Levels of SCS7/FA2H-Mediated Fatty Acid 2-Hydroxylation Determine the Sensitivity of Cells to Antitumor PM02734

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Abstract
PM02734 is a novel synthetic antitumor drug that is currently in phase I clinical trials. To gain some insight into its mode of action, we used the yeast Saccharomyces cerevisiae as a model system. Treatment of S. cerevisiae with PM02734 rapidly induced necrosis-like cell death, as also found for mammalian cells treated with its close analogue kahalalide F. We have screened the complete set of 4,848 viable S. cerevisiae haploid deletion mutants to identify genes involved in sensitivity or resistance to PM02734. Forty-five percent of the 40 most sensitive strains identified had a role in intracellular vesicle trafficking, indicating that the drug severely affects this process. A mutant strain lacking the sphingolipid fatty acyl 2-hydroxylase Scs7 was found to be the most resistant to PM02734, whereas overexpression of Scs7 rendered the cells hypersensitive to PM02734. To validate these findings in human cells, we did small interfering RNA experiments and also overexpressed the Scs7 human homologue FA2H in human cancer cell lines. As in yeast, FA2H silencing turned the cells resistant to the drug, whereas FA2H overexpression led to an increased sensitivity. Moreover, exogenous addition of the 2-hydroxylated fatty acid 2-hydroxy palmitic acid to different human cell lines increased their sensitivity to the cytotoxic compound. Taken together, these results suggest that the cell membrane and, in particular, 2-hydroxy fatty acid–containing ceramides are important for PM02734 activity. These findings may have important implications in the development of PM02734 because tumor cells with high FA2H expression are expected to be particularly sensitive to this drug. [Cancer Res 2008;68(23):9779–87]

Introduction
PM02734 is a synthetic cyclic depsipeptide related to natural kahalalides, especially to kahalalide F (KF), an antitumor compound isolated from the Hawaiian mollusk Ellysia rufescens (1, 2). PM02734 has shown in vitro activity against a broad spectrum of tumor types: breast, colon, pancreas, lung, prostate, etc. In addition, PM02734 shows statistically significant in vivo antitumor activity in several human cancer cell lines xenografted into mice. Based on these observations, and in view of its acceptable nonclinical toxicity profile, PM02734 has been selected for clinical development (2).

Although PM02734 has entered phase I clinical trials with a positive therapeutic index in advanced pretreated solid tumors, very little is known about its mechanism of action. To gain insight into the in vivo mechanism of the action of PM02734, we used the yeast Saccharomyces cerevisiae as a model organism. We found that the compound induces rapid necrosis-like cell death in yeast. We also provide evidence that PM02734 affects vesicle trafficking and mitochondrial functions because mutants affected in these processes were highly sensitive to the drug. Furthermore, we have identified the scs7 mutant as the most resistant strain to the cytotoxic action of PM02734. Moreover, increased levels of Scs7 turned the cells hypersensitive to the compound. These results have been validated in human cells. Scs7 and its human homologue, FA2H, are hydroxylases that introduce a hydroxyl group at the position 2 of fatty acids. 2-Hydroxy fatty acids are found almost exclusively as N-acyl chains within the ceramide moiety of a variety of sphingolipids. The functional role of fatty acid 2-hydroxylation has not been clearly established, but it seems to affect membrane conformation (3). We have found that 2-hydroxylated fatty acid–containing ceramides are involved in the mechanism of action of PM02734. The implications of all these findings in the treatment of tumors with PM02734 and patient selection are discussed.

Materials and Methods

Chemicals. PM02734 was obtained from PharmaMar, prepared as a 10 mg/mL stock solution in DMSO/ethanol (1:1), and kept at −20°C. The drug concentrations used for the experiments were in the range of those obtained after clinical administration of the compound. Syringomycin E was also supplied by PharmaMar and was dissolved in DMSO at 5 mg/mL. MMS was supplied by Fluka and SDS was obtained from Sigma.

Yeast strains and media. The 4,848 S. cerevisiae haploid deletion mutants in nonessential genes were obtained from Invitrogen. Yeast strains were grown at 30°C on yeast extract-peptone-dextrose (YPD) with geneticin (G418, Duchefa) or without geneticin when growing the wild-type strain.

Electron microscopy. Cells (2 × 106) were fixed in 3% glutaraldehyde for 1 h at room temperature and processed as described by Wonisch et al. (4). Finally, cells were resuspended in 500 μL of 100% Spurr resin and transferred to capsules where resin polymerized for 20 h at 60°C. Ultrathin sections were stained with lead citrate and uranyl acetate and examined in a transmission electron microscope.

Identification of yeast strains hypersensitive or resistant to PM02734. Deletion strains were grown in YPD with G418 at 200 μg/mL. Cells were resuspended and inoculated in YPD with 250 μg/mL PM02734. Resistant strains were identified as those with a higher absorbance after 24 h of growth. Sensitive strains were unable to grow after 48 h. Strains hypersensitive or resistant to PM02734 were isolated to new plates to...
determine their drug tolerance phenotype more accurately using different concentrations of the drug.

**Overexpression of SCS7 in S. cerevisiae.** Full-length SCS7 gene was placed under control of the GAL1 promoter using a PCR-based method (5). A PCR fragment containing 49 nucleotides 142 bp upstream of the SCS7 start codon together with the KanMX6 module and 50 nucleotides ending just downstream the start codon was amplified using the pair of oligonucleotides scs7-F4 (5'-AACGGGTTTGGCGGATTACT- GACCGTGACATCCTTTGGACAGAATGCTCGAGTCGTTAAAACGGA-3') and scs7-R2 (5'-GTACGGCTTTTTTGGAAAACAGTCTCCAGGAAG- TATTAGTGCAATTGATGATCGGTTT-3'). Plasmid pA6a-KanMX6-PGAL was used as template. *S. cerevisiae* BY4741 wild-type strain was transformed with the PCR fragment and selected in medium containing G418. Correct integration in the yeast chromosome was verified by PCR.

**Cell cultures.** HCT116 cells were grown in McCoy's 5A medium (Invitrogen). U937 and HeLa cells were grown in RPMI 1640. Media were supplemented with 10% calf bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained under humid conditions of 5% CO2 at 37°C.

**Overexpression experiments.** HCT116 cells were transiently transfected with pcDNA3 or pcDNA3-F2A (6) using Lipofectamine 2000 (Invitrogen). Briefly, plasmid/Lipofectamine 2000 complexes (0.5 μg/1.5 μL per well) were prepared in 100 μL Opti-MEM (Life Technologies, Invitrogen) and distributed in 24-well plates. HCT116 cells were trypsinized and resuspended in normal growth medium to 2.5 x 10⁴ cells per well. A positive control, the cells were incubated with 0.1 Ci/mL of [3H]thymidine for 24 h. As a positive control, the cells were incubated with 0.1 Ci/mL [3H]thymidine for 24 h. Assay, as described below.

To determine the incorporation of 2-hydroxy fatty acids into lipid species, the cells were labeled with 0.1 μCi/mL [3H]palmitic acid (specific activity, 50–60 mCi/mmol; American Radiocchemicals, Inc.) for 24 h. A positive contrast, the cells were incubated with 0.1 μCi/mL [3H]oleic acid (Amersham). Then, lipids were extracted according to the method of Bligh and Dyer (7) and separated by TLC using three different mobile phases: (a) diethyl ether/petroleum ether/acetic acid (95:5; to separate triacylglycerols from fatty acids); (b) ethyl ether/acetone (90:10; to separate polar lipids from nonpolar lipids; ref. 9); and (c) chloroform/formic acid (95:5; to separate ceramides from glycerophospholipids; ref. 10). Radioactivity in the plates was visualized by autoradiography. The position of the different radioactive spots was compared with that of commercial standards.

**Small interfering RNA knockdown experiments.** HCT116 cells were transfected with small interfering RNA (siRNA) oligonucleotides using Lipofectamine 2000 and following the recommendations of the Silencer siRNA Starter kit (Ambion). Briefly, siRNA/Lipofectamine 2000 complexes were dispensed in 24-well plates and then mixed with 5 x 10⁴ cells per well. Three different siRNAs (Ambion) against E2F2 were tested for the reduction of mRNA levels 24, 48, or 72 h after transfection. The most effective was 5'-GGGAUUCUGUACAGCAGA-3' (sense) and 5'-CGCAACCUCCUGGAGGTT-3' (antisense) at 30 nmol/L 48 h after transfection.

**Protein extracts and Western blots.** Cells transfected with pcDNA3 or pcDNA3-F2A or stable clones were washed twice with PBS and lysed with 100 μL of lysis buffer [10 mM HEPES (pH 7.4), 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 25 μg/mL phenylmethylsulfonyl fluoride]. Protein concentrations were measured using the bicinchoninic acid protein assay kit (Pierce). For E2F2 detection, 30 μg of total protein extract were run on a 10% SDS-PAGE gel, transferred to nitrocellulose, and incubated with anti-human FA2H antibody (1:1000, ref. 6) for 2 h at room temperature. Horseradish peroxidase–linked donkey anti-rabbit IgG antibodies were used as secondary antibodies at 1:2,000 dilution. Immunoblots were incubated for 1 h at room temperature and developed using enhanced chemiluminescence Western blotting detection reagents (Amersham). For caspase-3 and poly(ADP-ribose) polymerase (PARP) detection, 30 μg of protein from HCT116 or U937 cells were used. Anti-caspase-3 antibody (Cell Signaling Technology) and anti-PARP antibody (Sigma) were used at 1:1,000 and 1:2,000, respectively.

**Cell viability assays.** Determination of cell viability was carried out following the recommendations of the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega Biotech Iberica). Cells seeded in 24-well plates at the indicated concentration in the overexpression or siRNA experiments were treated with different concentrations of cytotoxic compounds. To determine viability, 100 μL of a combined 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium salt/phenazine methosulfate solution were added to each well containing 500 μL of culture medium. After 3 h of incubation, the formation of the formazan product was assessed by measuring absorbance at 490 nm in a 24-well plate reader (Tecan Ultra Evolution).

**RNA extraction and Northern blots.** Total RNA from cells was obtained following the recommendations of the RNasey Mini kit (Qiagen). Five micrograms of each sample were separated on a agarose formaldehyde gel and Northern blotting was carried out following the protocol of the ExpressHyb Hybridization Solution (BD Biosciences). The FA2H cDNA probe was an 862-bp fragment obtained by PCR using the plasmid pcDNA3-F2A as template (6) and the oligonucleotides FA2H-F1 (5'-GCGGCGCTC- CATGGAGAACAGCCTGTAGC-3') and FA2H-R1 (5'-TGGGAATGGTGT- CACTCCGTCTTCAGGTTGG-3'). Probes were labeled with [α-32P]dCTP using the Rediprime II Random Prime Labeling System kit (Amersham).

**Results**

PM02734 inhibits cell growth in budding yeast. To analyze whether PM02734 affected the growth of *S. cerevisiae* cells, we constructed growth curves using different concentrations of the drug (Fig. IA, left). The minimum concentration of PM02734 observed to completely inhibit cell growth over at least 24 hours was 250 μg/mL (157 μmol/L). To determine whether the effect of PM02734 in yeast cells was cytostatic or cytotoxic, a survival test after incubation with several concentrations of PM02734 was done (Fig. IA, right). Viability declined with increasing concentrations of PM02734, indicating that the compound was cytotoxic to yeast cells.

PM02734 rapidly induces necrosis in yeast cells. Following lethal injury, two modes of cell death can be distinguished in mammalian cells: apoptosis and oncosis, or primary necrosis (11). During the development of oncosis, membrane integrity is lost and cells become permeable, first to small molecules such as propidium iodide (PI) and then to larger molecules such as high-molecular weight dextrans (12). To test whether PM02734 permeabilized yeast to PI, we treated cells with concentrations of PM02734 ranging from 50 to 350 μg/mL and then did PI treatment. Flow cytometry analysis revealed that PM02734 permeabilized cells to PI because a population of PI-positive cells appeared (Fig. IB). PM02734-dependent PI permeation was found to be very quick (PI-positive cells appeared after 1 hour of PM02734 treatment) and concentration dependent. The percentage of PI-positive cells did not increase after incubation times longer than 5 hours. PI-positive cells corresponded to dead cells, as shown by methylene blue staining (Fig. IC, dark cells). In summary, PM02734-induced cell death results in the uptake of methylene blue and PI, suggesting that the cells become metabolically inactive and lose their integrity, which is different from mammalian apoptosis and yeast apoptosis-like cell death, in which only cell viability is lost (13). Different studies carried out with the PM02734 analogue, KF, have indicated that the compound induces cell death with no markers of apoptosis but
clear features of oncosis (14–16). One of the signature events of cell death by apoptosis is the activation of a cascade of proteolytic enzymes, the so-called caspases, which are synthesized as zymogens (procaspases) and undergo proteolytic maturation. To examine the features of PM02734-induced HCT116 cell death, analysis of procaspase-3 and its active fragment was studied by immunoblot. Figure 1D shows that treating the cells with PM02734 did not induce cleavage of caspase-3. As a control of these experiments, we used cells exposed to 25 μmol/L bromoenol lactone, a compound that induces apoptosis in a variety of cells by interfering with membrane phospholipid metabolism (17, 18), and caspase-3 active fragments were readily detected (Fig. 1D, left). These data suggest that PM02734-induced HCT116 cell death is not of the apoptotic type. To confirm this, cleavage of the caspase-3 substrate PARP was studied as well. The digestion fragment of 85 kDa only appeared in the positive controls but not in PM02734-treated cells.

**PM02734 causes invaginations of the plasma membrane.**

To gain further insight into the alterations caused by PM02734 in *S. cerevisiae* cells, we did electron microscopy studies (Fig. 2). Untreated cells displayed a large nucleus, one or more large vacuoles, and an intact membrane system (Fig. 2A). Surviving cells

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**Figure 1.** A to C, effects of PM02734 in *S. cerevisiae*. A, growth curves of *S. cerevisiae* in the presence of the indicated concentrations of PM02734 (left) and percentage of viable cells after 5 h of treatment with PM02734 (right). B, fluorescence-activated cell sorting analysis showing the permeability of yeast cells to PI. Cells (10⁶) were resuspended in 5 mL YPD and treated with PM02734 at the indicated concentrations, washed with water, and resuspended in 50 mmol/L sodium citrate containing 2 μg/mL PI. Abscissas show FL2-H values (orange-red fluorescence, log scale). PI-negative peaks (PI−) correspond to background fluorescence. PI-positive peaks (PI+) represent the number of cells that became permeable to PI due to the effect of PM02734. C, cells treated with 250 μg/mL PM02734 for 2 h were stained with PI and methylene blue. Dead cells are stained blue (dark cells in the black and white picture) and living cells are unstained. D, Western blots analysis of caspase-3 and PARP in extracts from HCT116 or U937 cells untreated or treated for the indicated doses and times with PM02734 and BEL. Actin was used as a loading control.
exposed to PM02734 exhibited the same characteristics as untreated cells (Fig. 2B, left). In contrast, cells killed by the action of PM02734 showed extensive cell disorganization and most intracellular structures were destroyed, which is characteristic of necrotic cells. The most remarkable effect of PM02734 in yeast cells was seen at the level of the plasma membrane, where we observed several membrane invaginations (Fig. 2B–E, arrows). Multiple membrane vesicles were also observed inside PM02734-treated cells (Fig. 2C–E).

Screening for PM02734-hypersensitive deletion mutants.
We did a genome-wide PM02734 sensitivity screening by replica plating the 4,848 deletion mutants into 96-well plates containing 250 μg/mL PM02734. By doing so, we identified 199 mutants that were hypersensitive to the compound (Supplementary Table S1; detailed information about the screening is supplied in Supplementary Data). Supplementary Table S2 shows the top 40 most sensitive strains to PM02734. When we analyzed the whole list of 199 hypersensitive strains (Supplementary Table S1), we found a total of 39 genes involved in vesicle-mediated transport, of which 25 were specifically involved in Golgi trafficking (underlined). We also found some other functional groups. One of them was formed by genes that participate in mitochondrial functions, including ATP5, ATP7, ATP4, or ATP15, which are involved in ATP biosynthesis, and COQ9, CAT5, and COQ4, with a role in the biosynthesis of ubiquinone (coenzyme Q). Another functional group included the following genes playing a role in cell wall biosynthesis: FLC2, CWH41, CCW12, ROT2, HOCl, CHS1, ALG6, YUR1, SKT5, and OST3. Finally, a group of genes participating in sphingolipid biosynthesis was found: CSG2 (this mutant was already found in the top 40), FEN2, SUR2, SUR1, or BST1.

Identification of PM02734-resistant mutants.
To identify strains resistant to PM02734, the whole collection of deletion mutants was grown in the presence of 250 μg/mL PM02734 for 24 hours (detailed information about this screening is supplied in Supplementary Data). Supplementary Table S3 shows the top 20 most resistant strains to PM02734. In contrast to the sensitive strains, these 20 mutants were not grouped in functional categories by the Gene Ontology Term Finder tool. To determine their degree of resistance more accurately, mutants in genes that presented a clear human ortholog (tcb3, ssc7, ste24, and ygr054w) were grown in rich medium, in the presence or absence of 250 μg/mL PM02734, and growth curves were obtained (Fig. 3). We found that ssc7 was
the strain most resistant to PM02734 because it exhibited the lowest degree of growth inhibition. tcb3 was also found to be highly resistant, whereas ste24 and ygr054w were found to be only slightly resistant to the compound.

SCS7 encodes a sphingolipid fatty acid 2-hydroxylase, located in the endoplasmic reticulum, which introduces an α-OH into the characteristic very long chain fatty acids of yeast sphingolipids (19, 20). Scs7 is a well-conserved protein and FA2H has been identified as its human ortholog (6). tcb3 belongs to the yeast family of tricalbins. Tricalbins are thought to be the yeast orthologs of mammalian synaptotagmins (21), a family with at least 14 mammalian variants involved in membrane-trafficking events (22). The high level of resistance of the scs7 mutant to PM02734, together with its putative role in defining membrane conformation (3) and its high conservation in mammalian cells (FA2H), prompted us to focus on Scs7/FA2H as a putative target of PM02734.

Sensitivity of S. cerevisiae cells to PM02734 depends on Scs7 levels. Because SCS7 deletion led to resistance to PM02734 in S. cerevisiae cells, we hypothesized that overproduction of Scs7 could lead to hypersensitivity. To test this hypothesis, we first constructed a S. cerevisiae strain in which the SCS7 gene was placed under the control of a regulatable GAL1 promoter. This promoter is repressed by glucose and induced in the presence of galactose (23). Scs7 overexpression resulted in increased sensitivity to PM02734, as revealed by spot assays (Fig. 4, YPGal panel), whereas repression of Scs7 resulted in resistance to the drug, like the scs7 mutant (Fig. 4, YPGlc panel). These cells were found to be resistant to PM02734 and impermeable to PI after treatment with the compound (data not shown). The drugs syringomycin E, MMS, and SDS were used as controls. Syringomycin E is an antifungal cyclic lipodepsinonapeptide produced by Pseudomonas syringae (24). Interestingly, the scs7 mutant has also been found to be resistant to this compound (3). As expected, PGALSCS7 cells grown on glucose behaved like the scs7 mutant in terms of resistance to syringomycin E (Fig. 4, YPGlc panel) and Scs7 overexpression slightly increased the sensitivity of the cells to syringomycin E (Fig. 4, YPGal panel). As controls, we used MMS, a DNA alkylating agent, and SDS, a detergent that causes the cell membrane to break down. As shown in Fig. 4, cells lacking or overexpressing Scs7 behaved as the wild-type in terms of sensitivity to both MMS and SDS. These results indicated that levels of Scs7-mediated lipid hydroxylation specifically affect the sensitivity of the cells to syringomycin E and PM02734.

Overexpression of FA2H increases the sensitivity of mammalian cells to PM02734. To validate the results found in yeast in human cells, we decided to look at the effect of FA2H overexpression and FA2H knockdown on the sensitivity of mammalian cells to PM02734. It has previously been shown that COS7 cells transfected with pcDNA3-FD2H contain higher levels of 2-hydroxyceramides and 2-hydroxy fatty acids compared with control cells, which indeed shows that the FA2H gene encodes a fatty acid 2-hydroxylase (6). Transient overexpression of FA2H in HCT116 cells leads to an increased sensitivity to PM02734 but has no effect on sensitivity to the alkylating agent MMS nor to syringomycin E (see Supplementary Fig. S1A). Similar results were obtained in two additional mammalian cell lines: COS1 and embryonic A293T (data not shown).

To examine in more detail the effect of FA2H overexpression on the sensitivity of human cells to PM02734, we generated stable clones overexpressing FA2H not only in HCT116 cells but also in HeLa cells (Fig. 5). Clones overexpressing FA2H were identified by Western blot (Fig. 5A) and examined for sensitivity to PM02734. As

Figure 3. Growth curves of S. cerevisiae strains in the presence or absence of PM02734.

Figure 4. Sensitivity of S. cerevisiae strains to different drugs. The SCS7 gene was placed under control of the regulatable GAL1 promoter. In the presence of galactose (YPGal), the promoter is on and PGALSCS7 cells express high levels of Scs7. When cells are grown in glucose, the promoter is off and the PGALSCS7 strain behaves like the scs7 null mutant. WT, PGALSCS7, or scs7A cells were grown in rich medium with either glucose or galactose. Five microliters of 4 × 10⁸ cells/mL and 1:10 serial dilutions were spotted onto plates containing either YPGal or YPGlc.
shown in Fig. 5B, these clones were clearly more sensitive to the drug in both cell lines. Microscopic examination of these cells after 1 hour of treatment showed a swollen phenotype also reported for cells treated with the PM02734 analogue KF (15, 16, 25). In conclusion, these experiments indicate that overexpression of FA2H greatly increases the sensitivity of mammalian cells to PM02734.

Exogenous addition of 2-hydroxy fatty acids increases the sensitivity of mammalian cells to PM02734. In view of the results obtained in the overexpression experiments, we decided to investigate whether increasing the cellular levels of 2-hydroxylated lipids, by incubating different cell lines (HCT116, U937, and HeLa) with exogenous 2-hydroxylated fatty acids, would result in an enhanced sensitivity to PM02734. Exogenous addition of fatty acids (2-hydroxylated or not) up to 50 μmol/L did not compromise cell viability even in the absence of serum (data not shown). HCT116 cells that had been incubated in the presence of 2-hydroxy palmitic acid showed a marked increase in their sensitivity to PM02734 compared with cells incubated with palmitic acid or without any fatty acid (Fig. 5D, left). This effect was specific for PM02734 because the addition of 2-hydroxy palmitic acid did not change the sensitivity to MMS. Similar results were obtained with HeLa and U937 (data not shown).

To study the metabolic fate of 2-hydroxy palmitic acid in the cells, 14C-labeled fatty acid was used and the different lipid products into which the fatty acid could potentially incorporated were separated by TLC. Interestingly, 2-hydroxy palmitate incorporated almost exclusively into the ceramide fraction in the three cell lines used. Results with HCT116 cells are shown (Fig. 5D, right). Essentially no 2-hydroxy palmitic acid remained in the cell as free fatty acid or bound to phospholipid. This distribution strongly contrasts with that of a nonhydroxylated fatty acid, such as palmitic or oleic acid, where, as expected, most of the label appeared in the phospholipid and triacylglycerol fractions. These results indicate that cell membranes with an increased pool of 2-hydroxy fatty acid in ceramides are more sensitive to PM02734.

Knockdown of FA2H increases the resistance of HCT116 cells to PM02734. To address whether FA2H depletion had an effect on the sensitivity of the cells to PM02734, we did RNA interference experiments. Three different siRNAs were tested for a reduction in FA2H mRNA levels at 24, 48, or 72 hours after transfection. Northern blot analysis revealed a significant reduction in FA2H mRNA after 48 hours in HCT116 cells transfected with one of the tested oligos versus the nontargeting control siRNA (data not shown). HCT116 cells transfected with this siRNA were assayed for sensitivity to PM02734, MMS, or syringomycin E (Fig. 6A and B) and analyzed by Northern blot (Fig. 6C). Survival assays showed that FA2H knockdown significantly increased the resistance to PM02734 (Fig. 6A). This effect was only observed for PM02734 because treatment with MMS or syringomycin E did not affect HCT116 sensitivity to these drugs. Figure 6B shows that cells transfected with control siRNA cells were completely swollen when treated with a high dose (7 μmol/L) of PM02734 for 1 hour. In contrast, the majority of the cells transfected with FA2H siRNA had the same appearance as the untreated cells, which visually confirmed that depletion of FA2H results in increased resistance to PM02734.

Discussion

In the present study, we took advantage of the cell biology and genetics of the budding yeast S. cerevisiae to clarify the mechanism of action of the antitumor drug PM02734. Here, we report that PM02734 induces a rapid necrosis-like cell death in S. cerevisiae and HCT116 cells, as also found for other human cell lines treated with the closest PM02734 analogue, KF. Yeast cells were found to be sensitive to PM02734 at concentrations much higher than human cells. This difference might be due to the presence of the yeast cell wall because some of the mutant strains hypersensitive to PM02734 were deleted in genes involved in cell wall organization and biogenesis.

The alterations caused by the PM02734 analogue, KF, in human cells (cytoplasmic swelling, vesiculation of cytoplasmic organelles, vacuolization, and mitochondrial and endoplasmic reticulum damage) prompted Suarez et al. (15) to suggest that the compound induces changes in the osmotic balance of the cell. Accordingly, the mechanism of action of KF has been postulated to be similar to those of other cytotoxic peptides, which induce cell death through the formation of new ion channels in the membrane and/or by changing the activity of existing channels (14, 26). In particular, Garcia-Rocha et al. (25) suggested that KF may act similarly to monensin, a Na+/H+ ionophore produced by Streptomyces cinna-

monensis. This compound slows down intracellular transport, principally within the Golgi complex (27, 28). We found that 39 of the top 199 strains most sensitive to PM02734 were deleted in genes involved in intracellular vesicle trafficking, 25 of them in vesicle targeting to, from, or within the Golgi apparatus. These results strongly indicate that PM02734 also affects intracellular transport in yeast and that it might cause damage to the Golgi compartment, as also found for monensin. However, other organelles, such as mitochondria, may also be subject to the effect of PM02734 treatment because mutants in genes exerting a role in mitochondrial functions were also found to be hypersensitive to PM02734. Mitochondrial dysfunctions have been associated with cancer, and drugs targeting mitochondria have been suggested to be important to inhibit cancer cell proliferation (29). If PM02734 does affect mitochondria in human cells, this might explain, at least in part, its higher effectiveness in cancer cells because the rapid and continuous growth of tumor cells is highly energy dependent.

Interestingly, many of the yeast genes identified that participate in intracellular transport are highly conserved in humans (see Supplementary Data). Some of them, such as AP3D1 or SYNJ2, are expressed at low levels in certain tumors (30, 31). It will be very interesting to analyze whether lowering the levels of the corresponding genes in human cells leads to increased sensitivity to PM02734, as found in yeast. If this turns out to be the case, the prediction will be that tumors expressing low levels of these proteins will show a good response to PM02734 treatment.

Although PM02734 could interact with the lipid bilayer, it is unlikely to form pores by itself because the molecule is too small to directly span the lipid bilayer. A minimum of 20 amino acids is required for this action (26) and PM02734 is formed by only 14 amino acids. One possibility is that PM02734 forms multimers. It is also possible that the drug interferes with some component of the membrane, resulting in the formation, or modification, of ion channels. It is known that the composition of the phospholipid bilayer strongly influences the formation of channels by other cytotoxic peptides (26). In the present work, we have shown that sensitivity to PM02734 both in yeast and humans largely depends on the levels of Scs7/F2A2H-mediated fatty acid hydroxylation. A role for FA2H in membrane conformation has been suggested based on the observation that 2-hydroxylation participates in
Figure 5. Increased sensitivity to PM02734 due to FA2H overexpression. A, level of FA2H protein in stably transfected clones of HCT116 and HeLa. B, cell viability of HCT116 and HeLa clones after 1 h of treatment with PM02734. Survival rates were calculated as percentages of untreated cells. Points, mean of the experiment done in triplicate; bars, SD. C, phase-contrast micrographs of HCT116 and HeLa clones treated with PM02734 for 1 h. D, left, effect of fatty acids on the survival rate of HCT116 cells treated with PM02734 (top) or MMS (bottom). The cells were incubated with 50 μmol/L palmitic acid (●), 50 μmol/L 2-hydroxy palmitic acid (▲), or neither (control; □) for 24 h. After this time, the cells were washed and exposed to the indicated concentrations of PM02734 or MMS, and viability was assayed as described in Materials and Methods. Right, TLC separation of 14C-labeled 2-hydroxy palmitate (left) and 14C-labeled palmitate (right) lipids from HCT116 cells. The plates were developed in the indicated solvents (top of each figure), and radioactivity was visualized by autoradiography. The position of authentic standards is indicated. FFA, free fatty acid; PL, phospholipids; DAG, diacylglycerol.
intramolecular and intermolecular hydrogen bonding (32, 33). Interestingly, it has previously been shown that sensitivity to another cyclic peptide, the pore-forming antifungal agent syringomycin E, also depends on the presence of fatty acid 2-hydroxylation because the scs7 mutant was also found to be resistant to this drug (3). One hypothesis, proposed by Hama et al. (3), is that 2-hydroxylation would enhance the formation of sterol/sphingolipid-rich membrane domains (lipid rafts), which may serve as sites for syringomycin E binding and channel formation. This hypothesis is supported by the fact that mutants in other genes involved in sphingolipid biosynthesis are also resistant to the compound. However, this is not the case for PM02734. We found that mutants in sphingolipid biosynthesis were hypersensitive to the compound, indicating that disruption of lipid rafts by the depletion of sphingolipids does not lead to resistance but to hypersensitivity to PM02734. In other words, lipid rafts may constitute a barrier to the action of PM02734 and not a platform for its action. Another difference between syringomycin E and PM02734 is that the levels of FA2H-mediated sphingolipid fatty acid 2-hydroxylation did not affect the sensitivity of human cells to syringomycin E. Based on our results, we propose one of the two following scenarios to explain the mechanism of action of PM02734: (a) fatty acid 2-hydroxylation allows PM02734 hydrogen bonding directly to sphingolipids to facilitate ion channel formation or permeability to the drug and (b) 2-hydroxylation influences the formation of some kind of membrane microdomain, not necessarily lipid rafts, present in the lipid bilayer that is important for drug-membrane association. In this regard, we have shown that PM02734 induces the formation of plasma membrane invaginations in yeast (Fig. 2). These structures may represent the theoretical microdomains.

Our results highlight the importance of the study of 2-hydroxylation in membrane conformation. The role of FA2H has only been investigated in the brain, where FA2H is a myelination-associated gene (34), and in the skin, where it participates in the formation and function of epidermal permeability barrier (35). Sphingolipids containing 2-hydroxy fatty acids are uniquely abundant in the brain, skin, intestinal tract, and certain cancers (36). Our results may have important implications in PM02734 therapy because tumor cells with high levels of sphingolipid fatty acid 2-hydroxylation are expected to be highly sensitive to this drug. FA2H could then become a marker of PM02734 activity and help guide patient selection.

Figure 6. Increased resistance to PM02734 by FA2H knockdown. HCT116 cells were transiently transfected with a siRNA against FA2H or with a nontargeting control siRNA. Twenty-four hours after transfection, medium was replaced by fresh medium, and 48 h after transfection, cells were assayed for sensitivity to different drugs. A, survival after treatment with PM02734, MMS, or syringomycin E was determined as described in Fig. 5B. B, phase-contrast micrographs of transfected cells untreated or treated with 7 μmol/L PM02734 for 1 h. C, effect of siRNA on FA2H mRNA levels was analyzed by Northern blot.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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