Effects of anticancer alkyl-lysophospholipids on cell death and survival
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Chapter 4

Anticancer alkyl-lysophospholipids inhibit the phosphatidylinositol 3-kinase - Akt/PKB survival pathway

G.A. Ruiter, S.F. Zerp, H. Bartelink, W.J. van Blitterswijk, and M. Verheij

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**Introduction**

Synthetic membrane-permeable alkyl-lysophospholipids (ALPs) represent a promising class of anti-tumor drugs with a broad range of clinical applications. For example, Et-18-OCH$_3$ (1-Octadecyl-2-O-methyl-rac-glycerol-3-phosphocholine; Edelfosine) has been used as a purging agent in autologous bone marrow transplantation [1,2]. Topical application of HePC (hexadecylphosphocholine; Mitelfosine) was shown to be an effective therapy for skin metastases of breast cancer [3] and cutaneous lymphomas [4]. Oral administration of HePC is successfully used in the treatment of visceral leishmaniasis, a systemic protozoal infection [5]. A HePC analog, D-21266 (octadecyl-(1,1-dimethyl-piperidino-4-yl)-phosphate; Perifosine), has recently been evaluated as an oral anti-cancer drug in a clinical phase I study [6].

Although the mode of action of ALPs has not yet fully been established, their target(s) have to be sought at the level of the membrane. Due to their long alkyl chain, ALPs readily insert in the outer leaflet of the plasma membrane. Furthermore, because ALPs are resistant to phospholipase activities, these compounds accumulate persistently in cellular membranes [7]. As a consequence, ALPs cause a disturbance of the continuous, rapid turnover and biosynthesis of natural phospholipids [8-10]. We and others have previously shown that ALPs are capable of inducing apoptosis in a variety of tumor cells [11-16]. We also found that ALPs strongly enhanced apoptosis in the leukemic cell lines U937 and Jurkat T when combined with ionizing radiation [11].

The phosphatidylinositol 3-kinase (PI3K) regulated Akt/PKB pathway has recently emerged as a pathway that is essential for cell survival [15] and important for the regulation of apoptosis [17-20]. For example, overexpression of constitutive active forms of Akt/PKB or PI3K has been shown to inhibit apoptosis induced by growth factor deprivation, ultraviolet B irradiation or loss of matrix attachment [15,21-23]. Akt/PKB is activated downstream of PI3K in response to receptor stimulation [19,24,25]. PI3K catalyzes the phosphorylation of phosphatidylinositol(4,5)bisphosphate [PI(4,5)P$_2$] and phosphatidylinositol(4)phosphate [PI(4)P] into phosphatidylinositol(3,4,5)trisphosphate [PI(3,4,5)P$_3$] and phosphatidylinositol(3,4)bisphosphate [PI(3,4)P$_2$], respectively [26]. The pleckstrin homology (PH) domain of Akt/PKB binds to these membrane-associated phosphatidylinositols, leading to translocation of Akt/PKB to the membrane and its activation [24,27]. At the membrane, Akt/PKB becomes phosphorylated within its catalytic loop at Thr308 and C-terminally at Ser473 [28-31]. Phosphorylation of the Thr308 residue is catalyzed by the PI(3,4,5)P$_3$-dependent kinase PDK1 [31,32]. Although the kinase that is responsible for the phosphor-
ylation of the Ser473 residue has not been identified, several candidates have been suggested [33–35]. Both translocation of Akt/PKB to the membrane and its phosphorylation are believed to be required for a full activation of Akt/PKB [36]. Akt/PKB signaling promotes cell survival by phosphorylating and inactivating various pro-apoptotic proteins, including Bad. Forkhead family transcription factors, caspase-9 and ASK-1 [34,35,37–39].

Because ALPs act on membranes and it was shown that another synthetic apoptosis-inducing lipid (C2-ceramide) inhibits the Akt/PKB pathway [40], we investigated in the epithelial carcinoma cell lines A431 and HeLa the effect of ALPs on this signaling pathway. We show here that Akt/PKB activation is fully dependent on PI3K activity. Three clinically relevant ALPs [Et-18-OCH₃, (Edelfosine), HePC (Mltemfosine) and D-2166 (Penfloliny)] strongly inhibit the PI3K-Akt/PKB survival pathway. In addition, we demonstrate that inhibition of this pathway by the PI3K inhibitor wortmannin is associated with activation of the pro-apoptotic stress-activated protein kinase (SAPK/JNK) pathway.

Materials and methods

Materials

HePC was purchased from Sigma (Zwijndrecht, The Netherlands). Et-18-OCH₃ was from Biornol (Plymouth Meeting, PA). D-2166 was kindly provided by ASTA Medica (Frankfurt, Germany). ALPs were diluted in serum-free media. [γ³²P]ATP (3 mCi/mmol) was from Amersham (Little Chalfont, UK). Bio-benzimide (Hoechst 33258) was purchased from Sigma. Monoclonal antibodies against Akt/PKB were purchased from Transduction Laboratories (Lexington, KY). Polyclonal phospho-Akt (Ser473) antibodies were from New England Biolabs (Beverly, MA). Monoclonal anti-phosphotyrosine (PY-20) antibodies were purchased from Transduction Laboratories. Wortmannin (Sigma) was dissolved in dimethyl sulfoxide to a concentration of 2.3 mM and stored at −20°C. Prior to use, an aliquot was diluted in water to a final concentration of 1 mM. LY294002 was purchased from Calbiochem (Calbiochem-Novabiochem, Schwalbach, Germany). SAPK/JNK1 (C17) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

A431 and HeLa epithelial carcinoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Paisley, UK) supplemented with 8% heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin.

Apoptosis assays

Apoptosis was determined by staining with the DNA-binding fluorochrome bio-benzimide [41] to detect morphological nuclear changes.

Cells were washed once with phosphate-buffered saline (PBS) and resuspended in 50 µl of 3.7% paraformaldehyde. After 10 min at room temperature, the fixative was removed and the cells were resuspended in 15 µl of PBS containing 16 µg/ml bio-benzimide. Following 15 min incubation, a 10-µl aliquot was placed on a glass slide and 500 cells per slide were scored in duplicate for the incidence of apoptotic nuclear changes under an Olympus AH2-RFL fluorescence microscope using a UV1 exciter filter.

Analysis of Akt/PKB phosphorylation

After stimulation, cells were washed once with ice-cold PBS and lysed in lysis buffer [20 mM HEPES (pH 7.4), 2 mM EDTA, 50 mM β-glycerophosphate, 1% (w/v) Triton X-100, 2.5 mM MgCl₂, 1 mM Na₃VO₄, 5 µM leupeptin, 2.5 µM aprotinin and 400 µM phenylmethylsulfonyl fluoride] on ice for 15 min. Lysates were clarified by centrifugation at 14,000 r.p.m. for 10 min and normalized for protein content. For Western blot analysis, samples were boiled for 3 min in the presence of Laemmli sample buffer and subjected to SDS–PAGE (10%). Separated proteins were transferred to nitrocellulose membranes, blocked for 1 h with 5% (w/v) Nutrilone Premium (Nutricia, Zoetermeer, The Netherlands) in TBST (10 mM Tris–Cl, pH 7.5, 0.5 M NaCl and 0.05% Tween 20). Blots were incubated overnight at 4°C with polyclonal phospho-Akt/PKB (Ser473) or monoclonal Akt/PKB (for total Akt/PKB levels) antibodies in 1% Nutrilone premium in TBST. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies, proteins were detected with SuperSignal West Pico reagents (Pierce, OmniBulo, Breda, The Netherlands) and exposure to X-Omat AR films (Eastman Kodak, Rochester, NY).

In vitro PI3K assay

A431 cells were made quiescent by culturing them overnight in serum-free DMEM medium. Cells were incubated for 30 min with increasing amounts of ALPs and then stimulated with 10 µg/ml insulin for 5 min. Cells were then washed once with ice-cold phosphate-buffered saline and scraped into 250 µl lysis buffer (30 mM Tris, pH 8.0, 50 mM KCl, 10 mM EDTA, 1% NP-40, 1 µg/ml aprotinin, 1 µg/ml trypsin inhibitor, 1 mM Na₃VO₄ and 600 µg/ml phenylmethylsulfonyl fluoride) and solubilized on ice. The soluble cell lysates were clarified, by centrifuging for 10 min at 14,000 r.p.m. and normalized for protein content. Equal amounts of cell lysates were incubated with anti-phospho-tyrosine (PY-20) antibody for 1 h. The lysates were incubated for an additional hour in the presence of Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). The immunocomplexes were then precipitated and washed 3 times with IP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Tween 20 and 0.02% Na₂Az) and once in PIPKI N buffer (50 mM
Tris, pH 7.5, 10 mM MgCl₂, 20 mM KCl and 2 mM EDTA). The beads were resuspended in 90 μl PIPKI N buffer containing 5 μmol phosphatidylinositol. The reaction was started by adding 10 μl ATP-mix (200 μM ATP and 5 μCi [γ-³²P] ATP. After 20 min at 30°C, the reaction was stopped by the addition of 500 μl CHCl₃:MeOH (1:1) and 125 μl 2.4 N HCl. Samples were centrifuged and the lower organic phase was removed and applied to a silica gel 60 thin-layer chromatography plate that has been coated with 1% potassium oxalate. The plates were developed in CHCl₃:CH₃OH:H₂O:NH₄OH (25%) (45:35:8:2), dried, visualized and quantified by using a Fuji BAS 2000 TR PhosphorImager.

**In vitro SAPK/JNK assay**

SAPK/JNK activation was determined by an immune-complex kinase assay, using GST-c-Jun (1-135) as substrate. Cells were treated with Et-18-OCH₃ or wortmannin, washed and lysed in lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 2 μM leupeptin, 10 mg/ml soy bean trypsin inhibitor and 400 μM phenylmethylsulfonyl fluoride). Cells were allowed to solubilize on ice for 15 min and clarified by centrifuging for 10 min at 14 000 r.p.m. The nuclei-free supernatant was normalized for protein content and subjected to immunoprecipitation with anti-SAPKβ1/JNK1 (C17) conjugated to Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). To 41.5 μl SAPK/JNK-bound beads in assay buffer was added 4 μl 1 mg/ml GST-c-Jun (1-135), 4.5 μl Mg-ATP mix (20 mM MgCl₂/25 μM ATP) and 1 μl [γ-³²P]ATP. The reaction was terminated after 20 min at 30°C by addition of 15 μl Laemmli sample buffer and boiling, and the products resolved on 12.5% SDS–PAGE. The relative SAPK/JNK activity was determined by quantification of the levels of phosphorylated GST-c-Jun (1-135) after stimulation relative to the control situation, using a Fuji BAS 2000 TR PhosphorImager. **Results**

ALPs induce apoptosis in the human epithelial carcinoma cells A431 and HeLa

The apoptotic potential of three clinically relevant ALPs (Et-18-OCH₃, HepC and D-21266) was tested in the human epithelial vulva carcinoma cell line A431 and the human epithelial cervix carcinoma cell line HeLa. All three compounds tested induced a time- and dose-dependent increase in apoptosis in these cell types, as detected by bis-benzimide staining. Figure 1(A) shows a
bis-benzimide staining of untreated A431 cells. The first morphological changes characteristic of apoptosis were detected as early as 6 h after treatment (Fig. 1B and C). Apoptosis reached maximal levels of about 55–65% after 24 h incubation. A dose–effect relation was observed between 5 and 30 µM Et-18-OCH₃ with an ED₅₀ of approximately 15 µM (Fig. 1D). The ED₅₀ values of the three different ALPs in both cell lines are shown in Table 1.

ALPs inhibit the PI3K-Akt/PKB pathway
To determine Akt/PKB activation, we used antibodies against the phosphorylated Ser473 of Akt/PKB. Phosphorylation of Akt/PKB on this residue is indicative for its activation. Treatment of A431 cells with 10 µg/ml insulin resulted within 3 min in a sustained increase in phosphorylation of Akt/PKB at Ser473 (Fig. 2A). This phosphorylation was prevented by 20 min pretreatment with the PI3K inhibitors wortmannin (200 nM) and LY294002 (5 µM), implying that this event is fully dependent on the activity of PI3K in these cells (Fig. 2B). To investigate the effect of ALPs, cells were treated for 30 min with increasing concentrations of ALPs and subsequently incubated with 10 µg/ml insulin for 5 min. Akt/PKB phosphorylation was inhibited in a dose-dependent manner by Et-18-OCH₃ (Fig. 2C). C2-ceramide, another apoptosis-inducing sphingosine-based lipid, also inhibited insulin-induced Akt/PKB activity. In addition to insulin, Et-18-OCH₃ also inhibited LPA- and EGF-induced Akt/PKB activation (Fig. 2D). The two other ALPs, HePC and D-21266, inhibited Akt/PKB signaling as well (Fig. 3A). Similar results were obtained in HeLa cells (Fig. 3B).

Because Akt/PKB functions downstream of PI3K [24,42], we investigated the effect of ALPs on PI3K activity in A431 cells. Treatment of these cells for 5 min with 10 µg/ml insulin resulted in a more than 5-fold increase in PI3K activity. This activation was significantly inhibited in a dose-dependent manner by a 30 min pretreatment with Et-18-OCH₃ (Fig. 4). Similar data were obtained with HePC and D-21266 (not shown).

### Table 1

<table>
<thead>
<tr>
<th>ALP</th>
<th>A431</th>
<th>HeLa</th>
<th>HeLa (mean ± SD from three independent experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Et-18-OCH₃</td>
<td>15.4 ± 2.0</td>
<td>172 ± 3.0</td>
<td>23.1 ± 2.7</td>
</tr>
<tr>
<td>HePC</td>
<td>5.1 ± 1.6</td>
<td>8.1 ± 0.4</td>
<td>9.2 ± 1.8</td>
</tr>
<tr>
<td>D21266</td>
<td>17.2 ± 0.8</td>
<td>23.1 ± 0.7</td>
<td></td>
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Fig. 2

(A) Time course of insulin-induced Akt/PKB phosphorylation in A431 cells. Cells were stimulated with 10 µg/ml insulin for the indicated time points. (B) Effect of wortmannin and LY294002 on insulin-induced Akt/PKB activity in A431 cells. Cells were pretreated for 20 min with 200 ng/ml wortmannin or 5 µM LY294002 and subsequently stimulated for 5 min with 10 µg/ml insulin. (C) Effect of Et-18-OCH₃ and C2-ceramide on insulin-induced Akt/PKB activity in A431 cells. Cells were pretreated for 30 min with the indicated concentrations of Et-18-OCH₃ or C2-ceramide and subsequently stimulated for 5 min with 10 µg/ml insulin. (D) Effect of Et-18-OCH₃ on LPA, EGF and insulin-induced Akt/PKB activity in A431 cells. Cells were pretreated for 30 min in the presence or absence of 20 µM Et-18-OCH₃ and subsequently stimulated with 5 µM LPA, 2 ng/ml EGF or 10 µg/ml insulin.
Inhibition of PI3K-Akt/PKB by ALPs

(A) Effect of HePC and D-21266 on insulin-induced Akt/PKB activity in A431 cells. Cells were pretreated for 30 min with the indicated concentrations of HePC (upper panel) or D-21266 (lower panel) and subsequently stimulated with 10 μg/ml insulin. (B) Inhibition of Akt/PKB activity in HeLa cells. Cells were pretreated for 30 min with the indicated concentrations of Et-18-OCH₃ and subsequently stimulated for 5 min with 10 μg/ml insulin (upper panel) or with 2 ng/ml EGF (lower panel).

Inhibition of PI3K is associated with activation of SAPK/JNK

A recent report shows that activated Akt/PKB physically interacts with and phosphorylates SEK-1, thereby suppressing SEK-1-mediated signaling [43]. SEK-1 is the upstream activator of SAPK/JNK [44]. We have previously shown that ALPs rapidly activate the pro-apoptotic SAPK/JNK cascade. This SAPK/JNK activation appears to be an important event during ALP-induced apoptosis, as human myeloid leukemia cells (U937) stably expressing a dominant-negative c-Jun construct (TAM-67/U937 cells) failed to undergo apoptosis after ALP treatment [11]. To investigate whether the inhibition of the PI3K-Akt/PKB pathway might contribute to the activation of SAPK/JNK, we treated A431 cells for 20 min with 200 nM of the PI3K inhibitor wortmannin and subsequently measured the SAPK/JNK activity by an in vitro kinase assay. The wortmannin treatment resulted in a 2.7-fold increase activity of SAPK/JNK, which is of the same order of magnitude as the SAPK/JNK activity induced by 20 μM of the ALP Et-18-OCH₃, i.e., 4.8-fold (Fig. 5).
In summary, we have shown that three clinically relevant ALPs are potent inhibitors of the PI3K–Akt/PKB survival pathway. Our data further suggest that ALP-induced inhibition of the PI3K–Akt/PKB survival pathway contributes to the SAPK/JNK activation and apoptosis induction.

Discussion

Anti-cancer ALPs represent a heterogeneous group of unnatural lysophospholipids with different biological effects, including inhibition of tumor cell invasion [45], induction of cell differentiation [46] and apoptosis [11,13–15]. Unlike most classical chemotherapeutic drugs that target the DNA, ALPs mainly exert their effects on membranes [47].

We have previously shown that ALPs affect two important signal transduction pathways that originate from the plasma membrane, i.e. the MAPK/ERK and the SAPK/JNK pathways [11]. Apoptotic concentrations of ALPs prevented the activation of the MAPK/ERK pathway and rapidly activated the SAPK/JNK pathway. While sustained MAPK/ERK activation is important for mitogenesis [48], the SAPK/JNK pathway appears to play a major role in the induction of apoptosis [49–51]. For example, it was shown that dominant-negative constructs of components of the SAPK/JNK pathway significantly inhibited radiation- and drug-induced apoptosis [49,52]. We and others also demonstrated the importance of this pathway for ALP-induced apoptosis [11,53].

The PI3K–Akt/PKB pathway, which also originates from the plasma membrane, has more recently been implicated as an important regulator of cell death and survival [36]. The present study was initiated to test the hypothesis that ALPs may also interfere with this pathway: Our data indicate that in the epithelial carcinoma cell lines A431 and HeLa three clinically relevant ALPs (Et-18-OCH₃, HoPC and D-21266) [1–6] strongly inhibit Akt/PKB signaling at or upstream of PI3K. In addition, we demonstrated that inhibition of the PI3K–Akt/PKB pathway by the PI3K inhibitor wortmannin was associated with activation of SAPK/JNK.

Numerous studies suggest the existence of a balance between pro- and anti-apoptotic signal transduction pathways that controls apoptosis. Several groups have shown that inhibition of MAPK/ERK correlates with SAPK/JNK activation [11,54,55]. For example, in the presence of nerve growth factor (NGF) the MAPK/ERK pathway is activated whereas the SAPK/JNK pathway is suppressed. Conversely, NGF withdrawal resulted in MAPK/ERK inhibition and SAPK/JNK activation resulting in apoptosis [54]. A link between the PI3K–Akt/PKB survival pathway and the SAPK/JNK pathway has also been suggested in several studies [56,57]. In those studies it was shown that overexpression of a dominant-negative Akt/PKB mutant correlates with an activation of SAPK/JNK, while the ability of a constitutive active mutant to mediate cell survival correlates with inhibition of the SAPK/JNK pathway. More direct evidence of the existence of a cross-talk between both pathways came from recent work of Park et al. [43]. These investigators showed in 293T cells that insulin inhibited the anisomycin-induced activation of SEK-1 and its substrate SAPK/JNK. This inhibitory effect was prevented by the PI3K inhibitor LY294002, and expression of a constitutively active mutant of Akt/PKB inhibited the SEK-1 and SAPK/JNK activation. In addition, they demonstrated that Akt/PKB physically interacted with endogenous SEK-1 and that this interaction was promoted by insulin. Additional experiments revealed that Akt/PKB inhibited SEK-1-mediated apoptosis. Our own data showing that inhibition of the PI3K–Akt/PKB survival pathway results in the activation of SAPK/JNK are in line with these observations. Apparently, Akt/PKB suppresses SAPK/JNK signaling by targeting SEK-1 and this suppression is abrogated when the PI3K–Akt/PKB pathway is inhibited. We therefore speculate that ALP-induced inhibition of the PI3K–Akt/PKB survival pathway (at least partially) contributes to the SAPK/JNK activation and apoptosis induction.

Conclusion

In this study we have identified the PI3K–Akt/PKB pathway as a novel target of ALPs. Our data are consistent with the concept that cell survival and death is regulated by a delicate and dynamic balance between anti-apoptotic and pro-apoptotic signaling. We propose that ALPs induce apoptosis by shifting this balance by decreasing anti-apoptotic signaling (MAPK/ERK and PI3K–Akt/PKB) and increasing pro-apoptotic signaling (SAPK/JNK).

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