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The ether-phospholipid edelfosine, a prototype antitumor lipid (ATL), kills yeast cells and selectively kills several cancer cell types. To gain insight into its mechanism of action, we performed chemogenomic screens in the Saccharomyces cerevisiae gene-deletion strain collection, identifying edelfosine-resistant mutants. LEM3, AGP2, and DOC1 genes were required for drug uptake. Edelfosine displaced the essential proton pump Pma1p from rafts, inducing its internalization into the vacuole. Additional ATLs, including miltefosine and perifosine, also displaced Pma1p from rafts to the vacuole, suggesting that this process is a major hallmark of ATL cytotoxicity in yeast. Radioactive and synthetic fluorescent edelfosine analogues accumulated in yeast plasma membrane rafts and subsequently the endoplasmic reticulum. Although both edelfosine and Pma1p were initially located at membrane rafts, internalization of the drug toward endoplasmic reticulum and Pma1p to the vacuole followed different routes. Drug internalization was not dependent on endocytosis and was not critical for yeast cytotoxicity. However, mutants affecting endocytosis, vesicle sorting, or trafficking to the vacuole, including the retromer and ESCRT complexes, prevented Pma1p internalization and were edelfosine-resistant. Our data suggest that edelfosine-induced cytotoxicity involves raft reorganization and retromer- and ESCRT-mediated vesicular transport and degradation of essential raft proteins leading to cell death. Cytotoxicity of ATLs is mainly dependent on the changes they induce in plasma membrane raft-located proteins that lead to their internalization and subsequent degradation. Edelfosine toxicity can be circumvented by inactivating genes that then result in the recycling of internalized cell-surface proteins back to the plasma membrane.