Ubiquitin Pathway Proteins Influence the Mechanism of Action of the Novel Immunosuppressive Drug FTY720 in Saccharomyces cerevisiae*

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FTY720 is an immunosuppressive drug in clinical development for transplant graft protection in humans. This agent is of particular interest because, unlike currently available regimes, it acts to sequester lymphocytes without causing cytotoxicity or blocking differentiation and growth potential. In an effort to elucidate the mechanism of action of FTY720, and identify its downstream effectors, we have screened genomic libraries and spontaneous mutants of the model system Saccharomyces cerevisiae for resistance to FTY720. We identified several proteins and pathways as being involved in the mechanism of action of FTY720. We show specifically that the two amino acid transporters TAT1 and TAT2, the two ubiquitin proteases UBP5 and UBP11, and the heat shock protein CAJ1 confer growth resistance to FTY720 when overexpressed. Another amino acid transporter, GNP1, and the ubiquitin structural gene UBI4 as well as the ubiquitin ligase RSP5, and its binding protein BUL1 confer growth resistance in a mutated form. Supporting the importance of amino acid transport in the growth resistance phenotype of S. cerevisiae to the immunosuppressive agent FTY720, a prototrophic strain was more resistant to FTY720 than the isogenic auxotroph. To further explore these results, the effects on amino acid uptake and protein degradation were measured in the presence of FTY720. Due to the high conservation of these proteins and pathways between yeast and humans, these results may provide valuable insights into the mechanism of action of FTY720 in lymphocyte sequestration in humans.

Current agents used for immunosuppression following organ transplant in humans (cyclosporin A, FK506, rapamycin) function to block T-cell activation and maturation by interfering with intracellular signaling pathways (1–5). While these agents are effective in preventing transplant graft rejection, there is considerable room for improvement in extending graft survival before chronic graft rejection and in reducing the severe side effects associated with the depletion of immune system function. The novel compound FTY720 is unique in that it does not directly block T-cell activation, but indirectly prevents antigen recognition by causing the rapid and reversible sequestration of peripheral blood lymphocytes in the secondary lymph nodes and Peyer’s patches (6–8). Currently in clinical development for transplant graft protection in humans, FTY720 has already been demonstrated to be effective in prolonging the allograft survival of skin (9–11), heart (6, 12), liver (13), and kidney transplants (14), as well as slowing down disease progression of graft versus host disease (15) and autoimmune type I diabetes (16) in animal models. The novel and unique mechanism of action of FTY720 provides beneficial and synergistic effects in the context of current immunosuppressive regimes. Despite these successes and intensive research efforts, the specific mechanism of action of this compound in human cells remains to be determined.

FTY720 was first synthesized as a chemical derivative of myricin, a natural product of the fungus Isaria sinclairii (9, 35). Also an immunosuppressive agent, myricin is shown to inhibit ceramide synthesis in Saccharomyces cerevisiae, leading to a reduction in the rate of intracellular transport of glycosylphosphatidylinositol-anchored proteins (36). In the murine cytotoxic T lymphocyte cell line CTLL-2, myricin inhibited another lipid transferase, serine palmitoyltransferase. This inhibitory effect is suggested to be mediated by binding to murine LCB1 and LCB2, mammalian homologues of yeast proteins linked to sphingolipid biosynthesis (31, 32). The immunosuppressive activity of FTY720, however, is thought to be different from that of myricin as it does not inhibit serine palmitoyltransferase activity and, also unlike myricin, does not inhibit the mixed lymphocyte reaction (37–39).

In the present study, we screened genomic libraries for multi-copy suppressor genes, screened populations of spontaneous mutants, and performed biochemical analyses in the model system S. cerevisiae in a search for genes and genomic alterations capable of conferring resistance to FTY720-induced growth inhibition. We report that proteins involved in the ubiquitin protein degradation pathway, amino acid transport, and a heat shock protein are capable of reversing growth inhibition caused by exposure to FTY720 in yeast either when overexpressed or in a mutated form. Due to the high degree of homology between yeast and human genomes and the strong conservation of amino acid transport and ubiquitin-mediated protein degradation pathways between these species, these data may provide insight to the mechanism of action of this exciting drug in humans and aid in the development of novel immunosuppressive agents.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Materials—**Strains used in this study are listed in Table I and were maintained according to standard laboratory procedure (19). All strains except those marked with an asterisk are isogenic to JK9-3d. Cultivation and maintenance of cells were carried out in YPD medium or synthetic minimal medium (S.D.) complemented with the appropriate nutrients for plasmid maintenance. FTY720 was dissolved in distilled water to be 10 mM and diluted to the appropriated concentrations with medium used for yeast culture.

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Isolation of Multicopy Suppressor Genes Confering Resistance to FTY720—The strain JK9-3da was transformed with yeast genomic DNA libraries received from M. D. Rose (Princeton), M. Carlson (Columbia University, New York) and J. Heitman (Duke University, Durham, NC). They were constructed, respectively, with the URA3-based 2-μ vectors YEP24, pSEY18 and pRS426. FTY720-resistant transformants were selected on minimal medium lacking uracil in the presence of 30 μM FTY720. The total number of transformants was >20,000. After the first selection, FTY720-resistant transformants were re-selected in YPD medium (yeast extract, peptone, dextrose) in the presence of 30 μM FTY720 at 30 °C. Plasmids conferring resistance to FTY720 were recovered as described (12), amplified, and reintroduced into JK9-3ds for the confirmation of the resistance. The inserts from the plasmids that conferred resistance were sequenced using the standard oligonucleotides M13 forward and reverse (pSEY18 vector), T3 and T7 (pRS426 vector), and 5′-ACC CGC ACC TGT GCC CCC GG-3′ and 5′-ACT TGG AGC CAC TAT CGA CT-3′ that flanked the inserts in the parent Ye24 vector. The DNA sequence was compared with the Saccharomyces Genome Database. Candidate genes TAT1, TAT2, UBP5, UBP11, and CAJ1 were cloned from the initial multicopy suppressor clones into the 2-μ-based vector, pRS426 (Table I) using specific restriction enzymes and standard cloning procedures.

Construction of Genomic DNA Libraries from FTY720 Dominant-Resistant Mutants—Total genomic DNA from dominant mutants and JK9-3da parent strain was isolated using Genomic-tip System (Qiagen) according to the manufacturer's instructions. DNA was subjected to ligation with a double-stranded M13 DNA at 47 °C for 15 min, and a BamHI site of the URA3 CEN-based vector pRS416. The ligation mixture was transformed into Escherichia coli strain TOP10F (Invitrogen). More than 106 independent transformants were pooled, and plasmid DNA was isolated. The mean insert size was 7–10 kb.

Cloning of the Dominant Mutant Gene GNP1—JK9-3da was transformed with the yeast genomic DNA library constructed from the dominant mutant. FTY720-resistant transformants were selected in YPD medium in the presence of 30 μM FTY720. The minimal size of the DNA fragment that conferred resistance to the drug was 2.9 kb and corresponded to the region from 1469012 to 1469856 on chromosome 4. The full sequence of the GNP1 gene was compared with the wild type sequence.

Cloning of the Recessive Mutant Gene BUL1—FTY720 recessive mutant was transformed with the wild type genomic DNA library, and URA3 transformants were selected on minimal plates lacking uracil. About 600 independent transformants were picked and FTY720-sensitive cells were selected on minimal plates in the presence of 30 μM FTY720. Plasmid DNA was isolated from FTY720-sensitive transformants, amplified, and re-introduced into the recessive mutant for the confirmation of FTY720 sensitivity. Both extremities of the inserts were sequenced using the standard oligonucleotides T3 and T7 that flanked the inserts in the pRS416 vector. BUL1 gene was identified as the gene that could complement for the FTY720-resistant mutation. The mutation of BUL1 gene in the recessive mutant was confirmed by the determination of the DNA sequence.

Construction of Bul1-disrupted Strain—pCAW14 (pRS416::BUL1) was doubly digested with SpeI/Eco47-3 restriction enzymes, and a 1.4-kb fragment containing the BUL1 gene was replaced by a G418 cassette generated by PCR on pF6A-KanMX4, with the oligonucleotides 5′-gtc aag agt cag aag cag gag gga-3′ and 5′-atc gac gat ggt gaa ttc gag tgc gac gga-3′ containing sticky ends for SpeI and Eco47-3. The disruption fragment was generated by digesting this plasmid with XbaI and KpnI and was transformed into JK9-3da strain. G418-resistant clones were selected and their genomic DNA extracted for PCR screening to check site-specific recombination.

Lcb4, lcb5, and lcb4 lcb5 Mutants—The mutants were constructed using the PCR-based method as described previously (20) using pF6A (G418) plasmid (20) and pAg32 (hygromycin) plasmid (21).

DA1-TAT1 and HA-TAT2 Plasmids—TAT1 protein tagging with the hemagglutinin protein HA1 was constructed as described for TAT2 (22).

HATAT1 (in pRS426) encodes an N-terminal HA-tagged, fully functional TAT1 protein under the control of its own promoter. HA-TAT1 was constructed by ligating a 0.6-kb XhoI-XmaI-digested PCR product, containing the TAT1 promoter, 5′-untranslated region, initiation codon, and double HA-tag, to a 2.6-kb XmaI-NotI-digested PCR product containing the TAT1 open reading frame and 3′-noncoding region. The XhoI and NotI sites were natural sites of pRS426 where the TAT1 gene was subcloned for multicopy suppressor application flanking the TAT1 gene. The PCR primers used to generate the 0.6-kb fragment were 5′-CAGGTCATACCCGGGGTGCTATGCCGAGCAGCTCATAGGATTAGATAGGCCCGCATAGTCCAGGAAAGTCTGCTATTTGAGCCCTTTTTATTACGAG-3′ (Xmal site in bold italics, antisense 2′X HA open reading frame is underlined, and antisense initiation codon is shown in bold) and the M13 standard primer (−40). The primers used to generate the 2.6-kb fragment were 5′-CAGGTCATACCCGGGGTGCTATGCCGAGCAGCTCATAGGATTAGATAGGCCCGCATAGTCCAGGAAAGTCTGCTATTTGAGCCCTTTTTATTACGAG-3′ (Xmal site in restriction site in italics) and the M13 rev standard primer (−26). Pfu DNA polymerase (Stratagene) or Taq polymerase (Invitrogen) were used for PCR. Introduction of the HA tagging-encoding sequence in TAT1 was verified by restriction digestion and sequencing. The final product was labeled pCAW16 (Table II). Functionality of the HA-tagged TAT1 protein was checked by testing the ability of the cells containing the construction to grow in the presence of 30 μM FTY720.

HA-TAT2-tagged protein cloned into the 2-μ-based vector YEplac195 was kindly given by M. N. Hall (22).

Amino Acid Uptake—The rate of import of radiolabeled tryptophan and leucine in the absence and presence of FTY720 in the JK9-3ds and the bul1 mutant strains were measured as described previously (23). Cells were grown to early logarithmic phase in YPD medium and divided into two cultures. FTY720 was added to one of the two cultures at a final concentration of 30 μM. An equivalent volume of water was added to the control culture. The cultures were incubated with the drug for 4 h at 30 °C. Aliquots (3 x 0.5 ml) of the import reaction mixtures were withdrawn at time points 0, 5, 10, 20, 30, 45, and 60 min. The bound radioactivity was quantified using a scintillation counter and corrected for differences in cell density (optical density at 600 nm).

Protein Analysis—To prepare whole cell extract for SDS-PAGE and Western analysis, cells were grown and treated for 1 h with 30 μM FTY720 in YPD medium to early logarithmic phase, re-suspended in ice-cold extraction buffer (120 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) and lysed with glass beads in a bead-beater with five pulses of 30 s. Unbroken cells and debris were removed by a short spin at 500 × g, and protein concentrations were determined using the BCA Protein microassay (Pierce). Samples were denatured at 37 °C for 15 min, and a

The abbreviations used are: HA, hemagglutinin; PBS, phytosphingosine; PB, phenylbutyrate.

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**Table I**

| Strain | Genotype | Source/Ref. |
|--------|----------|-------------|
| JK9-3da | his4 leu2 trp1 ura3 MATα | Our collection |
| MH338—2a α | Complete protophot (random integration of URA3) MATα | This study |
| 27064 8,000 (*) | trp1 ura3* MATα | 59 |
| 27061b (*) | trp1 ura3 UBP5 MATα | 59 |
| MH272–Id a/s 3432/1 | his4 leu2 trp1 ura3 bul1::kanMX4 MATα | M. N. Hall (unpublished data) |
| Jtp5 lcb4 a | his4 leu2 trp1 ura3 lcb4::hygromycin MATα | This study |
| JK9-3d lcb5 a | his4 leu2 trp1 ura3 lcb5::kanMX4 MATα | This study |
| JK9–3d lcb4 lcb5 a | isd leu2 trp1 ura3 lcb4::hygromycin lcb5::kanMX4 MATα | This study |

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1 The abbreviations used are: HA, hemagglutinin; PBS, phytosphingosine; PB, phenylbutyrate.
Mechanism of Action of FTY720 in Yeast

RESULTS

FTY720 Inhibits Yeast Growth; Overexpression of the Genes TAT1, TAT2, CAJ1, UBP5, and UBP11 Rescue FTY720-induced Growth Inhibition—The minimum concentration of FTY720, which potently inhibited cell growth was 30 \( \mu \text{M} \) (Fig. 1). We then transformed JK9-3a strain with three independent yeast genomic DNA plasmid libraries and screened FTY720-resistant colonies by selection on SD(-URA) plates containing 30 \( \mu \text{M} \) FTY720. From these transformations, roughly 200 resistant strains were isolated. Genomic inserts from each resistant colony were isolated and sequenced for comparison with the yeast genome. Overlapping multicopy plasmids were grouped yielding four independent genes, TAT1, CAJ1, UBP5, and UBP11, and subcloned as single gene in the initial plasmid. TAT2 was identified in a subsequent screening in rich medium (data not shown).

TAT1 and TAT2 are low and high affinity tryptophan transporters, respectively (17, 18). CAJ1 is the yeast homologue of the  \( E. \text{coli} \) DnaJ heat shock protein (25) with as yet unknown function in yeast. UBP5 and UBP11 are ubiquitin C-terminal hydrolases of the de-ubiquinating enzyme family. These are reported to cause protein stabilization by the release of ubiquitin from target proteins. Conversely, these ubiquitin-specific proteases are proposed to stimulate protein breakdown by providing free and re-usable ubiquitin (26). The function of these two enzymes has not yet been clearly established in yeast.

Dominant Mutation in the GNP1 Gene Confers Resistance to FTY720-induced Growth Inhibition—In addition to screening multicopy suppressor genes, we screened and isolated genomic mutants that showed resistance to FTY720-induced growth inhibition (see “Experimental Procedures”). Resistant strains were sorted as containing dominant or recessive mutations by back-crossing with the parent strain of the opposite mating type. Of these, one dominant mutation was characterized. To identify the mutant gene, a genomic DNA library was constructed from the mutant strain in the CEN-based vector pCAW1 (pRS416) and transformed into the sensitive parent strain. Plasmids from the resulting resistant clones were isolated and sequenced. The plasmids causing FTY720 resistance contained a gene encoding the glutamine transporter GNP1 (27) with a single missense mutation at position 239 (W239L) (Fig. 2A). Analysis of this mutation using PEPPLOT software (Genetics Computer Group) predicted a change in the secondary structure of the protein (Fig. 2B). A mutation of the same residue (W239C) was previously demonstrated to alter the substrate specificity from glutamine to l-citrulline (28). Because this mutation is dominant for FTY720 resistance, the possibility that the mutation causes loss-of-function was excluded.

Disruption of the BUL1 Gene Confers Resistance to FTY720-induced Growth Inhibition—The recessive mutants resistant to FTY720 were classified into complementation groups to simplify the task of mutation characterization. The majority of mutants mapped into a single complementation group. From this group we focused on a single mutant for further study. Characterization of the mutation responsible for FTY720 resistance by genomic cloning identified BUL1 gene as the gene responsible for phenotype reversal. The mutation in this mutant turned out to be a partial deletion of the gene that started upstream from the promoter and extended roughly to the middle of the gene. Subsequent generation of a BUL1 gene-disrupted strain confirmed that the loss of function of BUL1 was responsible for resistance to FTY720-induced growth inhibition (Fig. 3A).

Initially isolated as a binding protein of ubiquitin ligase RSP5 (29), BUL1 is proposed to be involved in specifying ubiquitination substrates by binding RSP5 ligase under specific conditions (30).

Reduction of Expression of RSP5 Confers Resistance to FTY720-induced Growth Inhibition—Because of the increasing evidence that the ubiquitin pathway is involved in the mechanism of action of FTY720, and because it is proposed to be the binding partner of BUL1, we determined whether a reduction in RSP5 expression would also lead to resistance to FTY720 (Fig. 3B). A mutant expressing ~10% of endogenous RSP5 was resistant to FTY720, confirming that the BUL1-RSP5 complex was important in mediating FTY720 resistance. Both loss-of-function of BUL1 and reduction of RSP5 would lead to an overall reduction in the number of BUL1-RSP5 complexes and, presumably, a loss of ubiquitin ligase specificity and/or activity.

UBI4 Disruption Confers Resistance to FTY720-induced Growth Inhibition—\( S. \text{cerevisiae} \) ubiquitin is a 76-amino acid protein encoded by four structural genes named UBI1, UBI2, UBI3, and UBI4. While each of these genes are reported to be expressed during yeast exponential growth, only UBI4 is shown to be strongly inducible by stress such as starvation, heat, and DNA-damaging agents (31, 32). For this reason, we

| Collection | Plasmid | Description | Source/Ref. |
|------------|---------|-------------|------------|
| pCAW1      | PRS416  | CEN URA3    | ATCC company |
| pCAW5      | pRS426  | 2-µ URA3    | ATCC company |
| pCAW8      | pRS426::TAT1 | 3.2-kb fragment containing TAT1 in pRS426 | This work |
| pCAW7      | pRS426::TAT2 | 3.7kb EcoRI fragment containing TAT2 in pRS426 | This work |
| pCAW8      | pRS426::UBP5 | 4.5kb Smal-SalI fragment containing UBP5 in pRS426 | This work |
| pCAW9      | pRS426::UBP11 | 3.4kb KpnI-XbaI fragment containing UBP11 in pRS426 | This work |
| pCAW10     | pRS426::CAJ1 | 1.9kb KpnI-HindIII fragment containing CAJ1 in pRS426 | This work |
| pCAW14     | PRS416::BUL1 | Subcloning of a 3.2kb EcoRI-XbaI fragment containing wild type BUL1 | This work |
| pCAW15     | PRS426::HA-TAT1 | 3.2kb XhoI-NsiI fragment containing HA-TAT1 in pRS426 | This work |
| pAS55      | YEplac181::HA-TAT2 | HA-tagged TAT2 2micron plasmid | 22 |

**TABLE II**

List of the plasmids that were used in this study
chose to focus our studies on UBI4. A UBI4 deletion strain was tested for reduced sensitivity to FTY720. This strain also showed resistance (data not shown), further demonstrating the importance of ubiquitination in sensitivity to FTY720.

Exposure to FTY720 Reduces Tryptophan and Leucine Uptake—Because the overexpression of two amino acid transporters conferred resistance to FTY720, we speculated that FTY720 would affect amino acid import. The uptake of radiolabeled tryptophan and leucine was measured over time in the auxotrophic parent and bul1-mutant strains in the absence and presence of FTY720 (Fig. 4). Importing amino acids were normalized to the number of cells as determined by optical density of the culture. A striking inhibition of uptake of both tryptophan and leucine was observed. The BUL1 mutant showed slightly improved uptake of tryptophan (27 versus 6.8% of untreated) and leucine (30 versus 13% of untreated) when compared with the parent strain.

FTY720 Induces TAT1, but Not TAT2, Protein Degradation—The parent, bul1, and ubi4 mutant strains were transformed with HA-tagged TAT1 and TAT2 genes. Total protein was extracted from nontreated and FTY720-treated cultures (1 h) and analyzed by Western blot using anti-HA antibody. TAT1 protein levels decreased after treatment with FTY720 in both the parent and the bul1 mutant strains as well as in the mutant that does not express ubi4 (ubi4Δ) (Fig. 5A). Curiously, TAT2 protein levels were increased in the parental strain following FTY720 exposure and showed enhanced level in the BUL1 mutant strain with and without exposure to FTY720 (Fig. 5B). This could be directly related to the loss of ubiquitination specificity presumed in the bul1 mutant strain. Without adequate BUL1-RSP5 complexes, TAT2 protein turnover could be blocked, leading to accumulation of the protein. The absence of UBI4 protein prevented the increase of TAT2 observed in both the parent and bul1 mutant strains upon treatment with FTY720. In addition TAT2 protein level was higher in the ubi4 mutant than in the parent strain, indicating a potential regulatory role of ubiquitin on TAT2 protein level. Although both TAT1 and TAT2 genes are high copy suppressors of growth inhibition by FTY720, only the level of TAT1 is reduced by treatment with the drug.

Auxotrophic Strains Are More Sensitive to FTY720-induced Growth Inhibition than Prototrophic Strains—Analyses of growth inhibition were repeated with a prototrophic yeast strain. Growth inhibition of the prototroph required greater than 2-fold higher concentration of FTY720 (Fig. 6). In addition, the sensitivity of the auxotrophic strain could be abolished by relieving auxotrophy with the introduction of the corresponding biosynthetic markers (data not shown). Tryptophan and leucine supplement in the medium, however, were not sufficient to rescue growth inhibition by FTY720. Last, the growth sensitivity of the prototroph was not rescued by introduction of the multicopy suppressor genes and mutations that reversed sensitivity of the auxotrophic strain at 30 μM FTY720 (data not shown). We therefore conclude that the targets described in the present study are only a subset of the proteins that are involved in the response of S. cerevisiae to FTY720 exposure. Further studies are under way to identify these additional targets.

The Sphingosine Kinases LCB4 and LCB5 Are Not Involved in the Action of FTY720 in S. cerevisiae—Recently, FTY720 has been shown to be phosphorylated by sphingosine kinase in mammalian cells in vitro (33). FTY720-phosphate has been proposed to act through S1P receptors. In yeast, two sphingosine kinases, LCB4 and LCB5, which can phosphorylate the sphingoid bases sphingosine, dihydrosphingosine, and phytosphingosine, have been identified (34). Therefore the sensitivity to FTY720 of mutants lacking LCB4 and LCB5 activity were tested. These mutants turned out to be as sensitive to FTY720 as the parent strain (Fig. 7), indicating that either FTY720 does not need to be phosphorylated in the yeast to mediate its effect or there are other kinases that phosphorylate the drug.

**DISCUSSION**

This study demonstrates that growth inhibition conferred by FTY720 in S. cerevisiae is reversed by the expression of multicopy suppressor genes TAT1, TAT2, CAJ1, UBP5, and UBP11, and by mutation in the genes for BUL1, RSP5, and UBI4. Excepting the heat shock protein CAJ1, these genes are involved in either amino acid uptake or the ubiquitination pathway. We therefore envisioned a simple model where nutrient permeases either through their overexpression or due to a blockade in their degradation rescue FTY720-mediated growth inhibition (Fig. 8). While both TAT1 and TAT2 overexpression rescue FTY720 sensitivity, yeast cells respond differentially to FTY720 with respect to the levels of these proteins. TAT2 protein levels increase in FTY720-treated cells while TAT1 levels decrease. This suggest that TAT1-modifying proteins are different from those of TAT2 and BUL1 could be the target protein involved in the regulation of TAT2. In addition, TAT2
has been reported to be specifically ubiquitinated and targeted to the vacuole for destruction upon cell starvation in a process involving RSP5 (22). RSP5-induced ubiquitination is proposed to be required for not only targeting of proteins to the vacuole for destruction, but also the first step of the endocytic pathway, the internalization step of surface proteins such as pheromone receptors, transporters, and nutrient permease (40). BUL1, however, is not specifically responsible for turnover of TAT1, as the BUL1 mutant showed degradation of this protein equal to parental strain. BUL1 homologues such as BUL2 may be responsible for this process.

The observation that absence of UB14 by gene deletion and the down-regulation of RSP5 ligase contributes to survival in the presence of FTY720 indicate that enhanced ubiquitination is involved in the growth inhibition mediated by FTY720. Because of its lipid-like structure, FTY720 may also induce changes in the membrane lipid composition leading to an increased endocytosis of cell surface proteins. It has been shown that lipid rafts influence the assembly of the yeast plasma membrane H^+/H_1 ATPase PMA1 protein and a class of mutants of PMA1 was isolated in which the protein was exported from the ER but targeted for vacuolar degradation instead of delivery to the plasma membrane (41, 42). Thus, in addition to enhancing ubiquitination through proteins such as BUL1 and RSP5, FTY720 may preferentially direct these transporters to degradative pathways in lieu of assembly at the plasma membrane. While a consequence of ubiquitination can ultimately be degradation, it could also be that ubiquitination is sufficient to sequester these molecules in nonfunctional compartments providing the toxicity.

The heat shock protein homologue CAJ1 may also be reducing protein degradation by playing a chaperone role with the proteins targeted for degradation. GNP1-dominant mutation may compensate for the effects of FTY720 by increasing nutrient uptake as a consequence of its change in specificity. It also cannot be ruled out that the GNP1 mutation is influencing the ubiquitin machinery as proteins carrying missense mutation are often favored substrates for protein degradation.
The sphingosine-like molecule PHS also inhibits growth of S. cerevisiae in a manner similar, but not identical, to FTY720 (45). For example, tryptophan amino acid supplements could revert PHS sensitivity in tryptophan auxotrophic strains (45), whereas amino acid supplements were not sufficient to revert FTY720 sensitivity. This indicates that FTY720 is not affecting the affinity of the transporters and is not in competition for the uptake of these amino acids. Like in PHS studies, we found that overexpression of the amino acid transporters TAT1 and TAT2 could rescue FTY720-induced growth inhibition. Most importantly, we have now identified other genes and proteins involved in the action of FTY720, and it remains to be shown whether these novel targets also play a role in PHS action.

While these experiments were in progress, two novel compounds, an antineoplastic agent phenylbutyrate (PB) and the volatile anesthetic isoflurane, have been shown to have in yeast similar effects to the ones we have observed with FTY720. The overexpression of Tat1, Tat2, or Trp1, a tryptophan biosynthetic gene, has been shown to reverse PB-induced growth inhibition of yeast (46) much like the results obtained with FTY720. Bul1 deletion also made cells resistant to PB. PB blocked tryptophan transport, and a mutated bul1 gene that conferred PB resistance restored tryptophan uptake in the presence of PB. However, unlike FTY720, a high concentration (5 mM) of tryptophan in the growth medium reversed the growth effects indicating some differences with FTY720. The observations made with the volatile anesthetic isoflurane (47) are also similar to PB effects. It is interesting to note that in the case of inhibition by PB, the bul1 mutant did restore transport, while with isoflurane and FTY720, the transport could not be fully restored but growth sensitivity was reversed. In our experiments the inhibition of transport of tryptophan is 15-fold lower in the treated parent, but is only 3.5-fold decreased in the bul1 mutant, in response to FTY720 compared with untreated samples. The values for leucine transport inhibition are also in the same direction, i.e. 16-fold in the parent but only 5-fold in the bul1 mutant. According to data published with isoflurane (47), it is possible that these changes are sufficient to rescue the growth in the mutant strain.

Besides nutrient import, amino acid permeases have also been proposed to behave as drug pumps similar to the ATP-binding transporters found to be involved in pleiotropic drug resistance in yeast (48). If this applied to FTY720 and the other drugs PB and isoflurane, Tat1 and Tat2 overexpression would lead to an increased export of the immunosuppressor from the cells making them resistant. We have observed that introduction of Tat1 or Tat2 on multicopy plasmids in the prototrophic strain did not confer FTY720 resistance (data not shown). Palmer et al. (2002) have also similarly reasoned against the exporter functions of these permeases with respect to isoflurane. These results are consistent with the observation that the Tat1-deleted strain is PB-resistant, thus arguing against the extracellular exporter functions of Tat1. However, we cannot completely exclude the role of these permeases in the import of the drugs and the growth inhibition associated through direct competition with the substrates and downstream modifications of permeases due to their association with these drugs.

It has recently been demonstrated that FTY720 shares structural homology to the natural mammalian sphingolipid sphingosine, is phosphorylated by sphingosine kinase, and binds with high affinity to four members of the sphingosine 1-phosphate family of membrane receptors (S1P1–4) (33, 43). The S1P receptors are a family of closely related G-protein coupled receptors that can, depending on cell type and S1P receptor expression, alternately stimulate or inhibit migration in vitro and can cause dramatic rearrangements of the actin cytoskeleton through small GTPase activation and calcium release. Yeast structural homologues of S1P are dihydrosphingosine phosphate and phytosphingosine phosphate. Although yeast homologues of the S1P receptors have not been identified, phosphorylated PHS binds with high affinity to the mammalian S1P receptors (44). In addition, yeast cells have the machinery that can potentially modify FTY720 to form FTY720-phosphate in a manner similar to the modification that occurs in mammalian cells. Nevertheless, if FTY720 needs to be phosphorylated to induce yeast growth inhibition, we showed that the responsible enzymes are not LCB4 and LCB5 sphingosine

![Mechanism of Action of FTY720 in Yeast](image-url)
Mechanism of Action of FTY720 in Yeast

kinases. FTY720-phosphate does not block growth of *S. cerevisiae* (data not shown), although it could not be excluded that it does not enter the cells.

Our findings in yeast can be extended to mammalian cells in several ways. Trypophan starvation has been proposed as a mechanism for T-cell starvation and tolerance in pregnancy and inflammation (49–51). Mammalian cells cannot synthesize tryptophan and exclusively need to import it from the extracellular environment. The yeast ubiquitin system is closely related to the mammalian system. Most of the enzymes and proteins have conserved homologues, including RSP5 and UBI4, although the BUL1 homologue has not yet been identified. Two mammalian homologues of the yeast ubiquitin ligation RSP5p have been reported so far: Ned4 and Itch. Mutation of the Itch locus induces some immune disorders, including inflammation of the lung, stomach, and intestine and enlargement of the thymus, spleen, and lymph nodes (52). This suggests an essential role for this E3 ligase and for the ubiquitination in the immune system regulation.

In animal cells, several membrane proteins have been shown to be ubiquitinated and degraded both in the vacuole and by the proteasome as recently reviewed (53, 54). Our results can also be extended to mammalian cells in a model that would involve FTY720-stimulated system for the degradation of GRK kinases involved in the de-sensitization of surface receptor acting in cell trafficking. GRK2 kinase was shown to be a target for ubiquitin degradation (55). Alternatively, the addition of ubiquitin need not always be associated with degradation. The ubiquitination of a lymphocyte homing receptor at its extracellular extremity has been demonstrated (56), presumably happening prior to the assembly of the protein. The modification has been associated with the binding of lysocymes to lymph node and a role for ubiquitin in cell adhesion was proposed. Therefore, FTY720-stimulated ubiquitination could also explain the increased lymphocyte homing observed upon treatment with the immunosuppressive drug. Recently, several examples of the regulatory role of ubiquitin in nonproteasome-mediated pathways have been documented (53, 54, 57).

In summary, FTY720 is a small sphingolipid molecule and has the potential to be modified in the eucaryotic cells by many intracellular enzymes that are conserved in evolution and thus has the potential to be modified in the eucaryotic cells by many pathways have been documented (53, 54, 57).

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