$), miltefosine and perifosine increased radiation-induced apoptosis in human leukemic cells [18]. Furthermore, miltefosine and perifosine showed radiosensitizing properties in human squamous cell carcinomas [13,19]. These findings have led us to the design of a phase I trial in patients with solid tumors where radiotherapy will be combined with daily intake of perifosine [20].

Perifosine acts on multiple cellular targets that contribute to the mechanism of enhanced radiation-induced cell death. The increased apoptotic response of U937 leukemic cells and Jurkat T-cells treated with APLs has been shown to depend on the activation of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) [18]. Moreover, these drugs were found to interfere with signaling pathways crucial for cell survival, like the protein kinase B [21,22], protein kinase C [23,24], and mitogen-activated protein kinase [25,26] signaling cascades. More recently, perifosine was identified as a potent cyclin-dependent kinase 2 (Cdk2)-inhibitor, causing a p53-independent, but p21-dependent cell cycle arrest [27]. In this context, it has been suggested that inhibition of Cdk activity may promote apoptosis, depending on the cellular context [28].

Thus far, reports on an enhanced radiation response after combined treatment with APLs have been limited to *in vitro* studies. We recently showed a high degree of metabolic stability of perifosine after oral administration and a relatively high drug uptake in a panel of squamous cell carcinomas *in vivo* [29]. Here, we have studied the effect of perifosine treatment in combination with ionizing radiation on different determinants of cytotoxicity *in vitro* and anti-tumor response *in vivo*, using the alkylphosphocholine-responsive KB tumor model.

Material and Methods

Antibodies. Antibody against active-caspase 3 used for flow cytometry was purchased from BD Biosciences (San Jose, California), FITC-labeled goat anti-rabbit IgG antibody was purchased from Molecular Probes, Inc. (Eugene, OR), mouse anti-bromodeoxyuridine was purchased from Dako Cytomation (Glostrup, Denmark), anti-mouse IgG-FITC was derived from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), cleaved-caspase 3 (Asp[175])-specific antibody and labeled polymer-horseradish anti-rabbit used for immunohistochemistry were purchased from Cell Signaling Technology (Beverly, MA) and DakoCytomation (Carpinteria, CA), respectively.

Reagents. Perifosine and [2,6-^{14}C]perifosine (66.8 µCi/mg) were kindly provided by Zentaris AG (Frankfurt, Germany). Crystal violet and glutardialdehyde were obtained from Merck KgaA (Darmstadt, Germany). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture. The human head and neck squamous cell carcinoma cell line KB, routinely tested for absence of mycoplasma, was cultured in Dulbecco's-Modified Eagles Medium (DMEM), supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum (FBS).

Clonogenic survival assay. Cells (200-3,200) in 10 mL medium were plated in 8 cm diameter dishes, incubated for 4 h for the cells to attach, and irradiated using a Pantak X-ray machine, operating at 250 kV_p and 12 mA with a 0.6 mm Cu filter with a dose rate ranging from 0.9 to 1.7 Gy/min. Perifosine was added at a final concentration of 0.4 µmol/L, immediately before irradiation. After 3 days, medium was removed and replaced with either control medium or with medium containing 0.4 µmol/L perifosine. Cells were allowed to form colonies over a period of 14 days after irradiation, which were subsequently fixed and stained by 0.2% crystal violet/2.5% glutardialdehyde. The number of colonies were counted with a Colcount (Oxford Optronix, Oxford, United Kingdom) and visually confirmed under a light microscope to contain at least 50 cells. Cell survival was corrected for plating efficiency.

Sulforhodamine B cytotoxicity assay. 500 KB cells in 200 µL/well were plated in 96-well plates. After perifosine was added in serial dilutions, the plates were irradiated (0-8 Gy). After 5 days of incubation, cells were washed and stained with sulforhodamine B [30]. Extinction was measured at 540 nm with a microplate reader (Bio-Tek Instruments, Winooski, VT). The data were fitted to a sigmoidal concentration-response curve and IC_{50} calculation was done using GraphPad Prism version 4.00 for Windows, (GraphPad Software, San Diego, CA). For each radiation dose, control wells (medium) were set at 100% survival.

Apoptosis measurement. KB cells (1.25×10^4/well) were plated in 6-well plates in 2 mL medium and incubated overnight to allow the cells to attach. Perifosine was added and the cells were irradiated using a ^{137}Cs radiation source at an absorbed dose rate of ~1 Gy/min. After 120 h, cells and supernatant were collected, washed

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and resuspended in Nicoletti buffer (50 µg propidium iodine/mL, 0.1% sodium citrate, 0.1% Triton X-100) [31]. The apoptotic fraction was assessed as the percentage of cells present in the sub-G_1 population. To confirm the findings by nuclear staining, cells were alternatively stained for active-caspase 3. In brief, cells were fixed in 4% formaldehyde/PBS and permeabilized in 0.1% saponin/0.5% BSA/PBS. Thereafter, cells were incubated with a rabbit anti-active-caspase 3 antibody (1:50) and stained with goat anti-rabbit FITC (1:100). All measurements were done using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Cell cycle analysis. KB cells (2.5×10^5 per well) were plated in 6-well plates and incubated overnight. Treatment consisted of either addition of perifosine at a final concentration of 2 µmol/L, irradiation to 5 Gy or the combination. After 8, 24 and 48 h of incubation, cells were labeled with IUdR as described previously [32]. In brief, nuclei were isolated and incubated with a mouse anti-BrdU antibody, which also binds to IUdR (1:50), followed by 30 min of incubation with a FITC-conjugated anti-mouse antibody (1:50). Finally, the nuclei were incubated with propidium iodine to stain total DNA. Flow cytometry was carried out using a FACScan flow cytometer.

***In vivo* tumor growth delay assay.** Female BALB/c nude mice, 6-10 weeks old (18-28 g) were obtained from the animal department of the Netherlands Cancer Institute. Animals were kept and handled according to institutional guidelines complying with Dutch legislation under a 12/12 h light/dark cycle at a temperature of 22°C, receiving a standard diet and acidified water *ad libitum*. Mice were injected s.c. at the lower back with 3×10^6 KB cells in 50 µL PBS, and tumor volume was measured regularly, using calipers. Tumor size was calculated using the formula: volume = π/6 x length x width x height, where tumor volume at the start of treatment was normalized to 100%. When the tumor reached a mean diameter of ~6 mm (measured in 3 orthogonal directions), treatment was started. Four treatment groups (n=5-9 animals/group) were distinguished: Control (no perifosine, no radiation), perifosine (oral administration), radiotherapy (local tumor irradiation), and combined therapy (oral administration of perifosine and local tumor irradiation). *Drug administration*: Mice received, by way of gastric intubation, 1-3 oral doses of 40 mg/kg perifosine every 48 h. Control animals received PBS, orally. *Irradiation:* Animals treated according to a combined treatment schedule were irradiated 48 h after the first perifosine administration. This time interval corresponds to approximately the t_{max} in tumor tissue after a single administration of 40 mg/kg. For

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irradiation, mice were immobilized in custom designed jigs, which allowed specific irradiation of the dorsal tumor while shielding the rest of the animal. Irradiations were carried out using a Pantak X-ray machine, with a dose rate of ~4 Gy/min. To ensure homogeneous dose distribution, mice were rotated through 180° half way during the irradiation procedure.

Histopathological analysis. At 96, 120, and 144 h after start of treatment, animals were sacrificed, and tumors were excised, fixed in ethanol/acetic acid/formol saline fixative (40:5:10:45 v/v/v/v), embedded in paraffin and sectioned at 3 to 4 µm onto slides. Sections were stained using an antibody against cleaved-caspase 3 (1:100) and a labeled polymer-horseradish peroxidase anti-rabbit, according to standard protocols. The percentage of cells expressing active-caspase 3 was determined by counting immuno-reactive cells in 3 different optical fields. Because the KB tumors grow rapidly and show central areas of necrosis when untreated, these analyses were done on the peripheral rim of vital tumor tissue.

Tumor and normal tissue pharmacokinetics. Mice bearing s.c. KB tumors (with an initial mean diameter of at least 6 mm) received 1-3 doses of 40 mg/kg perifosine, traced with [^{14}C]perifosine (0.05 µCi/g) dissolved in PBS in a volume of 5 µL/g body weight. At various time points after administration, mice were anaesthetized, and blood was collected by way of a heart puncture and sacrificed by cervical dislocation. Blood was centrifuged at 14,000 rpm for 5 min (4°C), and plasma was collected. Tumors and major organs were excised and dissolved in 1-6 mL Solvable (Packard Instrument Co., Groningen, The Netherlands) at 60°C overnight, bleached with 30% hydrogen peroxide, and diluted in Ultima Gold scintillation liquid (PerkinElmer, Wellesley, MA). All [^{14}C]perifosine measurements were done using a TRI-CARB liquid scintillation analyzer. The area under the curve (AUC) up to the last measured concentration-time point was determined by applying the linear-logarithmic trapezoidal method.

Results

In vitro results
Perifosine-induced radiosensitization is dependent on prolonged drug exposure. The impact of drug exposure time on the clonogenic capacity of KB cells after radiation was determined by applying a 3-day and a 14-day exposure to

0.4 µmol/L perifosine. Continuous exposure to this drug concentration reduced the plating efficiency by 36 ± 11% compared with the untreated cells. Incubation of the cells with perifosine for 14 days significantly reduced clonogenic survival after irradiation. At doses $\geq$ 6 Gy, this reduction was statistically significant. This prolonged exposure time seemed to be essential, because removal of perifosine 3 days after irradiation led to loss of this radiosensitizing effect (Fig. 1A).

Radiation increases sensitivity of KB cells to perifosine. To test whether irradiation enhanced the sensitivity of KB cells to perifosine, dose-response curves and corresponding IC_{50}'s of KB cells treated with increasing doses of irradiation were obtained using the SRB cytotoxicity assay. Whereas non-irradiated cells showed an IC_{50} of 0.38 ± 0.04 µmol/L after 5 days of incubation, radiation induced a dose-dependent decrease of the IC_{50} down to 0.23 ± 0.02 µmol/L for KB cells treated with 8 Gy irradiation. This corresponds to a maximum decrease of 39 ± 1% (Fig. 1B).

Perifosine enhances radiation-induced apoptosis. The effect of perifosine, radiation and the combination on apoptosis induction was assessed using flow cytometry. Both nuclear fragmentation with propidium iodine staining and caspase 3 staining using an active-caspase 3-specific antibody were measured. Both perifosine and radiation induced a significant dose-dependent apoptotic response. When radiation and perifosine were combined, the number of apoptotic cells was strongly increased and resulted in a more than additive effect in the dose range between 0.3 and 0.6 µmol/L perifosine (Fig. 1C). Similar results were obtained when cells were treated with perifosine, radiation, or the combination, and stained with an active caspase 3-specific antibody (Fig. 1D). It should be noted that the steep dose-response relationship of KB cells after treatment with perifosine or radiation hampers the calculation of a supra-additive interaction between both stimuli over the full dose ranges according to the concept of Steel and Peckham [33].
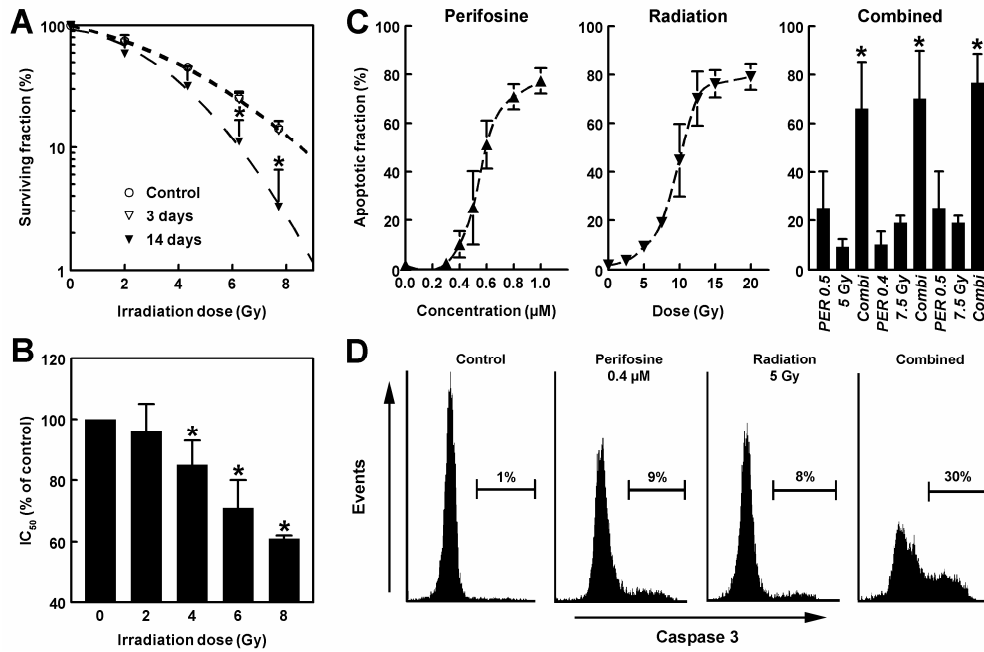
Figure 1. *In vitro* cytotoxicity induced by perifosine combined with radiation. *(A)* Radiosensitization is dependent on a prolonged exposure time after radiation. KB cells were irradiated in the absence (control), or in the presence of 0.4 µmol/L perifosine and exposed to the drug for either 3 days or 14 days. At day 14, cultures were fixed and stained for assessment of colony formation. Note: Markers representing survival of untreated cells (control) and cells exposed to perifosine for 3 days almost completely overlap. *(B)* Radiation increases the sensitivity of KB cells to perifosine. Dose-response curves were generated after a 5-day incubation period with a serial dilution of perifosine, combined with 0-8 Gy radiation. Expressed is the relative perifosine sensitivity after irradiation (IC_{50} irradiated cells/IC_{50} non-irradiated cells). *(C)* Perifosine enhances radiation-induced apoptosis. KB cells were treated with perifosine, radiation or a combination at doses indicated. After 5 days, cells were stained for DNA content by propidium iodine and nuclear fragmentation was quantified using flow cytometry. *(D)* Apoptosis analyzed by the detection of active-caspase 3-positive cells, 5 days after treatment. All values are means ± SD (*$P < 0.05$, one-tailed Student's *t*-test).

Perifosine prolongs radiation-induced cell cycle arrest, mainly in G_2. Cell cycle perturbations induced by treatment with either perifosine, radiation or a combination were analyzed using IUdR labeling and flow cytometry. Representative dot plots at 24 h after treatments are shown in Fig. 2A. At this time point, the most pronounced cell cycle arrest was observed after combined treatment. Both perifosine and radiation caused a block in G_2-M and a decrease in S phase. The S-phase population was reduced by ~80%, whereas the G_2-M population increased with >300% compared with control cells. At 48 h after treatment, the cell cycle distribution after irradiation was restored, whereas

perifosine and combined treated cells still displayed an impaired cell cycle progression (Fig. 2B).
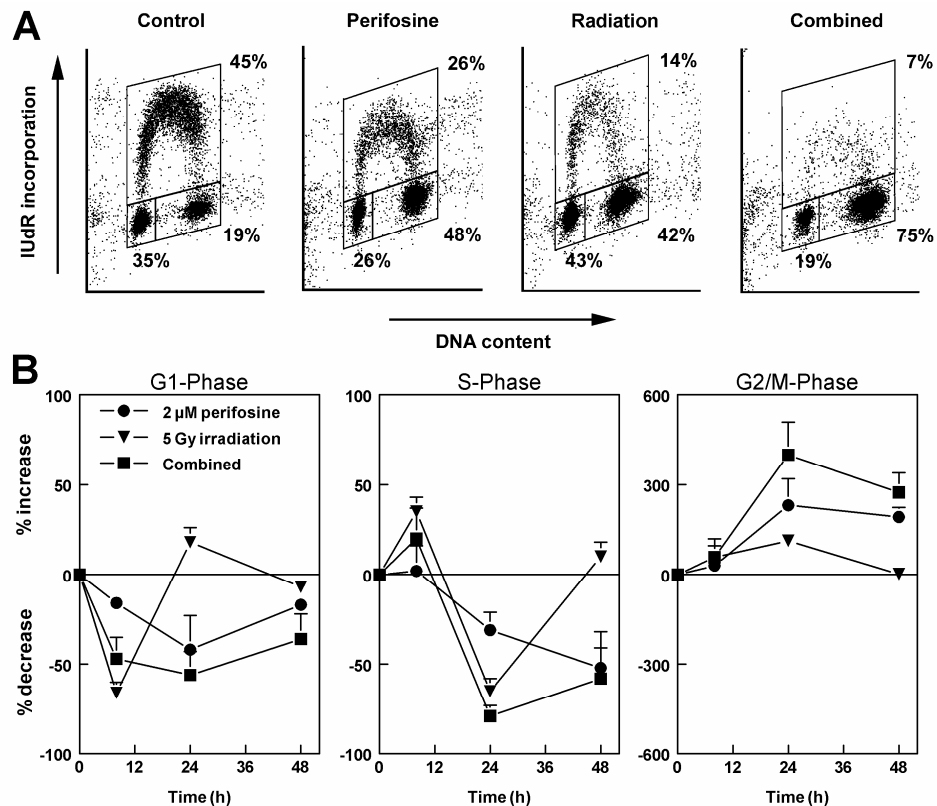
Figure 2. Analysis of cell cycle progression after treatment with either 2 µmol/L perifosine, 5 Gy radiation or the combination. *(A)* **Representative cell populations collected 24 h after treatment. Cells were labeled with IUdR for determination of S-phase fraction (upper region, positive for IUdR), and stained with propidium iodine for distinguishing G1 (lower left region) from G2/M cells (lower right region).** *(B)* **Time-course representation of cell cycle progression of KB cells after the different treatment schedules. Cell cycle changes are represented relative to untreated cells. Data are means ± SD.**

In vivo results

Perifosine enhances the anti-tumor effect of radiation. To investigate whether perifosine improves the tumor response after radiation *in vivo*, BALB/c nude mice bearing KB tumor xenografts were treated with either perifosine, radiation, or with both modalities, and tumor size was measured regularly. Table 1 shows the normalized tumor growth delay. Three treatment schedules were used to determine the *in vivo* enhancement of radiation by orally administered perifosine.

Table 1. *In vivo* antitumor effect of perifosine, radiation, and the combination

Treatment	No radiation	2 x 5 Gy	1 x 10 Gy	1 x 13 Gy
No perifosine	0	18.7 ± 6.8 (*n* = 9)	21.3 ± 4.7 (*n* = 7)	33.9 ± 10.2 (*n* = 8)
1 x 40 mg/kg	1.6 ± 3.9 (*n* = 8)	ND	20.2 ± 7.3 (*n* = 5)	ND
2 x 40 mg/kg	8.3 ± 9.5 (*n* = 9)	ND	∞	ND
3 x 40 mg/kg	12.3 ± 6.6 (*n* = 9)	∞	ND	ND

NOTE: Normalized tumor growth delay is expressed as the number of days necessary to reach 250% of the initial tumor volume minus the number of days necessary to reach 250% of the initial tumor volume for untreated animals (± SD). ∞, complete and sustained tumor regression. Abbreviation: ND, not determined.

In the first schedule, 10 Gy irradiation induced a growth delay of ~21 days; a single dose of 40 mg/kg was ineffective. This drug dose did not lead to enhancement of the radiation effect (Fig. 3A), although 1 complete remission occurred. The second schedule involved 2 administrations of 40 mg/kg with a 48 h interval; 10 Gy irradiation was applied immediately after the second administration. Two doses of 40 mg/kg perifosine led to a substantial growth delay of 8 days. Again, a radiation dose of 10 Gy induced a substantial growth delay (21 days). Two administrations of perifosine combined with 10 Gy irradiation led to complete remission of the KB tumor in 6 of 7 animals (Fig. 3B). The combined therapy of 10 Gy irradiation and perifosine was more effective than a single irradiation dose of 13 Gy, which ultimately resulted in regrowth of the tumors in 6 of 8 animals (Fig. 3B). This corresponds with an enhancement factor of at least 1.3. The third schedule involved a split dose radiation consisting of 2 fractions of 5 Gy on day 2 and 4. Perifosine was administered in 3 doses of 40 mg/kg on day 0, 2 and 4. As expected, both perifosine treatment and radiotherapy as single modalities led to a substantial growth delay (12 and 19 days, respectively). Again, combined treatment led to complete tumor regression, which sustained for at least 90 days (Fig. 3C).

Toxicity after oral perifosine treatment and local tumor irradiation. Body weight of animals treated according to the treatment schedules described in the previous paragraph was monitored and used as an index for systemic toxicity. In all 3 experiments, no significant weight loss due to local tumor irradiation was observed. A single dose of 40 mg/kg perifosine resulted in a slight but reversible weight loss, which sustained for 10 days. Increased weight loss was observed after combined treatment; however, this was reversible and initial body weight was regained within 3 weeks (Fig. 3D). Although 2 oral doses of 40 mg/kg resulted in a reduction in body weight of 6% at day 4 after start of treatment, the initial body weight was regained after 2 weeks. When this dose schedule was combined with

10 Gy irradiation at day 2, weight loss up to 8% on day 7 was observed. Again, this toxicity was reversible and lasted 18 days (Fig. 3E). Administration of 40 mg/kg perifosine at day 0, 2 and 4 did not result in increased toxicity, compared with 40 mg/kg perifosine administered only at day 0 and 2. Maximum weight loss was encountered when 3 doses of perifosine were applied with a split dose of 2 x 5 Gy irradiation. However, this did not exceed 10% of initial body weight and lasted for more than 3 weeks (Fig. 3F).
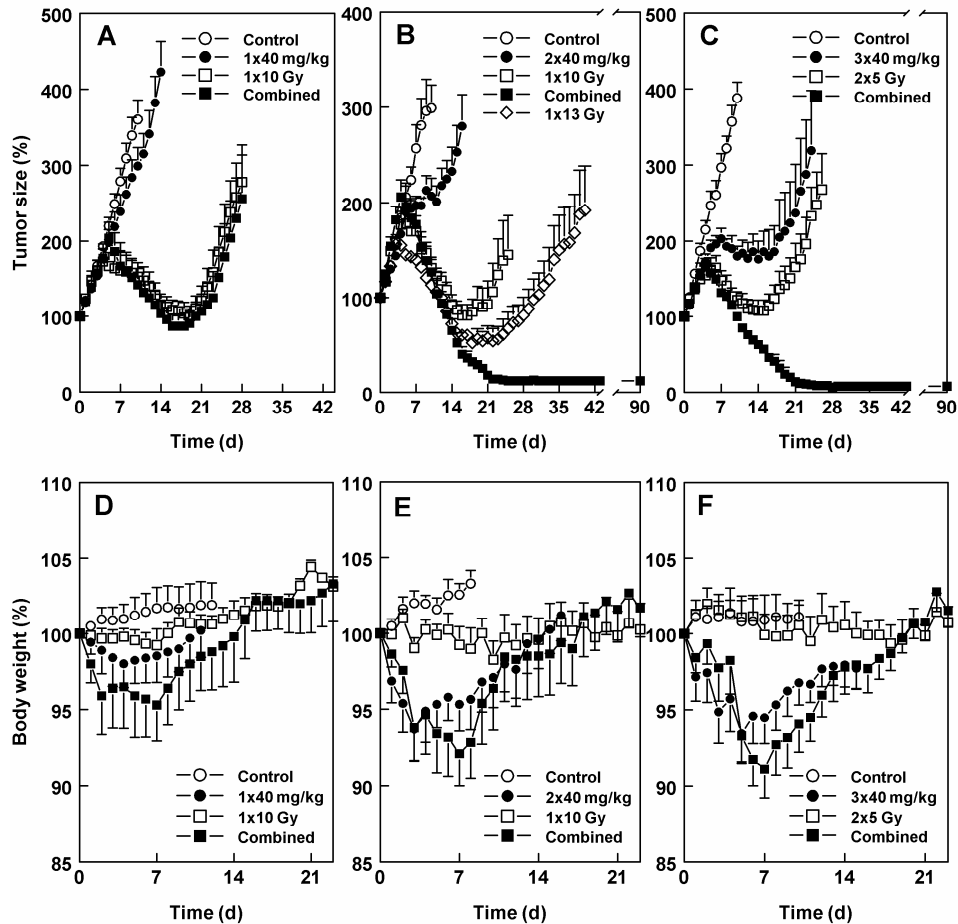
Figure 3. *In vivo* **efficacy and toxicity of treatment with perifosine, radiation and combined schedules. BALB/c nude mice bearing KB xenografts with a mean diameter of approximately 6 mm were treated with either perifosine p.o., radiation, or a combination.** *(A), (B) and (C)* **Animals treated with single or multiple doses of perifosine, and a single or split dose of γ-radiation, as indicated in the figure labels. Tumor size was measured at least 3 times a week (quantification of treatment efficacy, number of animals/group are summarized in Table 1).** *(D), (E) and (F)* **Treatment-induced toxicity, expressed as changes in body weight, after treatment as indicated in the figure labels.**

Histopathological analysis. KB tumors were excised at different time intervals (4-6 days) after treatment, and stained using a cleaved-caspase 3-specific antibody. Because untreated KB tumors grow rapidly and display central areas of necrosis, these analyses were done on the peripheral rim of vital tumor tissue. Compared with untreated tumors, an increase in the number of apoptotic cells was found after radiation or perifosine (Fig. 4A). The most prominent apoptotic response was observed after combined treatment with radiation and perifosine. Furthermore, enlarged nuclei were clearly visible in the radiation-treated and, to a lesser extent, combined-treated tumors. In untreated tumors the percentage of apoptosis varied between $3.2 \pm 1.3\%$ to $5.2 \pm 2.2\%$ (Fig. 4B). Radiation (1x10 Gy) induced a significant increase in tumor apoptosis ranging from $11.0 \pm 3.8\%$ on day 4 to $16.2 \pm 1.9\%$ on day 6. Tumors treated with perifosine only (2x40 mg/kg) also showed an increase in the amount of apoptosis, which was maximal at 5 days post-treatment ($23.0 \pm 9.0\%$; $P < 0.05$). The largest increase in the apoptotic response resulted from the combined radiation plus perifosine treatment. The percentage of apoptosis increased progressively from $13.8 \pm 1.0\%$ at 4 days to $30.5 \pm 7.4\%$ at 5 days and $38.2 \pm 13.1\%$ at 6 days. These numbers were also significantly higher than the amount of apoptosis induced by both treatments separately ($P < 0.03$ at 6 days).
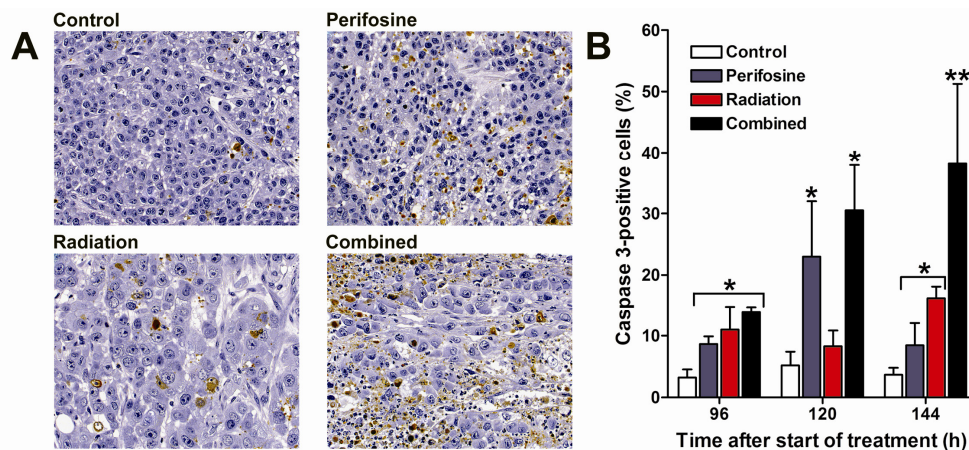
Figure 4. Histopathological analysis of KB xenografts. Tumor bearing animals received 40 mg/kg perifosine at 0 and 48 h; animals were irradiated at 48 h. At 96, 120 and 144 h after start of treatment, tumors were excised and stained for cleaved-caspase 3. *(A)* Representative sections of tumors from mice, 120 h after either no treatment (control), treated with perifosine, radiation, or the combination. Magnification 40x. *(B)* Quantification of the fraction of apoptotic cells present in tumor sections, harvested at various time points after start of treatment with perifosine, radiation or a combination. *P < 0.05, for separate treatments compared with controls; **P < 0.03, for combined treatment compared with separate treatments (one-tailed Student's *t*-test).

Tumor and normal tissue pharmacokinetics. To quantify tumor and normal tissue distribution after the various schedules, 1-3 doses of perifosine, together with tracer amounts of [^{14}C]perifosine, were administered to tumor bearing animals. A single dose of 40 mg/kg perifosine resulted in plasma levels of 5 µg/mL at 48 h after administration, which declined thereafter. A second 40 mg/kg dose at 48 h resulted in further increased plasma levels of 9 µg/mL at 96 h and 7 µg/mL at 144 h after the second administration. Finally, plasma levels exceeded 10 µg/mL after a third administration at 96 h (Fig. 5A).
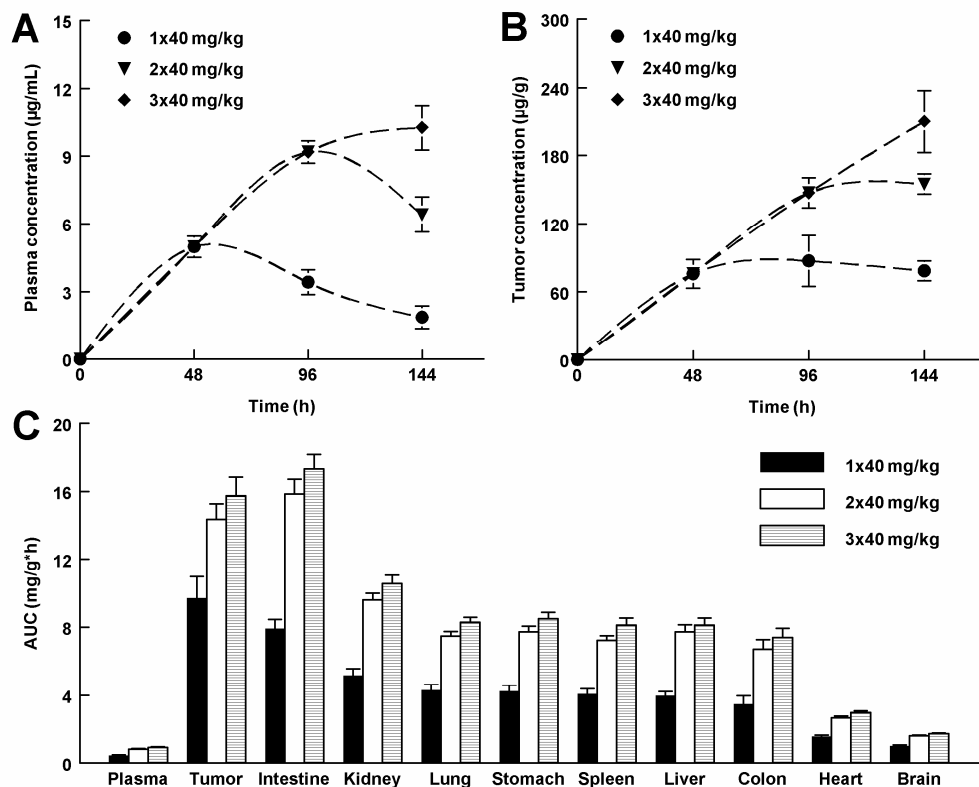
Figure 5. Tumor and normal tissue pharmacokinetics of 3 escalating doses of perifosine. Mice bearing KB tumor xenografts were administered 40 mg/kg perifosine/[^{14}C]perifosine p.o. on day 0 (1x40 mg/kg), day 0 and 2 (2x40 mg/kg) or day 0, 2 and 4 (3x40 mg/kg). On days 2, 4 and 6, plasma, organs and tumors were collected and drug exposure was determined. *(A)* Time-dependent plasma concentrations after oral administration of perifosine. *(B)* Time-dependent intra-tumoral drug concentrations. *(C)* Perifosine disposition in tumor and normal tissue. Represented is the area under the curve (AUC) up to 144 h, after 1-3 doses. All values are means ± SEM, 4-5 animals/group.

Steady state tumor concentrations of around 75 µg/g were measured from 48 until 144 h after a single administration. A second administration increased maximum tumor levels to ~150 µg/g at 96 to 144 h. A third dose of 40 mg/kg at 96 h resulted in tumor levels reaching ~200 µg/g at 144 h after start of treatment (Fig. 5B).

The area under the concentration-time curve was calculated from the concentration-time curves of the major organs (Fig. 5C). Perifosine was found in all organs, but the highest concentration was measured in tumor tissue and the small intestine. Higher drug accumulation was found after a second oral administration, whereas a third administration resulted in only a limited further increase in area under the concentration-time curve. To exclude an effect of radiation on drug uptake in the KB carcinoma, 1 group of animals was irradiated with 10 Gy, immediately followed by a first dose of 40 mg/kg perifosine and a second dose at 48 h after irradiation; 144 h after irradiation, similar tumor concentrations (170 µg/g) were measured, compared with tumors of animals that received perifosine without radiation (data not shown).

Discussion

In this study we show *in vitro* and *in vivo* enhancement of radiation-induced cell death by the APL perifosine in the KB squamous cell carcinoma. *In vitro*, perifosine reduced clonogenic survival, enhanced apoptosis, and blocked cell cycle progression after irradiation. *In vivo,* radiation or perifosine as single modality induced a dose-dependent tumor growth delay. However, multiple doses of perifosine combined with single or split dose irradiation resulted in complete and sustained remission of KB tumor xenografts.

To our knowledge, this is the first study in which the oral alkylphosphocholine analogue perifosine is shown to increase radioresponsiveness *in vivo*. Previous activity studies in animals with perifosine as single agent have already showed tumoristatic effects after long-term or high-dose administration of the drug [7]. Based on mechanistic insights collected over recent years by our group and others [13,18,21,26], we considered the likelihood that perifosine would increase the cytotoxic effect of radiation *in vivo.*

In the present studies, we tested the combination of perifosine and radiation in the human KB tumor grown *in vitro* and as xenograft in nude mice. To exclude any contribution of a perifosine-related metabolite to the cytotoxic effect, we tested the stability of the compound, both *in vitro* (not shown) and *in vivo* [29]. No significant

degradation (<4%) of perifosine was measured.

From the *in vitro* experiments we can conclude that perifosine affects cellular sensitivity to radiation, and that this interaction results in increased cytotoxicity as measured by both short-term and long-term assays. A more than additive apoptotic response was observed when radiation was combined with low concentrations of perifosine. The slow kinetics of apoptosis induction in these cells, associated with late (> 24 h) caspase 3 activation, are consistent with a post-mitotic or delayed type of apoptosis [34].

In a clonogenic survival assay, prolonged exposure to perifosine induced marked radiosensitization. This is in line with reports describing a treatment duration-dependent cytotoxicity by APLs [35,36]. Furthermore, radiosensitization by perifosine is suggested to be dependent on intense and prolonged PKB/Akt inhibition [37]. Along similar lines, prolonged inhibition of RAS-mediated survival pathways has been identified as a strategy to radiosensitize tumor cells [38-41].

Cell cycle-disrupting agents are considered attractive candidates to combine with radiotherapy [42]. One possible mechanism by which perifosine exerts its radiosensitizing effect might be by redistribution of cells in a radiosensitive phase of the cell cycle [43]. However, although pretreatment of KB cells with perifosine led to a relative accumulation in de sensitive G_2-M phase, we did not find a significant reduction in clonogenic survival after irradiation under these conditions (data not shown). Furthermore, these prominent cell cycle effects were observed at concentrations far exceeding those at which radiosensitization was found. Therefore, a major role of cell cycle redistribution in perifosine-induced radiosensitization is unlikely.

The steep dose-response relationship of perifosine in this tumor model *in vitro* was also evident *in vivo*, and seemed crucial for the radiosensitizing effect. A single dose of 40 mg/kg was ineffective by itself and did not enhance radiation-induced tumor growth delay. Multiple (2-3) administrations, however, resulted in significant tumor growth delay and, when combined with radiation, to complete tumor eradication.

The mechanism by which perifosine exerts its anti-tumor effect *in vivo*, either as single agent or in combination with radiation, remains uncertain. Based on our *in vitro* data, both apoptotic and non-apoptotic cell death contribute to the observed response. Furthermore, our histopathological analyses show a significant increase in apoptosis after treatment with perifosine or radiation. The largest increase in the amount of apoptosis was observed after combined therapy. Taken together, these

data support a significant role of apoptotic cell death in the anti-tumor effect of the combination treatment.

Tumor response was correlated with the degree of perifosine accumulation in tumor tissue. In fact, the amount of drug uptake by tumor cells after perifosine treatment as single or combined modality could well be the determining factor for treatment outcome. We measured plasma and tumor concentration at 48 h after the last oral administration, because perifosine uptake by the KB tumor has been shown to reach a plateau after this time interval [29]. Because this plateau was maintained for at least 168 h after administration, a prolonged tumor exposure is likely. Following a single oral dose of 40 mg/kg perifosine, mean maximum tumor levels of 87 µg/g were measured. This schedule was ineffective in enhancing the radiation response. Multiple perifosine administrations causing tumor growth delay and, in combination with radiation induced tumor regression, resulted in tumor levels ranging from 125 up to almost 300 µg/g. All these concentrations exceeded the levels of other alkylphosphocholines, such as miltefosine, octadecylphosphocholine and erucylphosphocholine measured in NMU-induced tumors in rats after oral administration of tumor growth inhibiting doses [44]. Importantly, the maximal perifosine plasma concentration measured in radiation-enhancing treatment schedules corresponded with clinically achievable plasma levels. In patients with advanced cancer, both steady state levels during treatment with a loading dose/maintenance schedule, and peak plasma levels measured in patients receiving 200 mg/day were in this range.

In conclusion, our data demonstrate that perifosine increases radiosensitivity *in vitro* and enhances tumor response to radiation. Multiple doses of perifosine were more effective than a single dose, and when given in combination with radiation, led to complete and sustained tumor regression. These studies also show that the tumor response after combined treatment is mediated, at least partly, by induction of apoptosis. Based on these findings, perifosine is an attractive candidate for evaluation as a radiosensitizer in clinical studies.

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Clinical study

Chapter 4.1

Phase I and pharmacokinetic study of combined treatment with perifosine and radiation in patients with advanced solid tumours

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Abstract

Purpose: Perifosine is an orally applicable, membrane-targeted alkyl-phosphocholine analogue with anti-tumour activity and radiosensitising properties in preclinical models. The purpose of this phase I study was to determine the feasibility and tolerability of concurrent daily perifosine and radiation in patients with advanced cancer. **Patients and methods:** Starting dose of perifosine was 50 mg/day; dose escalation was in steps of 50 mg. Daily administration commenced 2 days before radiotherapy and was continued throughout the radiation treatment. At least 3 patients were entered at each dose level; at the 150 mg/day level 10 patients were included. Pharmacokinetic sampling was performed weekly pre-dosing. Twenty-one patients were entered. Tumour types included NSCLC (n=17), prostate, oesophageal, colon and bladder cancer. Most patients (16/21) had received prior chemotherapy; none radiotherapy. Median number of daily perifosine administrations was 31 (range 24-53). Mean radiation dose (BED_{10}) was 59.8 Gy (range 50.7 to 87.5 Gy in 13-28 fractions). **Results:** Major drug-related toxicities according to CTC criteria were nausea in 57%, fatigue in 48%, vomiting in 38%, diarrhoea in 38% and anorexia in 19%. No bone marrow toxicity was observed. DLT (nausea/vomiting) was encountered in 2 of 5 patients at the 200 mg/day dose level. Dose-dependent steady-state plasma levels were reached after 1 week. Major radiotherapy-related acute toxicity consisted of dysphagia in 38% and pneumonitis in 29%. **Conclusion:** Perifosine can be safely combined with fractionated radiotherapy. A dosage of 150 mg/day, to be started at least 1 week prior to radiotherapy, is recommended for phase II evaluation.

Introduction

Alkylphospholipids (APLs) comprise a heterogeneous group of synthetic compounds with anti-tumour activity *in vitro* and *in vivo* [1]. Two classes of APLs can be distinguished: (1) alkylphosphocholines (APC), such as hexadecylphosphocholine (miltefosine), erucylphosphocholine and the APC analogue octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate (perifosine), and (2) alkyl-lysophospholipids (ALP), such as 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (edelfosine) and 1-hexadecylthio-2-methoxy-methyl-*rac*-glycero-3-phosphocholine (ilmofosine). APLs accumulate preferentially in sphingolipid- and cholesterol-enriched microdomains in the plasma membrane, so-called "lipid rafts" [2]. Here, they interfere with normal phospholipids turnover and affect multiple signal transduction pathways involved in cell survival, proliferation and apoptosis. APLs exert a strong cytotoxic effect against a variety of human tumour cell lines *in vitro*. The underlying mechanism of this anti-tumour activity is complex and includes interference with phosphatidylcholine biosynthesis, inhibition of phospholipase C and PKC activity, stimulation of the SAPK/JNK pathway and inhibition of MAPK/ERK and Akt/PKB signalling [3-5].

Perifosine is a recently developed APC with a favourable toxicity profile upon oral administration in preclinical models compared to other APLs [6]. In several clinical phase I studies, one of these performed in our institute, gastrointestinal adverse effects including nausea, vomiting and diarrhoea were reported as dose limiting toxicity [7,8]. As single agent, perifosine has shown only limited anti-tumour activity in phase II studies [9]. The combination of classical anti-cancer regimens with novel biological response modifiers, has emerged as an attractive strategy to further increase tumour response and limit normal tissue toxicity [10,11]. Given their potential to modulate signal transduction pathways mediating apoptosis, proliferation and survival, APLs are rational candidates for such a combined modality approach [11,12]. Indeed, perifosine demonstrates (supra-) additive cytotoxicity *in vitro* when combined with other drugs [13-16]. In addition, several APLs have been shown to enhance radiation-induced cell death in a variety of tumour types *in vitro* [4,11,12,17,18]. Recently, we demonstrated complete and sustained tumour regression of the xenografted KB squamous cell carcinoma after combined treatment of radiation and multiple doses of perifosine [19]. Based on these experimental and clinical findings we designed the current phase I trial. The objectives were (1) to test the feasibility and tolerability of the combined treatment

consisting of fractionated radiotherapy and concurrent daily oral perifosine in patients with advanced solid tumours, (2) to determine the dose limiting toxicity and maximum tolerated dose, (3) to identify the recommended dose schedule for further clinical testing, (4) to explore pharmacokinetics of perifosine in relation to toxicity, and (5) to document any anti-tumour activity of the combination.

Patients and methods

Eligibility criteria. This study, conducted at The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, was reviewed and approved by the Medical Ethics Committee of the institute. All patients signed a written informed consent. Patients were eligible for this trial if they had a histologically or cytologically proven inoperable solid tumour for which standard local/systemic treatment was not or no longer available. Patients had to be 18 years of age or older, have an ECOG-WHO performance status of 0-2 and a life expectancy of at least 12 weeks. Other eligibility criteria included adequate bone marrow, liver and kidney function. Exclusion criteria consisted of prior irradiation of target lesion within 1 year prior to entry; concomitant or recent (within 4 weeks) treatment with other anti-cancer agents; prior treatment with perifosine; history of haemolytic events; any condition classified as grade > 1 (NCI Common Toxicity Criteria, version 2.0) except if caused by the underlying malignant disease; symptomatic brain metastases or leptomeningeal disease; breast feeding, pregnancy or inadequate contraception.

Treatment plan and study design. *Perifosine:* Perifosine was supplied by Zentaris GmbH (Frankfurt am Main, Germany) as a 50 mg film-coated tablet, soluble in gastric juice. Patients commenced daily intake 2 days before the start of radiotherapy and continued this intake throughout the entire radiation treatment 4 hours prior to each fraction. Starting dose was 50 mg/day and dose escalation was in steps of 50 mg/day up to 200 mg/day. This dose scheme was based on the maximum-tolerated dose (MTD), previously established in a phase I study of perifosine alone [7]. For each dose level 3 patients were entered. At the highest dose level or at the dose level to be recommended for future studies, if this was lower than 200 mg/day, a minimum of 6 patients were included. Decisions on further escalation were made no sooner than 4 weeks after completion of a dose level. If no dose limiting toxicity (DLT) was observed within this 4 weeks interval,

the next higher dose level was opened for the next 3 patients. *Radiotherapy:* Fractionated external beam irradiation was given to a Biologically Effective Dose ($\alpha/\beta=10$) of 40-70 Gy in 4-7 weeks at 2.0 Gy per fraction with a minimum of 4 fractions per week. Fraction size should not exceed 3.0 Gy. The radiation dose was specified according to the ICRU 50 guidelines. Planning target volumes (PTV) encompassed the areas of macroscopic tumour including areas of radiologically evident involvement, with a margin of normal tissue of 1.5 cm at the 95% isodose. PTV was irradiated by AP-PA opposed fields or by multiple field arrangement. A simulation procedure was mandatory for all fields; shielding was by customised blocks or multileaf collimator.

Patient evaluation. Pre-treatment evaluation included a complete medical history and complete physical examination. Indicator lesions were measured by CT scan before start of treatment and repeatedly during the study for efficacy assessment. Response evaluation was performed according to the RECIST criteria. Prior to and at a weekly basis during therapy full haematology, serum chemistry, creatinine clearance and urine analysis were performed. Radiation-induced acute and late toxicity was graded according to the Southwest Oncology Group (SWOG) and the LENT SOMA Toxicity Scales, respectively. Perifosine-related toxicity was recorded according to the CTC. DLT was defined as an adverse event which is likely related to the study treatment with an intensity of drug-related CTC grade $\geq$ 3 (non-haematological toxicity, excluding alopecia and untreated nausea and vomiting) or CTC grade 4 platelets, CTC grade 4 ANC $\geq$ 5 days or grade 3 plus fever (haematological toxicity), or an intensity of radiotherapy-related SWOG (acute) and LENT SOMA (late) toxicity scale grade $\geq$ 3 despite symptomatic/prophylactic treatment, or discontinuation of intake of perifosine due to any grade of probably drug-related toxicity for more than 20% of planned treatment days. The MTD was defined as the dose level of perifosine where 2 or more out of 6 patients experienced a DLT.

Pharmacological studies. Whole blood for pharmacokinetic analysis was sampled before the start of treatment, weekly during therapy, before oral intake of perifosine (pre-dosing) and at the end of treatment. Samples were frozen and stored at –20°C until analysis by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), using miltefosine as an internal standard, as previously described [7,20]. The lower detection limit was 4.0 ng/mL using a 250 μL sample

volume. The assay was validated up to a concentration of 2000 ng/mL [20].

Results

Patients. Twenty-one patients with an indication to receive radiotherapy were entered in this study (Table 1). There were 17 males and 4 females with a median age of 59 years (range 43-82 years). All patients had advanced solid tumours, the majority NSCLC (17/21; 81%). NSCLC subtypes were: adenocarcinoma (n=9), large cell undifferentiated carcinoma (n=6) and squamous cell carcinoma (n=2). Prior treatment consisted of chemotherapy (n=16; 76%), hormonal therapy (n=1) and surgery (n=3). Two patients had received no prior treatment and none had previous radiotherapy. The median number of oral perifosine administrations was 31 (range 24-53; Table 2).

Table 1. Patient characteristics	
	Patients (*n*)
Total	21
Sex	
Male	17
Female	4
Age (years)	
Median (range)	59 (43-82)
Performance status	
0-1	19
2	2
Tumour type	
Lung (NSCLC)	17
Bladder	1
Oesophagus	1
Prostate	1
Colon	1
Previous therapy	
Chemotherapy	16
Hormonal therapy	1
Surgery	3
Radiotherapy	0
None	2

Table 2. Treatment characteristics	
Number of daily perifosine administrations	
Median (range)	31 (24-53)
Radiation schedule	
13 x 3 Gy	NSCLC (n=6)
	Prostate (n=1)
	Colon (n=1)
	Oesophagus (n=1)
17 x 3 Gy	NSCLC (n=8)
17 x 2.5 Gy	NSCLC (n=2)
25 x 2.4 Gy	Bladder (n=1)
28 x 2.5 Gy	NSCLC (n=1)
Mean total BED_{10} in Gy (range)	59.8 (50.7-87.5)
Mean number of fractions (range)	16 (13-28)
Mean fraction size in Gy (range)	2.9 (2.4-3.0)

BED_{10}, biologically effective dose calculated with an α/β ratio of 10

The administered daily doses ranged from 50 to 200 mg. Three patients were included at the 50 and 100 mg dose levels. Because at the 200 mg dose level 2 out of 5 patients developed DLT, the 150 mg dose level was expanded to a total of 10 patients to better define the tolerability and pharmacokinetic parameters of this dose level.

Fractionated radiotherapy was given with radical or palliative intent in a schedule

that depended on the stage and locoregional extension of the disease. AP-PA opposed fields were applied in 9 patients, a multiple field arrangement in 12 patients. Most patients (17/21; 81%) were irradiated to a total dose of 39-51 Gy in 13-17 fractions of 3.0 Gy, 4 fractions/week (Table 2). In 4 patients a different fractionated schedule was applied; 17 x 2.5 Gy (n=2), 25 x 2.4 Gy and 28 x 2.5 Gy (4 fractions/week), respectively. The calculated mean Biologically Effective Dose ($\alpha/\beta=10$) was 59.8 Gy (range 50.7-87.5 Gy; Table 2). All patients completed the radiation treatment as scheduled.

Toxicity. No bone marrow toxicity was observed in any of the patients treated. Non-haematological drug-related toxicity (Table 3) was mainly gastrointestinal and consisted of nausea (57%), including 2 dose limiting nausea at the 200 mg dose level, vomiting (38%), including 2 dose limiting vomiting in the same patients at the 200 mg dose level, anorexia (19%), diarrhoea (38%) and fatigue (48%). Both the frequency and the severity of these side effects increased with increasing drug dose. The 2 patients who developed DLT terminated the perifosine intake on day 15 and 17, respectively, but completed the radiation treatment as planned.

Table 3. Toxicity						
Daily dose (mg)		50	100	150	200	Total
Patients (n)		3	3	10	5	21
	CTC Grade					
Nausea	1-2	-	1	7	2	10
	3-4	-	-	-	2 (DLT)	2
Vomiting	1-2	1	-	3	2	6
	3-4	-	-	-	2 (DLT)	2
Anorexia	1-2	1	-	1	2	4
	3-4	-	-	-	-	-
Diarrhoea	1-2	1	1	4	2	8
	3-4	-	-	-	-	-
Fatigue	1-2	1	1	3	5	10
	3-4	-	-	-	-	-

Toxicities were graded according to the Common Toxicity Criteria (CTC); DLT, dose limiting toxicity

None of the patients demonstrated significant changes in blood glucose levels. The perifosine associated gastrointestinal side effects were most frequently observed from the second week of treatment onwards and did not always respond satisfactorily to standard anti-emetic regimens (metoclopramide and/or dexamethasone). One patient at the 100 mg dose level, 4 patients at the 150 mg

dose level and 4 patients at the 200 mg dose level needed 5HT-3 antagonists as well during treatment.

Radiotherapy-related acute toxicity consisted of skin erythema grade 1 (n=1), dysphagia grade 1-2 (n=6), grade 3 (n=2) and pneumonitis grade 1-2 (n=6). Patients who developed grade 3 dysphagia were treated with i.v. fluids and did not require tube feeding; 4 patients that developed grade 2 pneumonitis required temporarily steroids, but no oxygen. No radiation-induced acute toxicity grade 4 was observed and there was no clear relationship between acute radiation toxicities and the dose of perifosine. When analysing those patients with NSCLC (n=14) who received comparable radiation schedules and treatment fields (Table 2), a similar toxicity profile emerged.

Given the 2 DLTs at the 200 mg dose level the number of patients at the 150 mg dose level was expanded up to 10 to better document the tolerability and pharmacokinetic parameters of this dose level. Because none of these patients experienced DLT, the 150 mg dose level was identified as the MTD in combination with radiotherapy and recommended for further phase II evaluation.

Pharmacological studies. Fig. 1A shows representative plasma concentration versus time curves for each of the 4 dose levels. These data illustrate that steady-state plasma levels are reached approximately at 1 week after the start of perifosine intake. Mean pre-dose concentrations of perifosine on days 7, 14, 21 and 28 are provided in Table 4. A positive correlation was observed between the dose and trough plasma concentration (Fig. 1B).

Table 4. Weekly pre-dose plasma concentrations of perifosine								
Daily dose (mg)	Day 7	n	Day 14	n	Day 21	n	Day 28	n
50	1718 (445)	3	2191 (925)	3	1865 (926)	3	1627 (512)	3
100	2016 (516)	3	4076 (1073)	3	3181 (386)	2	3631 (659)	2
150	5664 (2337)	10	5508 (1550)	10	6160 (2949)	10	8399	1
200	5296 (656)	5	7183 (2651)	4	10170 (3861)	2	10490 (3267)	2

Data are expressed as means and (standard deviations) (ng/mL).
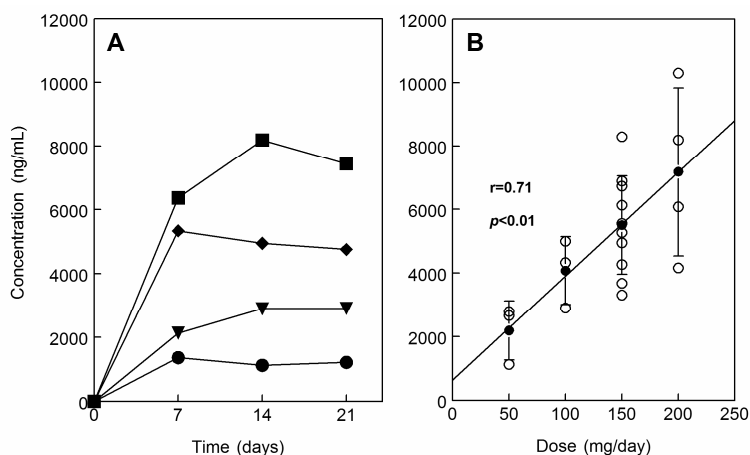
Figure 1. *(A)* **Trough plasma concentrations of perifosine versus time. Representative curves for each dose level: 50 mg (circles), 100 mg (triagles), 150 mg (diamonds) and 200 mg (squares).** *(B)* **Trough plasma concentrations of each patient on day 14 versus administered dose of perifosine. Solid symbols represent mean values. Blood samples were collected weekly pre-dosing and analysed by LC-MS/MS.**

The plasma concentration of perifosine of each patient on day 14 including the mean of these values versus the administered dose are presented in Fig. 1B. Regression analysis revealed a linear relationship (correlation coefficient = 0.71; $P < 0.01$).

Fig. 2 illustrates the plasma concentrations measured in patient number 9 who was recruited at the 150 mg dose level. This patient, receiving 28 fractions of 2.5 Gy combined with daily perifosine over a total period of 7 weeks, experienced only mild toxicity (grade 1 nausea and diarrhoea).

Figure 2. Weekly measured pre-dosing plasma concentrations of patient number 9 treated for 7 weeks with radiation (28 x 2.5 Gy) combined with daily 150 mg perifosine.

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Response data. Although the primary objective of this study was to test the feasibility and tolerability of concurrent perifosine and radiotherapy, clinical responses were also documented. Based on preclinical studies by us an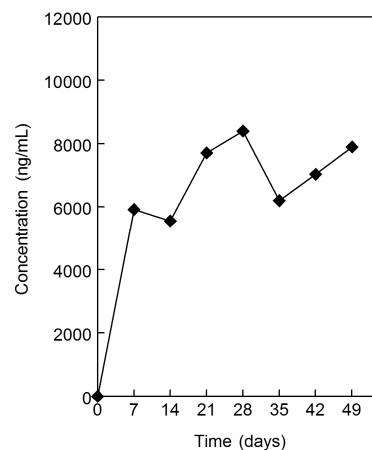d other groups [4,11,12,17-19], we consider perifosine as a potential radiosensitiser, increasing the anti-tumour effect of radiotherapy. Therefore, we focused this analysis on locoregional responses within the radiation portals (Table 5). After a median follow up of 10 months (range 1-39) we observed an infield response rate of 52% (11/21), consisting of 5 partial (24%) and 6 complete responses (28%). In 10/21 (48%) patients a stable disease was documented. After completion of treatment, half of the patients (11/21; 52%) showed metastatic disease progression outside the irradiated area at a median of 3 months (range 1-17). Patient number 9 with stage IV NSCLC and who was included at the 150 mg dose level, demonstrated a CT- and PET-confirmed complete remission within the radiation field. Two years later, he developed a solitary metastasis in the right adrenal gland, which was removed surgically. This patient shows at 3½ years after his initial treatment no evidence of disease.

Table 5. Pattern of response			
Dose group (mg)	n	response	tumour type
50	3	SD (n=2)	
		CR (n=1)	Bladder
100	3	SD (n=2)	
		PR (n=1)	NSCLC
150	10	SD (n=2)	
		PR (n=4)	3 NSCLC; 1 prostate
		CR (n=4)	3 NSCLC; 1 oesophagus
200	5	SD (n=4)	
		CR (n=1)	NSCLC

Responses within the radiation fields were evaluated according to the RECIST criteria after a median follow up of 10 months (range 1-39); SD, stable disease; PR, partial response; CR, complete response; NSCLC, non-small cell lung cancer

Discussion

Perifosine is an orally applicable, membrane-targeted alkylphosphocholine analogue with anti-tumour activity and radiosensitising properties in preclinical models [4,17,19]. Based on these favourable biological properties, this drug is considered to be an attractive candidate for combined use with radiotherapy [11,18]. In the present phase I clinical trial, escalating doses of perifosine were

given concurrently with fractionated radiotherapy in patients with advanced solid tumours in order to identify the DLT and MTD and to establish the recommended dose for further clinical testing. In addition, relevant pharmacokinetic data were generated for optimal dose scheduling. Our study shows that perifosine can be safely combined with radiation at a daily dose of 150 mg. The median number of daily perifosine administrations was 31, corresponding with a drug exposure time of 4½ weeks. The most frequently observed side effects included nausea (57%), fatigue (48%), vomiting (38%), diarrhoea (38%) and anorexia (19%) and appeared to increase both in incidence and severity with increasing doses. This is in agreement with toxicity profiles reported in other studies where perifosine was tested as single agent [7-9]. Although there was some variation in radiotherapy schedules and fields, the subgroup of NSCLC patients with comparable treatment parameters showed a similar toxicity profile as the total study population. Thus, the addition of radiation did not significantly enhance the acute side effects associated with perifosine. In 43% of the patients 5HT-3 antagonists were required besides standard anti-emetic regimens to treat gastrointestinal side effects. Despite these measures, DLT was seen in 2 out of 5 patients at the 200 mg dose level. Both these patients terminated perifosine intake, but were able to complete the radiation treatment as planned. Subsequently, the number of patients at the 150 mg dose level was expanded to a total of 10; no additional DLT was encountered. The MTD of perifosine in combination with radiation and recommended for subsequent phase II testing, was therefore established at 150 mg/day. The pharmacological studies showed that at day 7 dose-dependent steady-state plasma concentrations were reached, indicating the need for a run-in period of 1 week before the start of radiotherapy. Furthermore, a statistically significant correlation was found between plasma concentration and administered dose. All plasma concentrations ranged between 1.7 ± 0.4 µg/mL at the 50 mg dose level at day 7 and 10.5 ± 3.3 µg/mL at the 200 mg dose level at day 28. Previous preclinical studies have demonstrated that perifosine exerts its radiosensitising and anti-tumour effect *in vitro* at culture media concentrations between 0.2 and 11.3 µg/mL [6,19,21] and that *in vivo* plasma concentrations can be reached between 5 and 10 µg/mL [19]. Importantly, these plasma concentrations resulted in intratumoural drug accumulation varying from 60 to 200 µg/g, depending on the tumour model studied [19,21]. In line with these recently obtained animal data, which indicated continuous tumour accumulation after administration, 1 week of perifosine treatment prior to radiotherapy is recommended for phase II studies. We expect that this fine-tuning

of treatment protocol will not influence the tolerability. The plasma concentrations achieved in the present and other trials are biologically relevant, since they fall within the range of concentrations that induce tumour regression and radiosensitisation *in vitro* and *in vivo*.

In this trial we also looked at treatment efficacy. The significance of these data, however, is limited for several reasons. First of all, the design of phase I trials generally precludes statistical assessment of treatment efficacy, although it has been argued that these studies generate relevant information on response rates [22]. Moreover, radiotherapy alone will induce a clinical response in a number of patients irrespective of the addition of perifosine. Nonetheless, an overall locoregional response rate of 52% observed in the total study population and 47% in the subgroup of NSCLC patients, compares favourably with historical controls [23] and stimulates further clinical development of this novel combined modality approach. A multicentre randomised phase II study is currently being conducted in locally advanced NSCLC patients.

In conclusion, perifosine can be safely combined with fractionated radiotherapy. The dose limiting toxicity of this combined treatment is gastrointestinal. The recommended daily dose for clinical phase II studies is 150 mg to be started at least 1 week prior to radiotherapy. Pharmacological results showed that potentially active exposure to perifosine can be achieved to enhance radiation effects.

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Summary, discussion and conclusions

Chapter 5.1

Summary, discussion and conclusions

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-

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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

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 S49[AR] and S49[siSMS1] 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

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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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Chapter 5.2

Samenvatting

Samenvatting

Alkylfosfolipiden behoren tot een heterogene groep van synthetische fosfolipiden met een enkele (lange) koolwaterstofketen. Deze groep van anti-kankermiddelen is zeer effectief gebleken in preklinische modellen, maar de klinische toepassing is beperkt als gevolg van maag-darm toxiciteit. Doordat alkylfosfolipiden een werkingsmechanisme hebben dat verschilt van de klassieke anti-kankerbehandelingen (ze grijpen aan op celmembranen en niet op het DNA), zijn deze middelen aantrekkelijk om te combineren met andere modaliteiten zoals radiotherapie. Bovendien remmen alkylfosfolipiden de activering van bepaalde signaal transductie routes, zoals PKB/Akt en MAPK, die tumorcellen kunnen beschermen tegen de beschadigende werking van bestraling. Inderdaad blijken alkylfosfolipiden onder sommige condities de effectiviteit van bestraling te kunnen verhogen. Dit proefschrift beschrijft het onderzoekstraject naar de klinische toepassing van alkylfosfolipiden als combinatiebehandeling met bestraling, van het mechanisme van drug opname door gekweekte tumorcellen, via effectiviteit- en toxiciteitstudies in proefdieren naar uiteindelijk een fase I patiëntenstudie. In het bijzonder hebben we perifosine bestudeerd, een alkylfosfolipide dat oraal kan worden toegediend en dus voor patiënt en arts gemakkelijk in gebruik is.

In *hoofdstuk 1* beschrijven we het historisch gebruik van de verschillende alkylfosfolipiden als anti-kankerbehandeling en de gedachtegang om deze middelen te combineren met bestraling. Bovendien bediscussiëren we de eerste onderzoeksresultaten van deze combinatie.

Om effectief te kunnen zijn, moeten alkylfosfolipiden opgenomen worden door de tumorcel. In de *hoofdstukken 2.1* en *2.*2 is het onderzoek beschreven naar de opname via endocytose (opname van extracellulaire deeltjes, vloeistoffen en organismen via instulping van de plasma membraan gevolgd door binnenwaartse afsnoering van membraanblaasjes). Uit eerder onderzoek is gebleken dat het alkylfosfolipide prototype edelfosine in belangrijke mate wordt opgenomen via zogenaamde 'lipid rafts'. Rafts (letterlijk 'vlotten') zijn microdomeinen van de plasma membraan die worden gekenmerkt door een hoog gehalte in cholesterol en sfingomyeline. Deze rafts zijn betrokken bij diverse processen zoals signaaltransductie en inductie van apoptose, maar vormen ook een opname route van onder andere micro-organismen en, zoals recent is gebleken, van edelfosine. Wij vonden dat opname van alkylfosfolipiden via lipid rafts sterk celtype-afhankelijk is. Terwijl opname van de diverse geteste alkylfosfolipiden in S49 lymfoma cellen in

hoge mate afhankelijk was van de aanwezigheid van functionele rafts, vonden we hier geen aanwijzingen voor in KB carcinoma cellen. De hoge opname van alkylfosfolipiden door KB cellen blijkt een sterk temperatuur- en energie-afhankelijk proces, wat helaas nog niet volledig opgehelderd is. In *hoofdstuk 2.3* beschrijven we de remmende werking van alkylfosfolipiden op angiogenese (het proces dat leidt tot de vorming van nieuwe bloedvaten). Deze eigenschap is gebaseerd op een selectieve opname van deze stoffen door delende endotheelcellen, een conditie die belangrijk is tijdens angiogenese. In 2 *in vitro* modellen vertonen alle geteste alkylfosfolipiden een remmende werking op de uitgroei van een tubulair netwerk van endotheelcellen, hetgeen erop duidt dat de remming van bloedvatvorming een mogelijk mechanisme is dat bijdraagt aan het anti-kanker effect van alkylfosfolipiden.

Hoofdstuk 3 omvat voornamelijk de proefdierexperimenten van dit proefschrift. In *hoofdstuk 3.1* is de farmacokinetiek van perifosine bepaald in de muis na orale toediening. Bovendien hebben we het tijdsverloop van perifosine-ophoping bepaald in 3 verschillende subcutane plaveiselcel carcinomen (KB, A431 en HNXOE). Perifosine blijkt niet afbreekbaar en heeft een lange halfwaardetijd in het lichaam. Relatief veel perifosine wordt teruggevonden in de geteste tumoren, vooral in het KB tumor model. Het effect van perifosine behandeling in combinatie met bestraling is bestudeerd in *hoofdstuk 3.2*. Het blijkt dat perifosine niet alleen het celdodend vermogen van bestraling versterkt op KB cellen in kweek, maar ook op KB tumoren geïnduceerd in naakte muizen. Terwijl perifosine behandeling en bestraling afzonderlijk slechts tijdelijk de tumorgroei remde, bleek een combinatie van beiden te leiden tot een complete en langdurige tumorremissie. Hoe de tumorcellen dood gaan is voor een groot deel nog onduidelijk; wel blijken er duidelijk meer apoptotische cellen aanwezig te zijn in tumoren na een combinatiebehandeling.

Tenslotte rapporteren we in *hoofdstuk 4* de eerste klinische studie naar een combinatiebehandeling van radiotherapie en perifosine. Kankerpatiënten met tumoren in een vergevorderd stadium (voornamelijk longkanker) bleken de behandeling goed te tolereren. De plasma concentraties perifosine die we bij deze patiënten hebben gemeten liggen in dezelfde orde van grootte als die gemeten zijn bij de dierexperimenten en die gebruikt worden in celkweekexperimenten. Een gerandomiseerde fase II studie is inmiddels gestart om te bepalen of de combinatiebehandeling daadwerkelijk effectiever is dan bestraling alleen.

List of publications

Curriculum Vitae

Dankwoord

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Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 15 februari 1976 te Zaanstad. In 1995 behaalde hij het VWO diploma aan het St. Michaël College te Zaandam. In 1997 begon hij aan de studie Biomedische Gezondheidswetenschappen aan de Medische Faculteit van de Katholieke Universiteit Nijmegen (tegenwoordig Radboud Universiteit Nijmegen). Zijn 1^e bijvakstage "geneesmiddelen-onderzoek" werd hier uitgevoerd, bij de afdeling Farmacologie & Toxicologie, onder leiding van prof.dr. F.G. Russel. De 2^e bijvakstage "pathobiologie" betrof onderzoek naar de rol van macrofagen in artrose, op de afdeling Reumatologie van het UMC St. Radboud Nijmegen, onder begeleiding van dr. P.L. van Lent. Als hoofdvakstage toxicologie deed hij onderzoek naar de verandering van genexpressie in tumorcellen na bestraling, onder begeleiding van dr. R. Kerkhoven, bij de microarray faciliteit van het Nederlands Kanker Instituut/Antoni van Leeuwenhoek Ziekenhuis te Amsterdam. In dit instituut werkte hij vanaf 1 oktober 2001 tot 1 oktober 2006 als onderzoeker in opleiding, op de afdeling Experimentele Therapie (H6). De resultaten van zijn onderzoek in het kader van het KWF project "improvement of tumor response by combined modality treatment: a translational approach", onder supervisie van prof.dr. M. Verheij, zijn gebundeld in dit proefschrift. Op 13 november 2006 trad hij in dienst als toxicoloog bij de afdeling Food & Chemical Risk Analysis van TNO te Zeist.

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aan het einde van de rit nog een handje geholpen hebben. FieldCamp, onze gezamenlijke liefde voor André H., het gerstenat en andere mooie zaken won het toch ruim van onze (maar vooral j☺uw) onhebbelijkheden. Jan tenslotte, bedankt voor de ruimte in jouw groep om op H6 dit onderzoek te kunnen doen.

H3, met de 'ALP/APC/APL'-groep (Arnold, Jeffrey, Menno, Shuraila, Robert Jan (de 'Lipid Man'), Marianne, Gerald), was toch een soort 2^e huis voor mij. Ik kwam er af en toe chemicaliën, medium, serum ('op H6 weer niet uitgevuld'), antilichamen etc. bietsen, maar ik heb er ook vele proefjes mogen doen. Arnold, het opname werk is in grote mate door jou een belangrijk hoofdstuk van dit proefschrift geworden. Met plezier herinner ik me onze gesprekken en discussies, de 'quick & dirty' proefjes en niet te vergeten… je koffie. Jeffrey, je hebt enorm geholpen met het publiceren van de laatste 2 papers. Na mijn afscheid lag mijn lot zelfs in jouw handen, gelukkig deed je vanaf dat moment de ene na de andere mooie proef (waarmee ik niet wil suggereren dat de proeven daarvoor minder mooi waren). Ik zal dan ook hier, als handballer, geen flauwe opmerking maken over het korfballen.

Na eerst een maand door het NKI te hebben gezworven, kwam ik dan uiteindelijk toch terecht op H6. En gelukkig maar, het was een zeer prettige afdeling om te werken. Adrian en Fiona (en hun groepen) namen me bovendien ook nog eens op in hun radiobiologie 'club'. Nuttig commentaar kwam vaak uit deze hoek tijdens de '(vaak geen) soep'-besprekingen. Adrian, bedankt voor de tijd die je onvoorwaardelijk in dit onderzoek hebt gestoken. Sommige H6 collega's hebben me wat extra bijgestaan, met me geluncht, of waren gewoon erg gezellig en wil ik hier dan ook met naam noemen: Jacqueline, Saske, Hans, Thea, Hilde, Ben, Ingrid, Els, Teun, Debbie.

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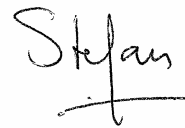
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Theo hielpen me de 'actieve' proeven soepeltjes te laten verlopen (zélfs die vreselijke ^{99m}Tc-annexin V scan!). Hetzelfde geldt voor Anita & Frank en Lenny & Lauran, met betrekking tot proeven met respectievelijk flow cytometrie en microscopie. Uiteraard vergeet ik niet de mensen van de proefdierfaciliteit die me in al die jaren het meest met de dieren geholpen hebben: Henk G., Henk S., Dick, Louis, Sjaak, Martin, Ton.

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Het is niet opgehouden bij het NKI, fijne collega's vind je gelukkig ook ergens anders zoals bij de toxicologen van TNO. Prof. Mulder (Sylvius Laboratories, Leiden) ben ik erkentelijk voor zijn rol als opleider met betrekking tot de registratie als toxicoloog.

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