Cytotoxicity of an Anti-cancer Lysophospholipid through Selective Modification of Lipid Raft Composition*

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Edelfosine is a prototypical member of the alkylphosphocholine class of antitumor drugs. Saccharomyces cerevisiae was used to screen for genes that modulate edelfosine cytotoxicity and identified sterol and sphingolipid pathways as relevant regulators. Edelfosine addition to yeast resulted in the selective partitioning of the essential plasma membrane protein Pma1p out of lipid rafts. Microscopic analysis revealed that Pma1p moved from the plasma membrane to intracellular punctate regions and finally localized to the vacuole. Consistent with altered sterol and sphingolipid synthesis resulting in increased edelfosine sensitivity, mislocalization of Pma1p was preceded by the movement of sterols out of the plasma membrane. Cells with enforced endocytosis and vacuolar protease activities prevented edelfosine-mediated (i) mobilization of sterols, (ii) loss of Pma1p from lipid rafts, and (iii) cell death. The activities of proteins and signaling processes are meaningfully altered by changes in lipid raft biophysical properties. This study points to a novel mode of action for an anti-cancer drug through modification of plasma membrane lipid composition resulting in the displacement of an essential protein from lipid rafts.

The synthetic lipid edelfosine (also known as 1-O-octadecyl-2-O-methyl-rac-glycerol-3-phosphocholine or ET-18-OCH3) is a prototypical member of the alkylphosphocholine class of cancer chemotherapeutic drugs. Alkylphosphocholines are effective drugs as they contain ether-linked fatty acids, as opposed to ester-linked fatty acids prevalent in endogenous phospholipids, and thus are much more resistant to cellular degradation by phospholipases (1–3). Because of their similarity in structure to phosphatidylcholine (PC), the main experimentations to determine a mechanism of action for edelfosine and other alkylphosphocholines have focused on PC metabolism. This course of action was supported by observations that only alkylphosphocholines with choline head groups, but not head groups found on other phospholipids such as ethanolamine or serine, were effective antitumor agents (1). Edelfosine and other choline-containing alkylphospholipids were found to inhibit PC synthesis and this correlated with inhibition of cell growth in various cancer cell lines (4–7).

Further metabolic labeling demonstrated that alkylphosphocholine drugs can inhibit the synthesis of PC-derived sphingomyelin, and this correlated with increased ceramide mass. Inhibition of de novo ceramide synthesis by the addition of the ceramide synthase inhibitor fumonisin B1 decreased ceramide levels and this was associated with increased resistance to alkylphosphocholines (8). As ceramide is a lipid second messenger whose accumulation can result in cytostasis or apoptosis (9, 10), an increase in cellular ceramide-mediated signaling was an alternate hypothesis proposed for alkylphosphocholine-mediated cell death (8).

To gain further insight into the mechanism of action of alkylphosphocholine-mediated cytotoxicity we performed a genetic screen in Saccharomyces cerevisiae to isolate genes that increased edelfosine susceptibility. The genetic screen provided evidence that ceramide structure and sphingolipid metabolism modulate edelfosine sensitivity and implicate lipid rafts as a site of action for edelfosine. Edelfosine addition resulted in the movement of plasma membrane sterols into the cell, suggesting that edelfosine-mediated cytotoxicity is through modification of the biophysical structure of lipid rafts. Analysis of lipid raft protein composition revealed that edelfosine was responsible for the selective partitioning of the plasma membrane lipid raft protein Pma1p from plasma membrane rafts to the vacuole. Yeast defective in endocytosis and vacuolar protease activity prevented edelfosine-mediated sterol movement, Pma1p loss from lipid rafts, and cellular cytotoxicity. Our results indicate a novel mode of action for an antitumor drug through drug insertion into lipid rafts and selective displacement of an essential protein from lipid rafts. These results are consistent with an emerging phenomenon whereby the activity of biological signal processes that assemble on lipid raft scaffolds (9, 10) are meaningfully altered by changes in raft biophysical properties.

MATERIALS AND METHODS

Plasmids and Yeast Strains—Yeast strain W303-1a (MATa ura3-1 his3-11,15 leu2-3,112 trpl-1 ade2-1 can1-100) was used for the genetic screen. Other strains used were RH1800 (MATa leu2 his4 ura3-52 bar1), RH3809 (MATa lcb1-100 leu2 his4 ura3 bar1), RH448 (MATa his4 lys2 leu2 ura3 bar1), RH732 (MATa his4 lys2 leu2 ura3 bar1 pep4::URA3), RH2765 (MATa his4 leu2 ura3 lys2 bar1 sla2-3 end4), RH2763 (MATa his4 leu2 ura3 bar1 pep4::URA3 sla2-1 end4), SEY6210 (MATa ura3-52 leu2-3,112 his3-100 trpl-100 lys2-801 suc2-9), SEY6210-PMA1-dsRFP (MATa ura3-52 leu2-3,112 his3-100 trpl-901 lys2-801 suc2-9 PMA1::tdimer2 (12)::kanMX4), and BY4741 (MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0). Multicopy PMA1 plasmid pCM10.4 was kindly provided by Dr. Rafael Schaffrath, Martin-Luther University, Germany (48). The AST1 gene ± 500 bp was amplified with
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specific primers (AST1-forward, 5'-GACTTGGCAGCGCAAGGCA-GAAAAAGGTTTTG-3', and AST1-reverse, 5'-GCCATATCTCTCAGACTCTGAATCTTTTGCTTG-3') using BY4741 genomic DNA as template. The PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen) and then subcloned into pRS426 ( HindIII and NotI sites). Yeast were grown at 30 °C unless otherwise indicated.

Yeast Genetic Screens—Yeast cells were transformed with a high copy genomic library and transformants were subsequently replated to plates with or without 80 μg/ml edelfosine (the kind gift of Medmac Pharma GmbH). We should note that we and others (11) have observed that the effect of lipids on cell growth in solid media can be affected by cell density. Plasmids were isolated from yeast cells on the master plates that corresponded to the same yeast strain that did not grow on plates containing edelfosine. The recovered plasmids were transformed into yeast to ensure that edelfosine sensitivity was because of plasmid borne gene(s) using a serial dilution approach. Plasmids from colonies that displayed increased sensitivity to the drug were isolated and their DNA sequence determined. Out of 10° colonies only 2 displayed increased sensitivity to the drug. Plasmids from those colonies were isolated and their DNA sequence was determined. One plasmid contained only one complete gene identified as SCS7, whereas the other plasmid carried three different genes. The specific gene responsible for the phenotype is currently being identified.

Yeast lipid biosynthetic protein encoding deletion strains were also screened for edelfosine sensitivity in synthetic minimal medium using isogenic derivatives of BY4741 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and included ckl1, pmt1, cpl1, ek1, etc1, epl1, cot2, opn3, psl1, psd2, faa1–4, gat1, gat2, ayr1, bro1, dpp1, lpp1, ino1, ino2, ino4, op11, pgs1, crd1, ls6b, sac1, vps34, fab1, tsc1, spc1, ydc1, lag1, elo2, elo3, scs7, sur2, lcb3, ysr3, lcb4, lcb5, ipt1, dpl1, cog2, and all viable erg mutants including erg2, erg3, erg4, erg5, erg6, and erg24. These strains were obtained from EUROSCARF.

Lipid Raft Isolation and Western Blots—Yeast detergent-resistant membrane (DRM) isolation was conducted as described (12) with minor modifications. Log phase growing cultures were incubated with or without the indicated edelfosine concentrations for the indicated times and temperatures in the defined medium. In general, edelfosine had a concentration-dependent effect on cell viability, and induced a decrease in protein content/cell after 5 h. Cells (10–20 optical density units as 600 nm) were washed in cold 1M sorbitol containing 10 mM Tris-HCl, pH 7.4, 5 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture (Roche), and broken with the aid of Fastprep (four 15-s pulses, 5.5 speed) in the presence of glass beads at 4 °C. Unbroken cells were removed by a 500 × g spin for 5 min at 4 °C, and the resulting supernatant, total cell lysate, was centrifuged for 3 h at 4 °C in an SW40 rotor at 35,000 rpm (218,000 × g) to pellet cellular crude membranes. The pellet was resuspended in Mes-buffered saline (MBS; 25 mM Mes, pH 6.5, 150 mM NaCl) including 60% sucrose, and Triton X-100 was added to a final concentration of 1%. After 1 h at 4 °C, membranes were further disrupted with several strokes in a Potter-Elvejem tissue grinder. Then, 350 μl of the extracted membranes were mixed with 2.27 ml of 60% sucrose in MBS, placed at the bottom of an ultracentrifuge tube, and overlaid with a sucrose step gradient from 5 to 50% sucrose in MBS (0.89 ml at 5%, 0.89 ml at 30%, 1.78 ml at 40%, 1.78 ml at 45%, and 3.54 ml at 50%). Sucrose gradients were centrifuged in an SW40 rotor at 38,000 rpm (257,000 × g) for 16 h at 4 °C in a Beckman Optima LE-80K ultracentrifuge, and then 1-ml fractions were collected from the top of the gradient, and 20 μl of each fraction was subjected to SDS-PAGE, immunoblotting, and enhanced chemiluminescence detection.

For incorporation of edelfosine in lipid rafts, cells were incubated with 20 μg/ml [3H]edelfosine (5.3 × 10^5 dpm/μmol) for the indicated times and subjected to lipid raft isolation as above.

Microscopy—Cells were processed for indirect immunofluorescence microscopy for the localization of Pma1p as described (14) and analyzed using a Zeiss Axiovert 200M microscope fitted with a plan-neofluor ×100 oil immersion objective lens. Images were captured using a Zeiss Axio Cam HR and using Zeiss Axiovision 3.1 or 4.1 software. Adobe Photoshop 7.0 was used for image alignment and labeling. Cells carrying a genomic PM1 gene fused to double-stranded RFP (15) were viewed using an LSM510-Meta confocal microscope (Carl Zeiss, Jena, Germany) with a 100 PlanApochromat objective (numerical aperture 1.4). Fluorescence signals of RFP (excitation 543 nm, HeNe laser) were detected using long-pass 585-nm emission filters. Zeiss LSM 5 Image Browser and Adobe Photoshop 7.0 were used for image alignment and labeling of confocal images.

Quinacrine (Sigma) was freshly prepared as a 2 mM stock in YPD8 (phosphate-buffered YPD, pH 8.0). One A_500 of late log control cells (BY4741) or cells treated with 20 μg/ml edelfosine in YPD for 20 min at 30 °C were centrifuged and resuspended in YPD8 ± edelfosine containing 50 μM quinacrine for 5 min. Cells were imaged live using the fluorescein isothiocyanate channel of a Zeiss Axiovert 200M microscope.
fitted with a plan-neofluor ×100 oil immersion objective lens within 5 min of quinicrin addition.

Filipin (Sigma) was freshly prepared as a 1 mg/ml stock in ethanol. Filipin was added to live cells at a final concentration of 10 μg/ml. Cells were then concentrated by brief centrifugation and imaged live in the UV channel within 5 min of filipin addition (16). To avoid vibration of the cells for acquisition of stacks used in deconvoluted images, cells were placed in slabs of solid medium made as described (17).

Metabolic Labeling—Yeast cells were grown to mid-log phase and treated with the indicated edelfosine concentrations for 30 min at 30 °C, followed by a 1-h incubation with 10 μM [14C]choline (1 × 10^6 dpm/nmol, American Radiolabeled Chemicals). Subsequent to incubation with radiolabel the cells were concentrated by centrifugation and washed twice with cold 1 M sorbitol containing 10 mM NaN₃ and 10 mM NaF. Labeled metabolites were extracted, analyzed, and quantitated as described (2). [3H]Edelfosine (5.3 × 10^6 dpm/μmol) was used for both drug uptake and lipid raft incorporation. In both cases, cells were incubated with 20 μg/ml [3H]edelfosine for the indicated times, and after washing 3–5 times with phosphate-buffered saline containing 3% bovine serum albumin to wash off cell surface-bound edelfosine, drug uptake was determined in the cell pellet by radioactivity counting as previously described (18). [3H]Edelfosine was synthesized by tritiation of the 9-octadecenyl derivative (Amersham Buchler).

Pulse-Chase Assays—Exponential cultures of cells growing at 30 °C in supplemented minimal medium lacking methionine and cysteine were concentrated and resuspended in fresh medium and incubated at 30 °C for 1 h. Cells were then pulse labeled with 50 μCi per A₉₀₀ Expre³⁵S³⁵S protein labeling mixture (PerkinElmer Life Sciences) for 5 min and chased with subsequent addition of methionine and cysteine at a final concentration of 0.5% (w/v) each. Aliquots of cells (∼3 × 10^7 cells) were taken at different times and transferred to tubes containing ice-cold 10 mM NaF/NaN₃ in 1 M sorbitol. Isolation of DRMs was conducted as described above with loading of the gradient fractions with equal radiolabel between time points. DRM containing fractions were analyzed by either direct SDS-PAGE separation (30 μl aliquots) or immunoprecipitation. For immunoprecipitation analysis aliquots (200 μl) were incubated with 1% SDS at 55 °C for 5 min and diluted to a final concentration of 15 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, and 0.05% SDS. After clearing with Pansorbin cells (Calbiochem), Pma1p was immunoprecipitated from the supernatant using a Pma1p-specific polyclonal antibody and protein A-Sepharose (Amersham Biosciences). Proteins were separated by 8% SDS-PAGE and analyzed with a Molecular Imager FX system using Quantity One analysis software (Bio-Rad).

Mass Spectrometry—Following separation by SDS-PAGE the protein was digested in situ with trypsin in the gel slice using a Genomics Solutions ProGest robot and identified by liquid chromatography-tandem mass spectrometry. Briefly, extracted peptides (10% of total sample in 3 μl) were separated on a Dionex LC Packings Ultimate gradient system and PepMap C18 reversed-phase column (15 cm × 75 μm). Mass spectrometry analysis was performed on-line using a Q-TRAP mass spectrometer fitted with a Nanospray source (Applied Biosystems/Sciex). Proteins were identified from mass spectrometry data collected in the information-dependent acquisition mode by searching the SWISS-PROT database using Mascot search engine (Matrix Sciences).

Lipid and Protein Mass Determinations—Free sterols were quantified as previously described (31) using the Amplex red cholesterol assay kit from Molecular Probes (Eugene, OR) and a spectrofluorometer (PerkinElmer Life Sciences). Briefly, exponential cultures of cells growing at 30 °C in YPD medium were treated with 20 μg/ml edelfosine. Control and treated samples were removed after 1- and 3-h treatments.

Total lipid extraction was performed as described above. Free sterols were quantified after resuspending dried lipids corresponding to 1, 0.5, and 0.25 nmol of lipid phosphate in 1× reaction buffer. Measurements were performed in duplicates. Lipid phosphorus was determined as described by Ames and Dubin (19), and protein mass using the Lowry protocol (20). Densitometry of protein bands from silver-stained gels was performed using a Model 3000 VersaDoc imaging system (Bio-Rad).

RESULTS

The Role of PC Synthesis in Edelfosine-mediated Toxicity in Yeast—To analyze if the effect of edelfosine on wild type yeast cells was cytostatic or cytotoxic a test for viability after incubation with various con-
centrations of edelfosine was performed. Viability declined with increasing concentrations of edelfosine indicating that edelfosine was cytotoxic to yeast cells (Fig. 1A), with yeast cells susceptible to edelfosine at concentrations similar to those observed for mammalian cells (7, 21).

Using 20 μg/ml edelfosine we determined that yeast cells were viable for up to 60 min of drug exposure followed by a rapid decline in viability to 20% at 90 min and 3% after 3 h (Fig. 1, B and C).

It was previously observed in mammalian cells that edelfosine inhibited the CTP:phosphocholine cytidylyltransferase-catalyzed rate-limiting step of the CDP-choline pathway for PC synthesis. One hypothesis for the cytotoxic effect of edelfosine is through inhibition of PC synthesis. Upon the addition of edelfosine we observed a 90% decrease in the incorporation of radiolabeled choline into PC (Fig. 2A). There was also very little radiolabel found in the PC turnover product glycerophosphocholine (GPC), consistent with edelfosine inhibiting PC synthesis in vivo. In addition, the inhibition of PC synthesis corresponded with a concomitant accumulation of phosphocholine indicative of a block at the CTP:phosphocholine cytidylyltransferase step (Fig. 2B).

Yeast cells containing a genetically inactivated CTP:phosphocholine cytidylyltransferase (pct1Δ) showed no difference in their sensitivity to edelfosine in comparison to isogenic wild type cells (Fig. 2C). Unlike most mammalian cells, yeast cells are able to live without a functional

FIGURE 2. Phosphatidylcholine synthesis inhibition is not the direct cause of edelfosine toxicity. A, yeast were grown in the indicated concentrations of edelfosine for 30 min followed by a 1-h labeling period with [14C]choline. The total time treatment of 1.5 h in edelfosine did not affect cell viability during the metabolic labeling experiment. Lipids were extracted and radioactivity associated with the homogenate, organic (phosphatidylcholine, PC), and aqueous phases was determined. Values are mean ± S.E. of two independent samples from a representative experiment repeated three times. B, the PC biosynthetic pathway metabolites choline (cho), phosphocholine (P-cho), CDP-choline (CDP-cho), and the catabolite glycerophosphocholine (GPC) were separated by thin layer chromatography using the solvent system methanol, 0.6% NaCl, ammonium hydroxide (50/50/5). Radioactive spots were integrated using a radiolabel imaging scanner. C, isogenic yeast with the indicated genotypes were grown to mid-log phase in minimal glucose medium containing the required supplements for cell growth. Identical numbers of cells were serial diluted 1:10 (starting with A600 = 1) and plated on control or 10 μg/ml edelfosine plates and incubated for 2 days at 30 °C.
To investigate if alterations in membrane synthesis in general could alter edelfosine toxicity we evaluated the relative edelfosine sensitivity of a set of viable isogenic yeast strains containing individual deletions of each known lipid biosynthetic gene. We observed that strains defective in either Elo2p or Elo3p, the long chain fatty acid synthases responsible for the unique long chain fatty acid composition of sphingolipids, as well as cells lacking Erg3p, a sterol C-5 desaturase involved in the final steps of ergosterol synthesis, were by far the most sensitive (Fig. 3, C and D). As inactivation of genes involved in sphingolipid and ergosterol synthesis increased edelfosine sensitivity this indicated a specific role for these two lipid pathways, as opposed to alterations in lipid synthesis in general, as effectors of cellular sensitivity to edelfosine.

Edelfosine Selectively Alters Lipid Raft Protein Composition—Sphingolipids and sterols are thought to self-associate at the plasma membrane into specific microdomains referred to as lipid rafts (28, 29). In human leukemia cells edelfosine was found to partition the Fas death receptor/CD95 into lipid rafts resulting in the recruitment of pro-apoptotic molecules and subsequent cell death (30). Lipid rafts are analyzed in vitro through their isolation as DRM fractions on density gradients. To assess if edelfosine altered lipid raft protein composition we isolated DRMs (28, 29) from three different wild type strains of edelfosine-treated yeast, and analyzed their DRMs by SDS-PAGE and silver staining. In each strain, there was a specific loss of a predominant 100-kDa band after cells had been exposed to edelfosine (Fig. 4A). Previous studies had determined that a major 100-kDa yeast lipid raft protein was Pma1p (12, 31, 32), an essential 10-membrane spanning proton pump ATPase (31, 33). Pma1p is considered a paradigm of an extremely stable membrane protein with a half-life of 11 h (33). Western blot analysis confirmed that Pma1p disappeared from raft fractions upon treatment of cells with edelfosine (Fig. 4, B and C). In addition, analysis of DRMs isolated from a yeast strain whose endogenous PMA1 open reading frame had been replaced with the reading frame for a Pma1p-dimeric red fluorescent protein chimera (Pma1p-RFP) (15) conclusively determined that Pma1p was the major protein lost from lipid rafts. In this yeast strain the 100-kDa Pma1p band was replaced by a protein of ~200 kDa that was also lost from lipid rafts as observed by Western blot with Pma1p antibodies (Fig. 4C), protein [35S]Met/Cys pulse-chase analysis, and silver staining of the DRM fraction (data not shown). As there was no underlying 100-kDa band in cells where the endogenous Pma1p was replaced with Pma1p-RFP, we were able to routinely assess Pma1p levels through SDS-PAGE and protein detection by either [35S]Met/Cys or silver staining. Further analysis of the 100-kDa band by mass spectrometry confirmed its identity as Pma1p.

Analysis of the entirety of the fractions isolated by density gradient centrifugation revealed a shift in Pma1p distribution to lower density fractions (Fig. 4, C and D) reflective of a change in its lipid environment. Overexposed blots indicated the concomitant appearance of Pma1p proteolysis products in these fractions (data not shown) accounting for the apparent decrease in total Pma1p levels noticeable in edelfosine-treated samples. Previous studies determined that appearance of these proteolytic bands was only observed in cells in which Pma1p had been internalized (33), and in line with these previous assertions was our inability to observe these Pma1p break-down products in yeast cells defective in vacuolar protease activity (data not shown). The cytoplasmic protein Pgp1p was used as a loading control with respect to total gradient protein as well as to monitor DRM purity (Fig. 4E).

A time course was performed to address how quickly edelfosine resulted in the loss of Pma1p from lipid rafts. We observed a time dependent loss of Pma1p from DRMs, with a 53 ± 13% (mean ± S.E.) decrease in Pma1p in DRMs after 60 min and a 67 ± 8% (mean ± S.E.) decrease in Pma1p after a 3-h exposure to edelfosine (Fig. 5A). The loss of Pma1p from DRMs over time was again confirmed by Western blots using anti-Pma1p antibodies (Fig. 5B). The disappearance of Pma1p...
from DRMs subsequent to edelfosine addition was confirmed using a second protocol for DRM isolation (Fig. 5C), indicating that its loss from DRMs was not because of a specific DRM isolation protocol but is a consistent and highly reproducible event. Examination of another well characterized lipid raft marker, Gas1p (12, 34, 35), revealed edelfosine did not affect its association with DRMs in the same way as was
FIGURE 5. Kinetics of Pma1p dissociation from lipid rafts. Wild type yeast cells (RH448) were incubated in defined medium ± 20 μg/ml edelfosine for the indicated times at 30 °C. A, lipid rafts from whole cell extracts were enriched using an Optiprep gradient followed by SDS-PAGE and silver staining of the DRM fraction during the time of treatment. Percentage of the Pma1p band density relative to time 0 from two independent experiments is illustrated. B, all isolated fractions for each time point from A were analyzed by Western blot for Pma1p, Gas1p, and the cytosolic protein Pgk1p. Left panels show the relative abundance of Pma1 distributed along each gradient. C, aliquots of sucrose density gradient fractions for the isolation of DRMs from membrane-enriched samples were analyzed by Western blot for the presence of Pma1p, Gas1p, and Pgk1p. D, equivalent amounts of total protein was separated by SDS-PAGE and Western blots versus Pma1p and Pgk1p were performed.
observed for Pma1p. A slight but reproducible enrichment of Gas1p in DRM was observed after 3 h of edelfosine treatment (Fig. 5, B and C). Examination of whole cell extracts by Western blot determined that total Pma1p levels, as well as levels of the non-raft protein Pkg1p, did not change throughout the time course of edelfosine treatment (Fig. 5D).

Pma1p becomes associated with lipid rafts almost immediately after its synthesis in the endoplasmic reticulum and is subsequently trafficked to plasma membrane rafts within 1 h of de novo Pma1p synthesis. The loss of Pma1p from rafts could be reflecting either displacement of plasma membrane Pma1p from rafts or an inability to assemble newly synthesized Pma1p into lipid rafts. To determine whether edelfosine affected the ability of newly synthesized Pma1p to associate with lipid rafts, cells were pulsed with [35S] Met/Cys for 5 min and chased for the indicated times in the absence or presence of edelfosine. DRMs were isolated, and Pma1p was visualized by detection of the radiolabel associated with the 100-kDa band we previously determined to be Pma1p, and by Pma1p immunoprecipitation, followed by SDS-PAGE (Fig. 6A). Edelfosine treatment did not alter the ability of newly synthesized Pma1p to associate with lipid raft fractions. Furthermore, Pma1p from total protein extract was also immunoprecipitated and we did not observe any difference in the presence of edelfosine (Fig. 6A), indicating that edelfosine did not affect the stability of newly synthesized Pma1p. To analyze the effect of edelfosine on Pma1p that has become raft associated, [35S] Met/Cys-pulsed cells were chased for 60 min (a time that has been previously determined to allow association of Pma1p with lipid rafts (36) prior to the addition of edelfosine). In these experiments, edelfosine resulted in the loss of 40% of radiolabeled Pma1p from DRMs (Fig. 6, B and C), indicating that edelfosine affects Pma1p lipid raft affinity, and not its assembly into lipid rafts.

Immunofluorescence experiments confirmed and extended our DRM partitioning results. Pma1p was observed in the plasma membrane of control cells, and edelfosine treatment resulted in movement of Pma1p and Pma1p-RFP to intracellular punctate regions within 1 h of edelfosine addition in fixed and live cells, respectively (Fig. 7, A and B), with Pma1p eventually associating with the yeast vacuole. This alteration in subcellular location is consistent with our DRM Western blot observations that Pma1p was found in Triton X-100-soluble fractions containing high proteolytic activity (33) in edelfosine-treated cells but not in untreated cells (Fig. 4, C and D, and data not shown).

Depletion of Pma1p from its proper lipid environment and removal from the plasma membrane is expected to have severe consequences on intracellular pH and membrane potential maintenance. We have used the intracellular pH indicator quinacrine to evaluate the effect of edelfosine in yeast cells treated for 30 min, a period of time where viability is not yet compromised (Fig. 1) but a considerable fraction of Pma1p has already disassociated from DRMs (Fig. 5). Quinacrine is a weakly basic dye that accumulates in low-pH compartments, primarily the vacuole of wild type yeast cells (49). In conformity with the changes observed for Pma1p, edelfosine treatment evoked intracellular acidification as reflected by accumulation of quinacrine in the cytoplasm of treated cells compared with its vacuolar accumulation in control cells (Fig. 8A). These results imply that intracellular acidification is an early physiological consequence of edelfosine treatment.

The contribution of Pma1p to the deleterious effect of edelfosine was assessed by two different approaches aimed to try to revert edelfosine sensitivity of wild type yeast by increasing Pma1p cellular levels. First, edelfosine sensitivity of cells overexpressing Pma1p was evaluated. Expression of PMA1 from a multicopy plasmid consistently alleviated edelfosine sensitivity of wild type yeast cells (Fig. 8B). High copy expression of AST1, whose product is thought to facilitate Pma1p interaction with lipid rafts, also alleviated edelfosine toxicity. Simultaneous expression of both genes from multicopy vectors did not further rescue cells from edelfosine toxicity.

Second, we reasoned that if loss of Pma1p from plasma membrane lipid rafts was a major mediator of edelfosine toxicity, then stabilization of Pma1p within plasma membrane lipid rafts would provide resistance to edelfosine. Previous studies revealed that Pma1p could be stabilized in lipid rafts in cells with decreased End4p function, indicating that Pma1p degradation requires endocytosis from the cell surface, or in pep4Δ cells indicating that Pma1p degradation also requires vacuolar proteolytic activity (33, 34, 37). A conditional end4 allele was used for these studies as a complete block in endocytosis would prevent material from reaching the vacuole and would preclude testing of the combined role of defective endocytosis and loss of function of pep4 to stabilize Pma1p in lipid rafts and/or provide edelfosine resistance. To produce the partial endocytosis block all cells were tested for edelfosine sensitiv-
Stabilization of Sterols at the Plasma Membrane Prevents Pma1p Loss from Lipid Rafts and Edelfosine Toxicity—In mammalian cells edelfosine was determined to partition into lipid rafts over time (21). We performed similar experiments using radiolabeled edelfosine and essentially identical results were observed with 20% of edelfosine seen in DRMs within 1 min of its addition to cells, and increasing to 45% after 3 h incubation (data not shown). We tested if edelfosine was redistributing lipids within rafts by using filipin to assess ergosterol localization as filipin has previously been proven to be a specific probe for yeast ergosterol distribution (35, 39). As filipin itself can perturb biological membranes we restricted the exposure of cells to filipin to 5 min as this was a time frame that has been previously shown to not alter cellular lipid distribution in yeast (16). Filipin fluorescence was exclusively observed in the plasma membrane of both fixed and live yeast cells (Fig. 9A and B). In fixed cells, movement of ergosterol out of the plasma membrane and into the cell was apparent within 15 min of edelfosine addition and increased over time. In live cells, some ergosterol movement out of the plasma membrane was observed 30 min subsequent to edelfosine addition and was very apparent after 60 min. The exposure time for image acquisition had to be decreased as the time of edelfosine exposure increased as filipin fluorescence increased dramatically over time of exposure to edelfosine. Edelfosine treatment was accompanied...
by a large increase in filipin fluorescence. We determined ergosterol mass in edelfosine-treated cells to assess if the increase in filipin fluorescence was reflecting a change in ergosterol levels. Edelfosine treatment for 0–3 h did not alter the level of ergosterol in cells (data not shown). We speculate that the observed alterations in filipin fluorescence reflect changes in the packing of sterols within membranes.

The cells with decreased endocytosis because of defective (but not completely deficient) end4 in combination with an inactivated pep4Δ gene did not lose Pma1p from lipid rafts and were remarkably resistant to edelfosine (Fig. 9, A and C). If sterol redistribution was indeed resulting in edelfosine toxicity it would follow that in end4 pep4Δ cells edelfosine would not result in loss of sterol from the plasma membrane in these cells. We observed that ergosterol remained associated with the plasma membrane in end4 pep4Δ cells regardless of the presence of edelfosine (Fig. 10C). As edelfosine uptake was identical in wild type and end4 pep4Δ cells (Fig. 9B), we can also conclude that the increase in internal filipin fluorescence observed in wild type cells was not because of increased membrane permeabilization by edelfosine but indeed reflects sterol movement.

**DISCUSSION**

In this study we uncovered molecules required for stabilization of lipid and protein components within lipid rafts and in doing so have uncovered a novel mechanism of antitumor drug action. We observed that edelfosine, the prototypical member of the alkylphosphocholine class of anti-cancer drugs, resulted in a redistribution of ergosterol from the plasma membrane into the cell resulting in the selective loss of Pma1p, an essential proton pump ATPase from plasma membrane lipid.
rafts. This is consistent with studies that observed that a mutant form of Pma1p defective in stability was lost from lipid rafts and then subsequently internalized (33). A combination of decreased endocytosis and loss of vacuolar hydrolase activity prevented the alteration in ergosterol redistribution from the plasma membrane, stabilized Pma1p within lipid rafts, and prevented edelfosine cytotoxicity. As edelfosine uptake in the endocytosis-deficient and vacuolar hydrolase-defective cells was similar to wild type cells we are certain that edelfosine resistance is subsequent to uptake.

In eukaryotic cells sphingolipid metabolism is coordinately regulated with sterol metabolism and a tight association between relative sphingolipid versus ergosterol levels and cell viability is apparent (26, 40 – 42). Previous studies had observed that sphingolipids and ergosterol are required for correct intracellular targeting of raft-associated proteins (12, 31). Our observed hypersensitivity of elo3Δ, elo2Δ, and lcb1–100 strains to edelfosine would be predicted to arise from edelfosine exerting its effect on already defective lipid rafts. Indeed, there was impaired localization of Pma1p in elo3Δ and elo2Δ mutants and decreased association of Pma1p with lipid rafts in lcb1–100 cells (12, 31, 34, 36, 43, 47). These previous studies on the effect of sphingolipid synthesis clearly demonstrated a role for lipid rafts in de novo Pma1p sorting. Our data indicate that edelfosine affects Pma1p that resides within lipid raft structures at the plasma membrane rather than de novo synthesized Pma1p.

Alterations in the composition of lipids within plasma membrane rafts has been demonstrated to be essential for the initiation of signaling pathways (10). The conversion of sphingomyelin to ceramide within lipid rafts was triggered by Pseudomonas aeruginosa infection and was essential for P. aeruginosa internalization and induction of apoptosis (44). As well, the stimulation of many cell surface receptor pathways appears to require clustering of these receptors with recent data indicating clustering of the Fas receptor (CD95) taking place in lipid rafts (45, 46). Activation of acid sphingomyelinase to convert sphingomyelin to ceramide was essential for Fas receptor signaling and apoptosis, and this was prevented in acid sphingomyelinase-deficient lymphocytes and by destruction of lipid raft integrity. The addition of edelfosine to human leukemia cells resulted in the recruitment of the Fas receptor into membrane rafts followed by cell death through apoptosis (30, 46), indicating that edelfosine mimics the sphingomyelin to ceramide conversion with respect to Fas receptor recruitment to lipid rafts. Our data in yeast support and extend this unique mode of action for an anticancer drug. We observed that edelfosine could partition into lipid rafts and effected a redistribution of sterols from the plasma membrane into the cell. This redistribution of a major lipid raft component almost certainly altered the biophysical properties of the remaining plasma membrane lipid raft microdomains (9, 10) and points to a novel mode of action for an anti-cancer drug via the modification of plasma membrane lipid composition resulting in selective (dis)association of essential proteins with lipid raft scaffolds. The data are consistent with an emerging phenomenon whereby particular biophysical alterations in membrane microdomain composition alter growth regulatory protein signals that require a specific lipid raft scaffold for assembly and/or function.

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