Preclinical Therapy

**In vitro and In vivo Selective Antitumor Activity of Edelfosine against Mantle Cell Lymphoma and Chronic Lymphocytic Leukemia Involving Lipid Rafts**

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**Abstract**

**Purpose:** Mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL) remain B-cell malignancies with limited therapeutic options. The present study investigates the *in vitro* and *in vivo* effect of the phospholipid ether edelfosine (1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine) in MCL and CLL.

**Experimental Design:** Several cell lines, patient-derived tumor cells, and xenografts in severe combined immunodeficient mice were used to examine the anti-MCL and anti-CLL activity of edelfosine. Furthermore, we analyzed the mechanism of action and drug biodistribution of edelfosine in MCL and CLL tumor-bearing severe combined immunodeficient mice.

**Results:** Here, we have found that the phospholipid ether edelfosine was the most potent alkyl-lysophospholipid analog in killing MCL and CLL cells, including patient-derived primary cells, while sparing normal resting lymphocytes. Alkyl-lysophospholipid analogues ranked edelfosine > perifosine > erucylphosphocholine ≥ miltefosine in their capacity to elicit apoptosis in MCL and CLL cells. Edelfosine induced co-clustering of Fas/CD95 death receptor and rafts in MCL and CLL cells. Edelfosine was taken up by malignant cells, whereas normal resting lymphocytes hardly incorporated the drug. Raft disruption by cholesterol depletion inhibited drug uptake, Fas/CD95 clustering, and edelfosine-induced apoptosis. Edelfosine oral administration showed a potent *in vivo* anticancer activity in MCL and CLL xenograft mouse models, and the drug accumulated dramatically and preferentially in the tumor.

**Conclusions:** Our data indicate that edelfosine accumulates and kills MCL and CLL cells in a rather selective way, and set co-clustering of Fas/CD95 and lipid rafts as a new framework in MCL and CLL therapy. Our data support a selective antitumor action of edelfosine. Clin Cancer Res; 16(7): OF1–9. ©2010 AACR.

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL) are two major B-cell-derived neoplasias for which current therapy is not satisfactory, leading in most cases to relapse and eventually to a fatal outcome. This lack of efficient therapy underscores the need for a continued search for novel chemotherapeutic agents. CLL is the most common adult leukemia and is characterized by the progressive accumulation of mature CD5⁺ B lymphocytes in the peripheral blood, bone marrow, and secondary lymphoid organs. New treatment combinations have incorporated the use of purine analogue (fludarabine)-based regimens together with monoclonal antibodies rituximab (anti-CD20) and alemtuzumab (anti-CD52), leading to improved complete response rates and prolonged progression-free survival, but a long-term survival benefit has not been shown (1, 2). MCL is characterized by the chromosomal translocation t(11;14)(q13;q32), resulting in the overexpression of cyclin D1 in mature B.
lymphocytes that have a striking tendency to disseminate throughout the body (3). MCL is an aggressive lymphoma with a poor survival outcome and a median survival time of 3.5 years. Conventional chemotherapeutic regimens have been the standard treatment of MCL until the recent incorporation of rituximab, which increases overall survival as well as the response rate and duration. The introduction of stem cell transplantation improves survival, although this therapeutic modality is only applied to younger and fit patients (4). Currently, allogeneic bone marrow transplantation represents the only therapy with the potential for a curative approach, although it is associated with a high rate of complications (4). Therefore, development of novel therapeutic strategies is urgently needed to improve survival in patients with the above B-cell malignancies. In the last years, new strategies have been developed that target crucial cellular pathways, and proteasome inhibition with bortezomib has recently been approved in relapsed/refractory MCL (5). MCL and CLL cells share a relatively low proliferative index and a poor apoptotic rate (6, 7), and therefore, the transforming event is likely a failure in death regulation rather than a loss of growth control. This implies that a therapeutic potential for these diseases may lie in potentiating apoptosis.

Synthetic alkyl-lysophospholipid (ALP) analogues constitute a family of promising anticancer drugs, including miltefosine, perifosine, edelfosine, and erucylphosphocholine, which promote apoptosis in a variety of tumor cells. Here, we have found that edelfosine behaves as the most potent alkyl-lysophospholipid analogue in inducing cell death in MCL and CLL cells through co-clustering of Fas/CD95 and rafts. Edelfosine induced a higher apoptotic response than perifosine in MCL and CLL patient-derived cells. Oral administration of edelfosine showed a strong in vivo anti-MCL and anti-CLL activity in xenograft mouse models. The drug accumulated in a dramatic and preferential way in the tumor, leading to drastic tumor regression. Our data reported here show a rather selective action of edelfosine against tumor cells and provide the proof of principle and rationale for further clinical evaluation of edelfosine to improve patient outcome in MCL and CLL.

Translational Relevance

Mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL) lack effective therapy. Synthetic alkyl-lysophospholipid analogues constitute a family of promising anticancer drugs, including miltefosine, perifosine, edelfosine, and erucylphosphocholine, which promote apoptosis in a variety of tumor cells. Here, we have found that edelfosine behaves as the most potent alkyl-lysophospholipid analogue in inducing cell death in MCL and CLL cells through co-clustering of Fas/CD95 and rafts. Edelfosine induced a higher apoptotic response than perifosine in MCL and CLL patient-derived cells. Oral administration of edelfosine showed a strong in vivo anti-MCL and anti-CLL activity in xenograft mouse models. The drug accumulated in a dramatic and preferential way in the tumor, leading to drastic tumor regression. Our data reported here show a rather selective action of edelfosine against tumor cells and provide the proof of principle and rationale for further clinical evaluation of edelfosine to improve patient outcome in MCL and CLL.

Materials and Methods

Drugs. Edelfosine was from Inkeysa and Apointech. Miltefosine was from Calbiochem. Perifosine and erucylphosphocholine were from Zentaris.

Cell lines and primary cells. Detailed information on the cell culture conditions for human MCL (JVM-2 and Z-138) and CLL (EHEB) cell lines and primary cells from CLL and MCL patients is included in Supplementary Data.

Apoptosis assay. Quantitation of apoptotic cells was determined by flow cytometry as the percentage of cells in the sub-G1 region (hypodiploidy) in cell cycle analysis as previously described (26).

To analyze apoptosis in CLL/MCL patient-derived samples, $5 \times 10^5$ cells were incubated for 48 h with the indicated agents. Cells were then washed in Annexin-binding buffer and incubated in 50 μL Annexin-binding buffer with allophyocyanin-conjugated anti-CD3 and phycoerythrin-conjugated anti-CD19 antibodies from Becton Dickinson for 10 min in the dark. Cells were then diluted with Annexin-binding buffer to a volume of 150 μL and incubated with 1 μL FITC-labeled Annexin V (Bender MedSystems) for 15 min in the dark. A total of 10,000 stained cells were then analyzed by flow cytometry on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson).

Western blot. Proteins (50 μg) were separated on 12% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). Membranes were probed with anticyclin D1 (DCS-6, Cell Signaling Technology) and anti-β-actin (Sigma) antibodies. Antibody binding was detected using the enhanced chemiluminescence detection system (Amersham).

Confocal microscopy. Cells were settled onto poly-L-lysine–coated slides and analyzed with a Zeiss LSM 510 laser scan confocal microscope for membrane raft and Fas/CD95 visualization using FITC-labeled cholera toxin B subunit (Sigma) and anti-human Fas/CD95 SM1/1 IgG2a mouse monoclonal antibody (Bender MedSystems).
followed by CY3-conjugated anti-mouse antibody (Phar-}
macia), as described (15). Colocalization assays were
analyzed by excitation of both fluorochromes in the same
section. Negative controls, lacking the primary antibody or
using an irrelevant antibody, showed no staining.

**Edelfosine uptake.** Drug uptake was measured as
described previously (13) after incubating 10^6 cells with
10 nmol [3H]edelfosine for 2 h in RPMI 1640/10% fetal
bovine serum and subsequent exhaustive washing (six
times) with PBS + 2% bovine serum albumin to elimi-
nate the loosely cell surface–bound ether lipid. [3H]Edel-
fosine (specific activity, 42 Ci/mmol) was synthesized by
tritiation of the 9-octadecenyl derivative (Amersham
Buchler).

**Cholesterol depletion.** Cells (2.5 × 10^5/mL) were pre-
treated with 2.5 mg/mL methyl-β-cyclodextrin (MCD)
for 30 min at 37°C in serum-free medium. Cells were then
washed thrice and resuspended in complete culture medi-
um before edelfosine addition.

**Xenograft mouse model.** CB17–severe combined immu-
nedeficient (SCID) mice (Charles River Laboratories), kept
and handled according to institutional guidelines, com-
plying with Spanish legislation under a 12/12-h light/dark
cycle at a temperature of 22°C, received a standard diet

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**Fig. 1.** Induction of apoptosis in MCL and CLL cells by ALPs. MCL (JVM-2 and Z-138) and CLL (EHEB) cell lines were incubated for the indicated times with 10 μmol/L of the distinct ALPs edelfosine, perifosine, miltefosine, and erucylphosphocholine (Erucyl-PC; A) or for 24 h with different concentrations of the ALPs (B). Apoptosis was then quantitated as percentage of cells in the sub-G1 region by flow cytometry. Untreated control cells were run in parallel. Data are means ± SE of four independent determinations. C, cells were untreated or treated with 10 μmol/L perifosine or edelfosine for the indicated times and analyzed by Western blot with anti-cyclin D1 and anti-β-actin antibodies. Immunoblotting of β-actin was used as an internal control for equal protein loading in each lane. Blots are representative of three experiments done.
 были изолированы и адаптированы в условиях одиночных клеток. CB17-SCID мыши были инкубированы в течение 3-5 суток в отсутствие (контроль) или в присутствии образца в макросом L на омега-3 соотношении 1:3, или в присутствии образца in vitro (PERF), и затем визуализация клеток была проведена с использованием следующего стандартного метода: (the shortest diameter)² × (the longest diameter) × 0.5. Животные были перенесены на более длительный период времени, и максимальный диаметр был измерен при помощи металлических мерок 2-5 суток, и объем (мм³) был измерен с помощью металлических мерок. Так как объемы последовательных наблюдений в контрольных условиях были различны, то они были оценены с помощью калибровочной линейки и центрифугированы.

Кровь собирали и собирали для последующей экстракции в течение 21 дня (MCL) и 34 дней (CLL), а затем они были убиты. Состояние животных оценивалось по наличию признаков болезни. Лечение продолжалось до тех пор, пока не были достигнуты следующие критерии: диаметр опухоли достиг 3 см или когда была отмечена значимая токсичность. Животные были скоронированы, по смерти и определено количество различных органов.

**Statistical analysis.** Всегда, при сравнении групп, в каждой группе было вычислено статистическое значение с помощью теста Манна-Уитни или t-теста. Величина значения <0.05 считалась статистически значимой.

**Results.**

**Edelfosine is the most potent ALP inducing apoptosis in MCL and CLL cells.** Мы раньше обнаружили, что ALP edelfosine эффективен в индуцировании апоптоза в нескольких лейкемических клетках, состоящих из включений Fas/CD95 в клетках leukemia. Среди различных тестов, проведенных (Fig. 1A) и эксперименты по дозо-ответу (Fig. 1B), мы обнаружили, что ALP edelfosine > perifosine > erucylphosphocholine > miltefosine в качестве кандидата на апоптоз, оценивая как процент клеток в под-G1-области (28). Последовательные наблюдения показали, что MCL и CLL клетки.

**Table 1. Selective killing of patient-derived CLL cells by edelfosine, sparing normal cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19+ CLL cells</td>
<td>CD3+ T cells</td>
</tr>
<tr>
<td>Edelfosine (10 μmol/L)</td>
<td>60.3 ± 5.6</td>
</tr>
<tr>
<td>Edelfosine (20 μmol/L)</td>
<td>43.7 ± 4.9</td>
</tr>
<tr>
<td>Perifosine (10 μmol/L)</td>
<td>73.1 ± 4.8</td>
</tr>
<tr>
<td>Perifosine (20 μmol/L)</td>
<td>61.0 ± 3.7</td>
</tr>
<tr>
<td>Staurosporine (0.5 μmol/L)</td>
<td>17.8 ± 6.7</td>
</tr>
</tbody>
</table>

**NOTE:** Primary lymphocyte cultures from CLL patients were incubated with edelfosine or perifosine for 48 h at the indicated concentrations. Staurosporine was used as a positive control of apoptosis. Percentage of cell viability was measured as nonapoptotic CD3⁺/CD19⁻ T cells or CD3⁺/CD19⁻ B cells from CLL samples in Annexin V analysis by flow cytometry. Untreated control CD19⁻ and CD3⁺ cells were run in parallel and showed a cell viability of >93% and >98%, respectively. Data are shown as mean values ± SE of five independent CLL patients.
perifosine or edelfosine. As shown in Fig. 1C, we found only a decrease in cyclin D1 protein level in Z-138 cells after 24-hour treatment. However, we detected the cleavage of the typical caspase-3 substrate poly(ADP-ribose) polymerase, as a marker for apoptosis onset, by 6-hour treatment in edelfosine- and perifosine-treated Z-138 and JVM-2 cells (data not shown), whereas cyclin D1 protein level remained unaffected (Fig. 1C). It is noteworthy that despite the presence of t(11;14) in both JVM-2 and Z-138 cell lines, JVM-2 cells harbored a reduced basal cyclin D1 content when compared with Z-138, as assessed by protein and mRNA levels (Fig. 1C; data not shown). Thus, these results suggest that cyclin D1 modulation is not involved in edelfosine/perifosine antitumor activity in MCL cell lines and that the decrease in cyclin D1 protein level detected at 24 hours (Fig. 1C) was a consequence rather than a cause in edelfosine- and perifosine-induced apoptosis.

Edelfosine was also more active than perifosine against primary malignant cells derived from MCL and CLL patients (Fig. 2). Following a dose-response analysis, we found that edelfosine was significantly more cytotoxic \((P < 0.05)\) than perifosine against primary CD19\(^+\) CLL cells derived from patients, whereas normal resting CD3\(^+\)-T lymphocytes from the same patients remained rather resistant (Table 1). This selective action of both edelfosine and perifosine was highlighted when compared with staurosporine that induced apoptosis in both malignant and normal cells at a similar rate (Table 1).

**Edelfosine-induced apoptosis in MCL and CLL cells is mediated by lipid rafts.** By using the raft marker FITC-labeled cholera toxin B subunit that binds ganglioside G\(_{M1}\) (29), mainly found in rafts (30), we found that 10 \(\mu\)mol/L edelfosine induced coclustering of lipid rafts and Fas/CD95 in both JVM-2 and EHEB (Fig. 3). Furthermore, we found that MCL (JVM-2 and Z-138) and CLL (EHEB) cell lines took up high amounts of edelfosine (Fig. 4A), whereas normal resting lymphocytes incorporated negligible amounts of drug (<20 pmol/10\(^6\) cells after

![Edelfosine-induced coclustering of Fas/CD95 and rafts in MCL and CLL cells.](image-url)
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Fig. 4. Raft involvement in edelfosine uptake and edelfosine-induced apoptosis. A, JVM-2, Z-138, and EHEB cells, untreated or pretreated with 2.5 mg/mL MCD for 30 min, were incubated with 10 nmol [3H]edelfosine for 2 h, and then drug uptake was determined. B, JVM-2, Z-138, and EHEB cells, untreated or pretreated with 2.5 mg/mL MCD for 30 min, were incubated with 10 μmol/L edelfosine for 24 h, and then apoptosis was assessed by flow cytometry as the percentage of hypodiploid (sub-G1) cells. Data are means ± SE of four independent determinations. Asterisks indicate values that are significantly different from the corresponding MCD-untreated cells at *P < 0.05 (*) and **P < 0.01 (**) levels by Student’s t test.

2 h of incubation with 10 nmol [3H]edelfosine). Lipid raft disruption by MCD treatment inhibited drug uptake (Fig. 4A), edelfosine-induced apoptosis (Fig. 4B), as well as Fas/CD95 clustering (data not shown). These data suggest that lipid rafts are essential for edelfosine uptake and edelfosine-induced apoptosis in MCL and CLL cells.

Edelfosine accumulates in MCL and CLL tumors and inhibits human MCL and CLL cell growth in vivo. We next determined whether edelfosine showed in vivo anti-human MCL and CLL cell activity. CB17-SCID mice were inoculated with 107 Z-138 or EHEB cells for MCL or CLL xenograft animal models. The MCL animal model developed aggressive tumors that were tangible within 1 week of inoculation, and increased rapidly in a few weeks time (Fig. 5A), whereas CLL tumors grew more slowly (Fig. 5A). Following development of a palpable tumor, mice were randomized into drug-treated (30 mg/kg edelfosine, daily oral administration) and control (PBS vehicle) groups. Caliper measurements were done every 2 to 5 days to calculate tumor volume (Fig. 5A). Oral administration of edelfosine achieved significant MCL and CLL tumor regression (Fig. 5A-C). When tumor size reached ~10% of body weight, animals were killed for ethical reasons. This size was approximately reached by day 15 in some of the MCL tumor-bearing mice (Fig. 5A), but this animal model was extended 1 week more to obtain reliable and comparable data with drug-treated animals in which tumor size was decreased by that time (Fig. 5A). A comparison of tumors isolated from untreated control and drug-treated MCL- and CLL-bearing mice, at the end of treatment, rendered a remarkable anti-MCL and anti-CLL activity of edelfosine, with a reduction of >85% in tumor weight and volume in both MCL and CLL animal models (Fig. 5B). Organ examination at necropsy did not reveal any apparent toxicity (data not shown), and there was an evident difference between the highly vascularized tumors from drug-free mice and the pale poorly vascularized tumors from edelfosine-treated mice (Fig. 5C). In addition, MCL tumors were bulky in drug-free mice but resulted rather flat after edelfosine treatment (Fig. 5C). No significant differences in mean body weight were observed between drug-treated and control animals during the in vivo assay (3-5% of body weight loss in the treated groups versus control groups). A drug biodistribution study showed that edelfosine dramatically accumulated in the MCL and CLL tumors (Fig. 5D). Tumor/plasma concentration ratio of edelfosine in the tumor was significantly higher than that detected in both kidney and liver after completion of the experiment in MCL and CLL animal models (Fig. 5D), with a drug mean concentration in plasma of 5.64 μg/mL. In the CLL animal model, we examined the content of edelfosine in a wide variety of distinct organs and found that the drug was dramatically accumulated in the tumor compared with lung, heart, spleen, liver, intestine, or kidney (Fig. 5D). Taken together, our data indicate a preferential accumulation of edelfosine in the tumor.

Discussion
The data reported here show that edelfosine behaves as the most potent ALP in killing MCL and CLL cells via a raft-mediated process. Our data indicate that edelfosine is a powerful antitumor agent against MCL and CLL as assessed by in vitro, ex vivo, and in vivo evidences. In addition, we found a rather selective and dramatic accumulation of edelfosine in MCL and CLL tumor cells in animal models. Here, we found that edelfosine induces the recruitment of Fas/CD95 death receptor in raft aggregates in MCL and CLL cells. Raft disruption by cholesterol depletion in MCL and CLL cells inhibited both edelfosine uptake and drug-induced apoptosis, as well as Fas/CD95 clustering, thus suggesting a major role of rafts in the uptake and antitumor action of edelfosine. Previous reports have shown that MCL and CLL cells express Fas/CD95, but a deficient apoptotic response to the external stimulation of Fas/CD95 by agonistic anti-Fas/CD95 antibodies was reported (7, 31). Unlike the natural ligand FasL/CD95L or agonistic anti-Fas/CD95 antibodies that act through their interaction with...
the exogenous portion of the Fas/CD95 death receptor, edelfosine induces activation of Fas/CD95 from within the cell independently of its ligand (16, 32). We have previously found that edelfosine is even more efficient than FasL/CD95L in promoting programmed cell death through Fas/CD95 activation by its recruitment in membrane rafts enriched in downstream signaling molecules (14, 16–18, 25, 33). Thus, edelfosine might induce Fas/CD95 activation, although the receptor is not triggered by its natural ligand or agonistic antibodies.

Using distinct MCL and CLL xenograft mouse models, we found that edelfosine accumulates in high amounts in the tumor tissue and shows a remarkable antitumor activity, leading to dramatic tumor regression. In addition, we consistently found in the MCL and CLL xenograft animal models that tumors became smaller and poorly vascularized. This could be in agreement with reports showing an antiangiogenic effect of edelfosine (34, 35). Thus, further insight into the effect of edelfosine on angiogenesis and how this action affects cancer development is warranted.

Following edelfosine oral administration in non–tumor-bearing SCID mice, we have recently found a rather wide drug distribution pattern to several tissues, including lung, spleen, intestine, liver, and kidney (36). In this study, we also found that edelfosine showed a preferential accumulation in the tumor in a MCL-bearing mouse animal model (36). Now, we have largely extended this initial study and analyzed the in vivo effect of edelfosine in MCL and CLL animal models. Interestingly, we found here that when
SCID mice contained a MCL or CLL tumor, edelfosine distribution was dramatically and significantly shifted toward the tumor (tissue/plasma concentration ratios >16; P < 0.01), suggesting a preferential tumor location for edelfosine. Our herein reported in vivo data, together with our present and previous in vitro determinations in a wide number of malignant and normal cells (14, 16, 17), suggest a rather selective edelfosine uptake and cytotoxic action in tumor cells. The selective action of edelfosine on tumor cells supports its low toxicity. We did not find any apparent damage in the distinct organs analyzed following necropsy analysis in the in vivo studies reported here. Lack of toxicity of edelfosine in a rat model has been reported, including no significant cardiac toxicity, hepatotoxicity, or renal toxicity (37). Our biodistribution data in the murine models reported here showed a mean concentration of edelfosine in plasma of 5.64 μg/mL (10.77 μmol/L; edelfosine molecular mass, 523.7). Thus, the herein reported in vitro effects, rendered by 10 μm edelfosine, were detected at a pharmacologically relevant drug concentration.

Our data constitute the first in vitro and in vivo evidence for the antitumor action of edelfosine in MCL and CLL, two hematologic malignancies with poor survival outcome. Taken together, the results reported here provide the proof of principle and rationale for further clinical evaluation of edelfosine to improve patient outcome in MCL and CLL. The results reported here also highlight the involvement of lipid rafts in the action of edelfosine on B-cell malignancies, such as MCL and CLL.

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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