

Antifungal Activities of Antineoplastic Agents: *Saccharomyces cerevisiae* as a Model System To Study Drug Action

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INTRODUCTION

Much of the success of medicine over the past century in preventing and treating infectious diseases is directly attributable to the introduction of vaccines, improvements in sanitation and water quality, and antibiotics. However, compared to antibiotics for bacterial infections, advances in the treatment of established viral or fungal infections have been slower, in large part because the target cell is either an infected human cell or a eukaryotic cell similar in structure and function to mammalian cells. The problems in treating viral and fungal infections are in many ways similar to those faced in developing treatments for cancer. Moreover, the growing problem of drug resistance in fungal infections (25, 72, 179, 270, 339, 340) and in cancer chemotherapy are similar and in many cases involve overexpression of multidrug resistance pumps (5, 335). We review here the antifungal activities of antineoplastic agents and propose that existing and candidate chemotherapy agents, which have known potential to increase the risk of fungal infections, can paradoxically represent an excellent resource for the discovery of novel antifungal targets and agents.

Deep-seated, invasive mycoses have never been more com-

monly reported than over the last decade (6, 14). From the endemic mycoses and *Cryptococcus neoformans* to the human colonizing yeast *Candida albicans* and the ubiquitous mold *Aspergillus*, invasive mycoses have become a modern medical problem in the treatment of neoplasms (226, 326, 338). Two factors have produced a major contribution to this epidemic of fungal infections. First, the human immunodeficiency virus (HIV) pandemic enlarged the worldwide immunosuppressed populations and provided large populations of at-risk individuals for both neoplasms and these secondary fungal pathogens. Second, modern cancer chemotherapy with its known immunosuppressive qualities such as profound neutropenia and the prolongation of life with broad-spectrum antibiotics and a myriad of invasive catheters have added to the numbers of immunosuppressed or at-risk pool of patients.

With this background, we review in detail a major risk factor for fungal infections: antineoplastic agents. However, we also focus on the effects of antineoplastic agents as direct antifungal agents or their use in the development of antifungal targets. There have been no clinical studies which suggest that any of the antineoplastic agents presently used in clinical practice have a positive impact on either preventing or treating fungal infections. On the other hand, many of the targets for these drugs have homologs in the eukaryotic fungal pathogens, and a careful examination of these classes of drugs may lead to the development of novel selective antifungal drugs. An increase in the incidence of invasive mycoses has brought about a signif-

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icant expansion in the use of antifungal drugs in clinical practice and a need for new, broad-spectrum fungicidal agents that can be used prophylactically, empirically, and therapeutically in these immunosuppressed patients (103, 104, 121, 262). None of the presently available antifungal agents completely fill the medical need, since in some respect they have weaknesses in spectrum, potency, safety, or pharmacokinetics.

In addition, the development of both antifungal and antineoplastic agents can benefit from studies in model and pathogenic yeasts. These fungi have been valuable tools in our efforts to understand the mechanisms of action of certain drugs and identify the targets of these drugs and explore their unique actions in mammals and fungi. Remarkably, studies with the yeast *Saccharomyces cerevisiae* as a model system have provided invaluable insights into the actions of a diverse array of drugs and compounds with quite specific activities in both mammals and fungi. For instance, cytotoxic topoisomerase I inhibitors (camptothecin, topotecan, and irinotecan), immunosuppressants that block T-lymphocyte function (cyclosporin A, FK506, and rapamycin), the phosphatidylinositol (PI) kinase inhibitor wortmannin, the HSP90 inhibitor geldanamycin, steroid receptor antagonists including tamoxifen, and the angiogenesis inhibitors fumagillin and TNP-470 have been studied mechanistically in yeast. Furthermore, recent advances in genome sequencing, genome arrays, combinatorial chemistry, and the development of a novel yeast three-hybrid assay promise to further extend the utility of yeast as a drug discovery tool, both in the identification of candidate antifungal and antineoplastic agents and in the elucidation of their mechanisms of action. For many years, yeast has been touted as an ideal model eukaryotic cell; these recent findings reveal that yeast is a better model for mammalian cell biology than we might have ever dreamed.

TOPOISOMERASES AS TARGETS OF ANTINEOPLASTIC AND ANTIFUNGAL AGENTS

Topoisomerases are enzymes that control the topological state of DNA by introducing transient enzyme-bridged DNA breaks (single-strand DNA for type I and double-strand DNA for type II) that allow passage of DNA strands (330). Topoisomerase inhibitors stabilize the transient enzyme-DNA complexes, resulting in an inhibition of transcription and replication that ultimately leads to DNA damage and cell death (66, 195). Redinbo et al. recently solved the crystal structures of human topoisomerase I in both covalent and noncovalent complexes with DNA (279); a model for the interaction of the anticancer drug camptothecin with the human topoisomerase I-DNA covalent complex has been proposed (312) (Fig. 1).

Significant progress has been made on the potential for molecular modeling and development of topoisomerase I-specific drugs. Furthermore, the importance of topoisomerase I as a drug target is emphasized by the development of two new antineoplastic agents, topotecan and irinotecan, which target topoisomerase I and are being used for antineoplastic chemotherapy against a variety of different neoplasms. The topoisomerase II inhibitor etoposide is also in clinical use as an antineoplastic agent. Several reviews have covered either type I or type II topoisomerase inhibitors as antitumor agents (18, 328, 329). The success of topoisomerase inhibitors in anticancer chemotherapy in humans underscores the potential of fungal topoisomerases as targets for development of novel antifungal compounds.

There has been substantial work on the structure and function of topoisomerase I and II in fungi. In the yeast *S. cerevisiae*, topoisomerase I and II serve separate functions important

to cell transcription, replication, and recombination (111, 243). Topoisomerase II is essential for viability in *S. cerevisiae* (145), whereas topoisomerase I is not (111). Recent studies with the pathogenic basidiomycete *Cryptococcus neoformans* reveal that topoisomerase I is essential for viability (68). Previous studies with *C. albicans* (156) also suggest that topoisomerase I is essential in this common fungal pathogen. Because topoisomerase I is essential for viability in fungal pathogens, it is an ideal antifungal drug target.

Importantly, the fungal and mammalian topoisomerase I enzymes exhibit structural differences. The *C. albicans* and *Cryptococcus neoformans* topoisomerase I primary structures contain amino acid insertions not found in the mammalian enzyme (68, 317). These inserts are located in the linker domain of the fungal enzymes. For instance, in human topoisomerase I, the linker domain is 77 amino acids long, whereas in these two pathogenic fungi, the topoisomerase I linker domain consists of 155 amino acids. Redinbo et al. (279) have shown that human topoisomerase I is a multidomain enzyme that contains two conserved globular domains, the core and the COOH-terminal domains, which are essential for catalytic activity. Two other regions, the NH₂-terminal and the linker domain, are not required for catalytic or relaxation activity (279). The functions of the fungal inserts are not yet known but could possibly be exploited in the development of fungus-specific topoisomerase I inhibitors.

The structure of human topoisomerase I is of significant medical importance because this enzyme is the unique target of the antineoplastic drug camptothecin and its derivatives topotecan and irinotecan. Previous studies demonstrated that *C. albicans* and human topoisomerase I are differentially inhibited by several compounds, including quinizarin, 5-HIAA (5-hydroxy-1H-indole-3-acetic acid), and A-3253 (96, 97). One issue that should be addressed is the ability of these topoisomerase I inhibitors to traverse the fungal cell membrane.

We have examined the in vitro activity of camptothecin and a series of camptothecin derivatives against the *Cryptococcus neoformans* topoisomerase I enzyme; several compounds are potent inhibitors of the fungal enzyme. While irinotecan and topotecan are not very potent inhibitors of the *C. neoformans* enzyme, some camptothecin derivatives are extremely active (67). Irinotecan and topotecan are 10-hydroxycamptothecin analogs. Topotecan has a dimethylaminomethyl hydroxychloride group substitution at the 9 position, whereas irinotecan has a carbamate ester at the 10-hydroxy group. Although these substitutions increase solubility, topotecan is a weaker topoisomerase I inhibitor than camptothecin whereas irinotecan is inactive against topoisomerase I in cancer cell lines. Similarly, both of these compounds are inactive against yeast cells (MIC > 500 µg/ml). Other analogs, such as the 10,11-methylenedioxy derivative and the corresponding glycinate esters, potentiate the topoisomerase I-inhibitory activity of camptothecin analogs both in cancer cell lines and in yeast cells. However, only the glycinate esters are active against yeast cells (MIC < 6.25 µg/ml). These findings support the notion that the addition of the glycinate ester increases the transport of the compound into the cell (67).

Topoisomerase II has been less extensively studied as an antifungal target. The antineoplastic topoisomerase II inhibitor etoposide has weak antifungal activity for *Candida* species, with MICs of 50 to 100 µg/ml (288). However, the fact that topoisomerase II is essential in yeast and fungi makes it a potentially important target for the development of future selective inhibitors and possibly new antifungal drugs.

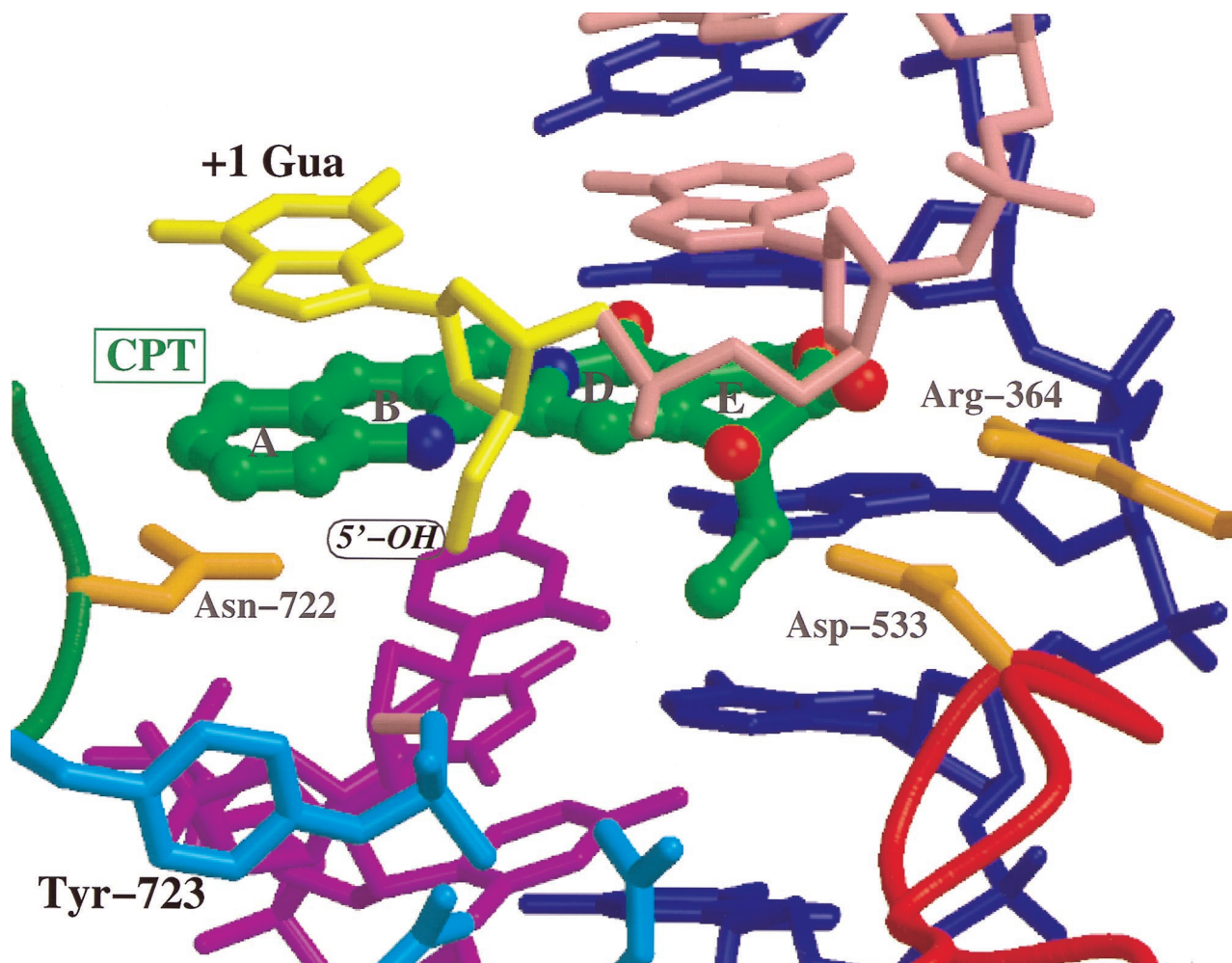


FIG. 1. Model of the human topoisomerase I-camptothecin interaction. A binding model of the three-dimensional structure of camptothecin (shown in green and labelled CPT) bound to the DNA-topoisomerase I complex is shown. Arg-364 and Asp-533 on the enzyme are hydrogen bonded to functional groups on the camptothecin E ring. A prominent feature of the model is that to accommodate camptothecin, the guanosine in the +1 position on the scissile DNA strand (labelled +1 Gua) is proposed to be flipped out of the DNA double helix. This could provide additional interactions, allowing the guanine ring to stack above the five-member ring system of the camptothecin molecule. This model will probably prove valuable in understanding the activity of camptothecin analogs and in developing novel analogs that specifically target human or fungal topoisomerase I enzymes. This figure is based on the X-ray structure of the DNA-topoisomerase I complex solved by Redinbo et al. (279) and was kindly provided by Matthew Redinbo and Wim Hol. It is important to note that this represents a binding model for the enzyme-drug-DNA complex and is not an experimentally determined binding mode for camptothecin association with the enzyme-DNA complex.

DIRECT ANTIFUNGAL EFFECTS OF CYTOTOXIC AND ANTIMETABOLIC CHEMOTHERAPY AGENTS

The direct effects of standard cytotoxic and metabolic anti-neoplastic agents against fungi have been studied *in vitro* by several investigators. The MICs of methotrexate, cyclophosphamide, vincristine, bleomycin, doxorubicin, and mitomycin C for three yeast strains (*C. albicans*, *C. tropicalis*, *C. kefyr*) are between 500 and 1,500 $\mu\text{g/ml}$ (108). In addition, bleomycin, carmustine, daunorubicin, doxorubicin, asparaginase, and 5-fluorouracil have MICs less than 100 $\mu\text{g/ml}$ by *in vitro* susceptibility testing (261). In fact, these compounds were always more active against *C. tropicalis* than against *C. glabrata* and *C. albicans*, and both 5-fluorouracil and asparaginase have MICs less than 1 $\mu\text{g/ml}$ for *C. tropicalis* (288). However, bleomycin is toxic to yeasts *in vitro* but has no antifungal activity in an experimental murine candidiasis model (115).

Direct antifungal activity of 5-fluorouracil against yeast is anticipated. Flucytosine is a known antifungal agent, in clinical use, which crosses the fungal cell wall and membrane and is

converted by cytosine deaminase to 5-fluorouracil within the fungus. This cellular pyrimidine is then incorporated into fungal RNA in place of uracil, resulting in a block to protein synthesis (267). The ability to use the less toxic precursor of 5-fluorouracil and to target a unique fungal enzyme has allowed flucytosine to be developed as an antifungal agent. Drawbacks of flucytosine as an antifungal drug are the ease with which resistance develops when it is used as a single agent and the limited spectrum of antifungal activity; nonetheless, flucytosine demonstrates the potential to exploit differences in fungal and mammalian metabolism. Another pyrimidine target for fungi and cancer chemotherapy has been dihydroorotate dehydrogenase. A series of 4-phenoxyquinoline agricultural fungicides which target this enzyme in mitochondria have been developed (51, 103, 125).

Despite the success of agricultural and medical fungicides such as benomyl and griseofulvin on tubulin formation in fungi (152), the microtubule aggregation inhibited by vincristine and vinblastine (288) and the disaggregation of microtubules by

paclitaxel appear to have little or no direct impact on fungal growth in vitro. In fact, paclitaxel antagonizes the activity of amphotericin B against fungi in vitro (230). On the other hand, depolymerization of microtubules by nocodazole produces a potent G₂-M cell cycle arrest in *S. cerevisiae*. There appears to be a definite difference in potency or activity between antifungal and antineoplastic agents at this target, which is similar to differences in antitubule drugs in their activity against certain tumors and fungi. For instance, griseofulvin can inhibit dermatophytes both in vitro and in vivo but has no impact on growth or survival of pathogenic yeasts such as *C. albicans*.

Ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis, is a favorite target of anticancer chemotherapy (220). For instance, eflornithine, which is an irreversible suicide inhibitor of ornithine decarboxylase, can inhibit the growth of *Cryptococcus neoformans* (265).

Leptomycins A and B, produced by *Streptomyces* sp. strain ATS 1287, are metabolites with both antitumor and antifungal activity. Interestingly, leptomycin B has in vitro activity against the commonly drug-resistant zygomycetes such as *Mucor* spp. and *Rhizopus* spp. In *Schizosaccharomyces pombe*, leptomycin B inhibits cell division, producing elongated cells with morphologically altered nuclei and cell plates (128, 129, 175, 176). Leptomycin B inhibits the cell cycle in both the G₁ and G₂ phases in rat fibroblasts and *S. pombe* cells (357). Furthermore, investigators performed bioconversion screenings with several bacterial and fungal strains to reduce the cytotoxicity profile of leptomycin B. Although the antiproliferative effect could be reduced by bioconversion, none of the metabolites possessed greater activity than the parental leptomycin B compound (180).

The target of leptomycin B has recently been identified as the Crm1 protein, (242), which is a component of the protein nuclear export complex consisting of Crm1, RanGTP, and proteins containing a nuclear export signal (95, 102, 254, 352). As a consequence, leptomycin B has a variety of different effects on proteins that shuttle out of the nucleus. For example, Wolff and coworkers have shown that leptomycin B inhibits HIV-1 Rev protein translocation and therefore may be useful as a novel antiretroviral therapy (180, 344). In addition, nuclear export is required for the degradation of the p53 tumor suppressor protein by either Mdm2 or papillomavirus E6-mediated mechanisms; leptomycin B blocks the nuclear export and degradation of p53, and might therefore serve to stabilize this tumor suppressor protein in some neoplastic cell types (100). The nuclear export of several other proteins involved in neoplastic transformation, including steroid receptors and the abl tyrosine kinase, are similarly inhibited by leptomycin B (314, 320). Thus, further studies of leptomycin B as a novel inhibitor of nuclear export via its action on the conserved Crm1 protein are warranted, and leptomycin B may find indications as a novel antineoplastic or antimicrobial agent.

Glidobactins A, B, and C represent a complex of new antitumor antibiotics produced by *Polyangium brachysporum*. A variety of glidobactin analogs which exhibit even better in vitro anticancer and antifungal activity have been isolated (217). However, the therapeutic activity of the main component A of these analogs against systemic experimental fungal infections was found to be marginal (247).

STUDIES ON CISPLATIN COMPOUNDS IN MICROORGANISMS

Cisplatin is a DNA-damaging agent that causes DNA strand cross-links and which has revolutionized the treatment of some solid tumors, most prominently testicular carcinoma. The ac-

tions of cisplatin have been previously studied in the yeasts *S. cerevisiae*, *Schizosaccharomyces pombe*, and *C. albicans*. Cisplatin was originally studied as a potential antimicrobial drug (280) and, along with two related metal-complex agents (palladium), was found to be a particularly effective inhibitor of mammalian cell proliferation (80, 173, 345). Studies have shown that these compounds can inhibit *C. albicans* when used at concentrations between 100 and 180 µg/ml (234). However, these levels are higher than those which could be achieved for any significant period in an infected host. In *C. albicans*, pretreatment of cells with the antifungal agent amphotericin B or miconazole increases their sensitivity to cisplatin in vitro, suggesting that these compounds might have synergistic antifungal activity in patients being given concomitant treatment (286), but clinical studies have yet to confirm any positive relationship.

Cisplatin has been used in studies on drug mechanisms in yeast. Studies in *S. cerevisiae* and *Schizosaccharomyces pombe* have focused on the toxic effects of cisplatin in DNA repair mutants and the mechanisms of repair of cisplatin-induced DNA lesions. The *SNM1* gene encodes a protein required for the repair of interstrand DNA cross-links induced by cisplatin and is transcriptionally induced in response to DNA damage by cisplatin or UV irradiation but not by methylating agents (346). Perego et al. examined the action of cisplatin and derivatives against a panel of 23 radiation-sensitive *Schizosaccharomyces pombe rad* mutants (260). Mutations in the *RAD1* and *RAD3* checkpoint genes and also in the *RAD18* DNA repair gene conferred marked hypersensitivity (28- to 58-fold) to cisplatin, whereas mutations in 15 additional *rad* genes conferred more modest (5- to 20-fold) sensitivity. These findings suggest that human tumors with specific checkpoint or DNA repair defects might be uniquely sensitive to cisplatin in comparison to nonneoplastic cells and, similarly, that if compounds that inhibit such activities were available, these agents might sensitize additional cell types to cisplatin toxicity.

Recent studies have identified a novel high-mobility group (HMG) DNA binding protein, Ixr1, that plays a central role in cisplatin toxicity in *S. cerevisiae*. The Ixr1 protein was identified by its ability to bind with high affinity to intrastrand DNA cross-links produced by cisplatin (34). Mutants lacking Ixr1 are viable and modestly resistant to cisplatin (twofold) and accumulate only one-third as many cisplatin-DNA cross-links as an isogenic wild-type strain expressing Ixr1 does. Subsequent studies revealed that Ixr1 binds with 10-fold-higher affinity to DNA containing cisplatin adducts than to undamaged DNA and that the Ixr1-DNA complex covers approximately 15 bp of damaged DNA (219). These studies suggest that the Ixr1 protein, or other DNA binding proteins, plays a role in the cellular responses to cisplatin, and may either cause toxic DNA-adduct-protein lesions, block the repair of DNA cross-links by other repair enzymes, or assist in the recognition and repair of DNA cross-links. Thus, specific inhibitors of cisplatin adduct repair enzymes might find use as novel antineoplastic or antifungal agents.

EFFECTS OF DRUG COMBINATIONS (ANTINEOPLASTICS AND ANTIFUNGALS)

Ghannoum et al. (109) took the examination of antineoplastic and antifungal agents even further. For instance, they performed interactive studies on the combination of antifungal and antineoplastic agents. Several findings were noted: (i) inhibitory drug combinations whose MICs were lower than those of the respective agents alone were found; (ii) interactive effects between antineoplastic and antifungal agents may be very

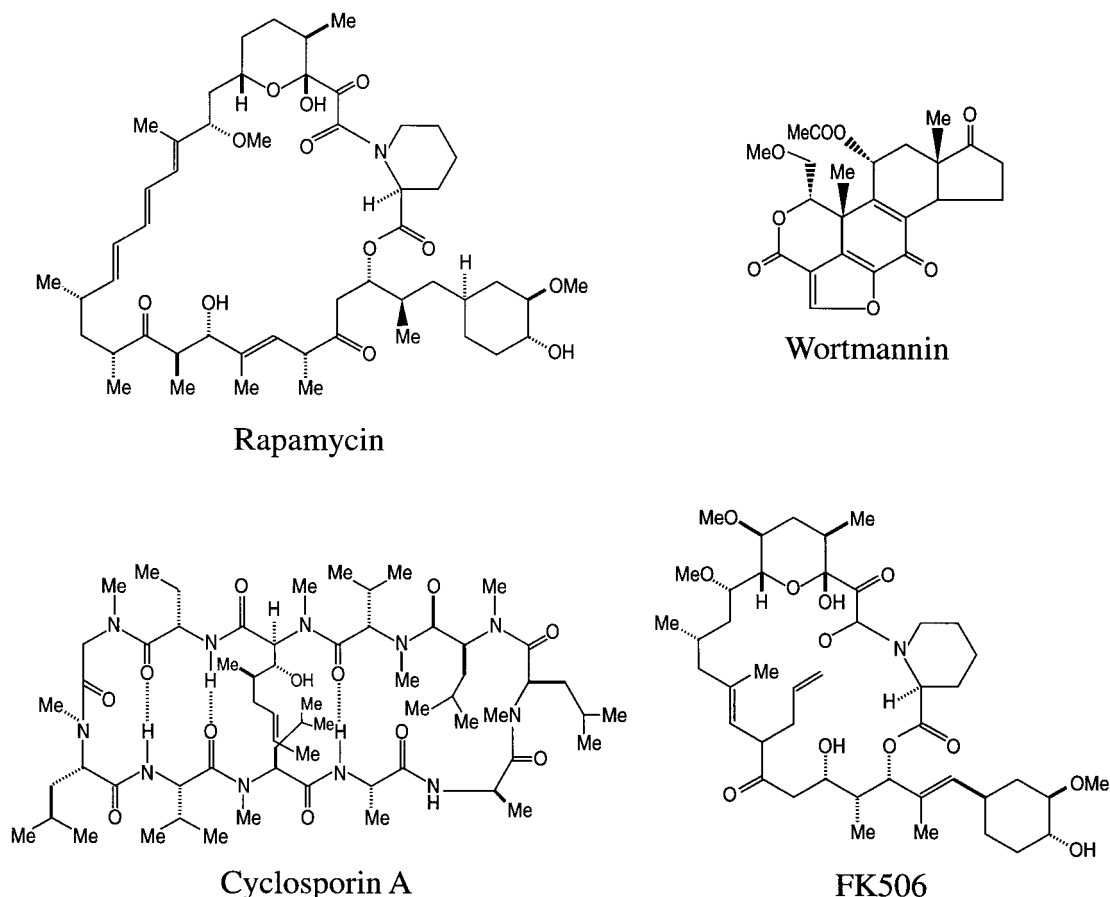


FIG. 2. Structures of rapamycin, wortmannin, CsA, and FK506. The molecular structures of several natural products that inhibit signal transduction cascades are depicted. CsA is a cyclic peptide, rapamycin and FK506 are structurally related macrolide antibiotics, and wortmannin is related to the steroids.

large; (iii) optimal combinations of drugs for yeast inhibition depend on both the relative and absolute concentrations of drugs in the mixture; and (iv) effective combinations were generally active against multiple fungal species, but the ratios of drugs may vary (109). In general, a polyene combined with an antineoplastic agent was the most potent combination against yeast growth. For example, the combination of amphotericin B and either methotrexate, mitomycin C, doxorubicin, or 5-fluorouracil had a positive interaction against yeast growth. This finding was partially explained by the membrane perturbation caused by amphotericin B, although it did not necessarily extend to another polyene, nystatin. While, as expected, 5-fluorouracil had the most positive antifungal interaction with amphotericin B, drugs such as cyclophosphamide and bleomycin antagonized the antifungal activity of the polyene (108).

The *in vitro* antifungal activity of camptothecin against yeast strains is greatly enhanced by mutations such as *erg6* that cause defects in cell membranes (67, 244). Thus, the use of certain drugs in combination with others could alter the cell membrane and enhance the antineoplastic and antifungal activity of antitumor drugs. For example, the use of amphotericin B and cisplatin together results in an *in vitro* synergistic antineoplastic effect against both sensitive and resistant ovarian carcinoma cells and human colon cancer cells (10, 166). In combination assays, amphotericin B can potentiate the activity of a few

antineoplastic agents against fungi (108), but the possible clinical relevance of this *in vitro* observation is not known.

RAPAMYCIN AND TORS IN THE YEAST, FUNGAL, AND MAMMALIAN CELL CYCLE

Rapamycin (Fig. 2) is a natural product with antimicrobial and immunosuppressant activities due to its ability to inhibit signal transduction cascades (reviewed in reference 44). Rapamycin is in late phase III clinical trials as an immunosuppressive drug for organ and bone marrow transplant recipients, and Food and Drug Administration approval is anticipated shortly. Rapamycin is also toxic to a number of tumor cell lines and thus may find additional indications as a novel chemotherapy agent. Finally, rapamycin is one of the most potent antifungal agents ever discovered, and nonimmunosuppressive analogs hold great promise as novel, potent, broad-spectrum antifungal agents (11, 324).

In both yeast and mammalian cells, the action of rapamycin is mediated by its association with a highly conserved binding protein, the peptidylprolyl isomerase FKBP12 (140, 174, 301). Remarkably, yeast mutants lacking FKBP12 are viable and resistant to rapamycin toxicity, indicating that both the protein and the drug are required for rapamycin action and providing strong support for a model in which the FKBP12-rapamycin complex is the active *in vivo* agent. Overexpression of FKBP12

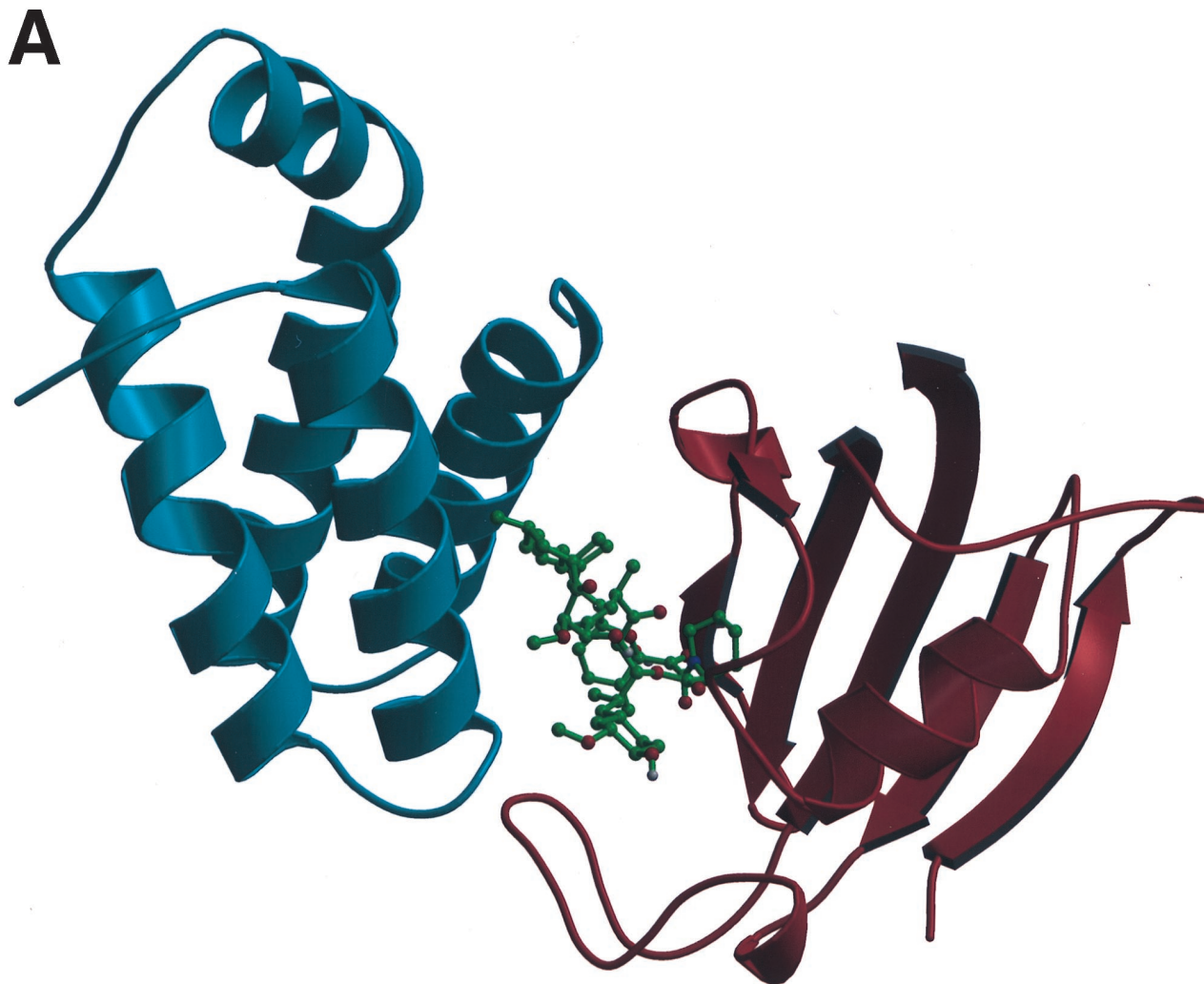


FIG. 3. Structures of the FKBP12-rapamycin-mTOR complex. (A) Structure of the ternary complex between the FKBP12-rapamycin binding domain on the FRAP/mTOR molecule (blue), rapamycin (green), and FKBP12 (red). (B) Detailed view of the FRB domain of FRAP/mTOR (blue) bound to rapamycin (green). Rapamycin binds into a hydrophobic pocket on the surface of TOR composed of the highly conserved residues Phe2108, Tyr2105, Trp2101, Phe2039, and Tyr2038 (side chains all in gold). These residues are conserved from yeast to humans and provide critical interactions with the inhibitor. This figure is based on the X-ray crystal structure of the FRB-rapamycin-FKBP12 complex solved by Choi et al. (54) and was kindly provided by Jon Clardy.

in mammalian cells increases sensitivity to rapamycin, and cell lines with reduced levels of FKBP12 are rapamycin resistant, providing evidence that FKBP12 is the conserved target of rapamycin action in yeast and mammals (28, 101).

The targets of the toxic FKBP12-rapamycin complex are novel kinase homologs, the TOR1 and TOR2 proteins in yeast and the mTOR protein in mammals, which are conserved from yeast to humans. Genetic studies in yeast, in which rapamycin-resistant mutants were isolated, first implicated the TOR1 and TOR2 gene products as targets of the FKBP12-rapamycin complex (140). Analysis of the cloned *TOR1* and *TOR2* genes revealed the potential to encode large (~280-kDa) proteins that have 67% overall identity (40, 141, 183). However, despite this remarkable similarity, TOR1 and TOR2 serve both shared and distinct functions. Deletion of the *TOR1* gene confers only a modest growth defect under most conditions, whereas deletion of *TOR2* is lethal. Subsequently, a mammalian TOR homolog (mTOR/FRAP/RAFT1/SEP/RAPT1) was identified by its ability to physically interact with FKBP12-rapamycin and was found to have ~50% identity to the yeast TOR proteins

(31, 52, 281, 282). The highest level of identity between the yeast and the mammalian TOR proteins is in the carboxy-terminal domain, which exhibits sequence identity to both lipid and protein kinases. The X-ray structure of FKBP12-rapamycin bound to a small portion of the mTOR protein (the FRB domain) has been solved (Fig. 3) (53). This structure revealed that rapamycin docks into a hydrophobic pocket on the surface of the TOR protein that has been highly conserved from yeast to humans. Few protein-protein contacts are apparent in the X-ray structure, but they should exist and play an important role in the complex, because rapamycin does not bind to the mTOR protein alone and thus FKBP12-TOR contacts should contribute to the ternary complex. Genetic studies had identified three residues that play a critical role in FKBP12-rapamycin binding to the yeast TOR1 and TOR2 proteins (205); these three residues, Ser1975, Trp2042, and Phe2049, are all conserved in the mammalian TOR protein and form the base and sides of the hydrophobic rapamycin binding pocket on mTOR (Fig. 3) (53).

The TOR proteins are members of a ubiquitous family of

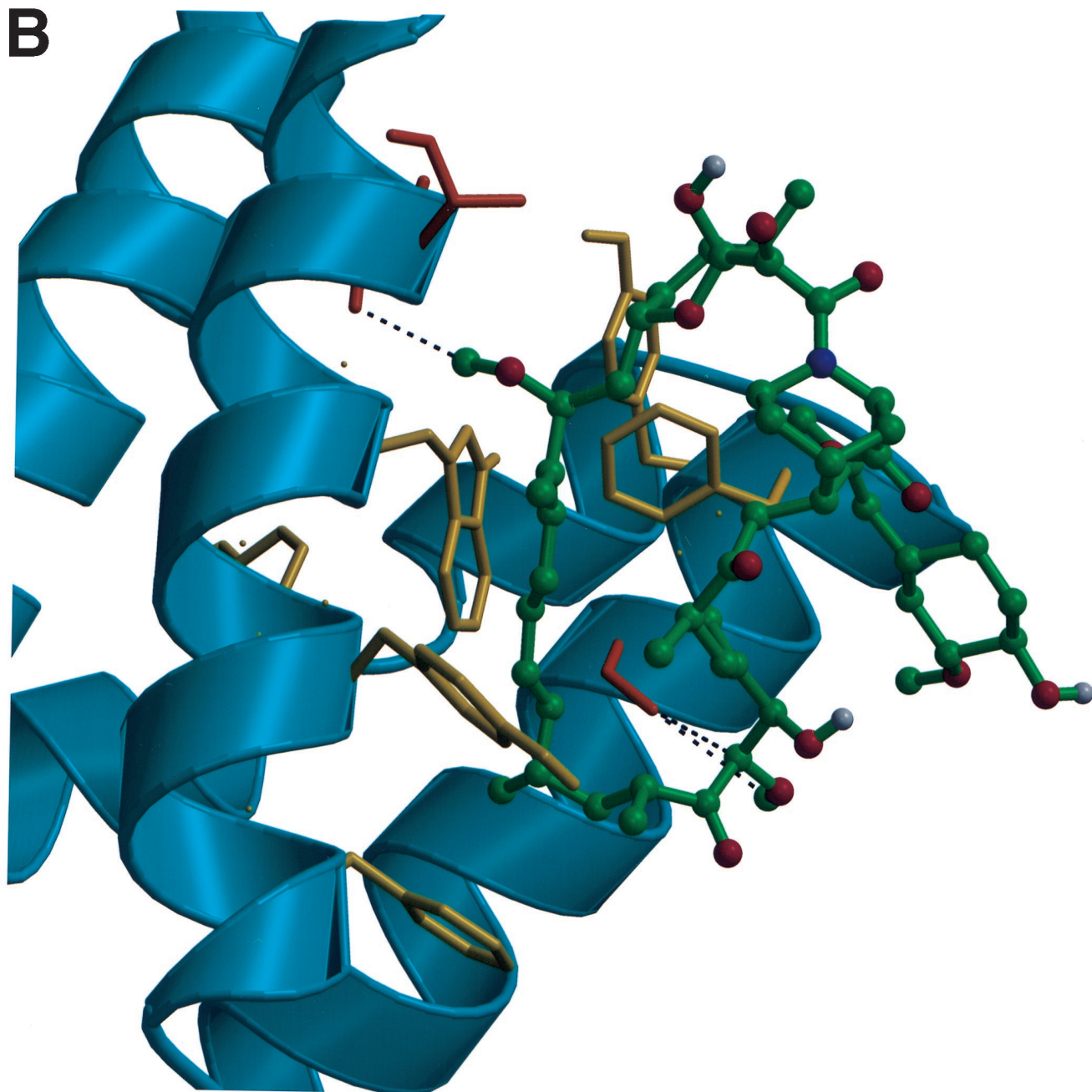


FIG. 3—Continued.

signalling proteins, which includes the yeast cell cycle check-point and DNA repair proteins TEL1, MEC1, and RAD3 and the lipid kinases PIK1 and STT4, the *Drosophila* MEI41 protein, and the mammalian ataxia-telangiectasia (AT) protein ATM and its related protein ATR, the catalytic subunit of DNA-dependent protein kinase, and several phosphatidylinositol 3-kinases (PI3-kinases), PI4-kinases (for reviews, see references 162 and 359). In general, these proteins play conserved roles in transducing signals, either in response to exogenous stimuli such as growth factors or in response to endogenous cellular signals such as DNA damage and telomere length.

Rapamycin is also toxic to a number of pathogenic yeasts and fungi, including *C. albicans*, *Cryptococcus neoformans*, and

Aspergillus fumigatus (59, 249, 347). In fact, rapamycin was originally discovered at Wyeth-Ayerst Pharmaceutical company in a screen for novel antifungal agents directed at *C. albicans* (304), and only much later was it appreciated to have potent and specific immunosuppressive activity, which is responsible for renewed interest in this compound. The FKBP12 homolog RBP1 has been identified and cloned in *C. albicans*, but studies demonstrating that this protein mediates rapamycin action in *C. albicans* have not yet been reported (89). Similarly, sequences derived from a TOR homolog are available in the systematic *C. albicans* genome-sequencing project, but no molecular studies have been published.

Rapamycin is also toxic to the opportunistic fungal pathogen

Cryptococcus neoformans (249), and recently TOR1 and TOR2 homologs were identified and cloned based on homology to degenerate PCR (TOR1) and random sequencing of an expressed sequence tag (EST) database (TOR2) (59). Moreover, a *C. neoformans* FKBP12 homolog was cloned based on its ability to interact with the TOR1 FKBP12-rapamycin binding domain in a novel two-hybrid screen (59). Importantly, disruption of the FKBP12 encoding gene *FRR1* by homologous recombination revealed that in *C. neoformans*, as in *S. cerevisiae*, mutants lacking FKBP12 are fully viable and resistant to rapamycin and to FK506. Spontaneous rapamycin-FK506-resistant mutants were also found to harbor FKBP12 mutations that prevent protein expression (59). Finally, a spontaneous rapamycin-resistant mutant was shown to have a mutation in the conserved serine residue in the FKBP12-rapamycin binding domain of TOR1 that is required for drug action in *S. cerevisiae* and mammalian cells (59). Taken together, these studies reveal that the antifungal activity of rapamycin is mediated via conserved complexes with FKBP12 and TOR homologs in *C. neoformans*. Furthermore, these studies suggest that nonimmunosuppressive rapamycin analogs have potential as novel antifungal agents.

One function of the TOR proteins that is shared by both *S. cerevisiae* TOR proteins and their mammalian homolog mTOR/FRAP/RAFT1 is required for signalling translational initiation and thereby cell cycle progression from G₀ or G₁ to S phase (13, 17). The second essential function of the yeast TOR2 protein is the control, via the RHO1 and RHO2 GTPases, of polarized distribution of the actin cytoskeleton during cell cycle progression (293, 294); however, it is not yet known whether this function is conserved in mammalian cells. The precise roles of the TOR proteins in either function are not yet well understood, and both the relevant substrates for TOR kinase activity and the upstream regulatory elements in these pathways are just beginning to be identified.

Genetic studies reveal that integrity of the TOR kinase domain is essential for TOR in vivo function in yeast and mammalian cells. Thus, TOR1 and TOR2 kinase-inactive mutants fail to complement *tor1* or *tor2* mutations in yeast (43, 360), overexpression of TOR kinase-inactive mutants is toxic in yeast (4, 360), and TOR kinase-inactive mutants are unable to function in a mammalian cell transfection assay involving TOR-dependent, rapamycin-sensitive activation of the p70 S6 kinase (32). The mammalian TOR protein (mTOR) is capable of autophosphorylation on serine residues, and this activity depends on the integrity of the kinase domain and is inhibited by both FKBP12-rapamycin and wortmannin (32, 36). Moreover, studies in which yeast TOR-mTOR chimeric proteins were expressed in wild-type or *tor* mutant *S. cerevisiae* strains revealed that the function of the kinase domain has been conserved between yeast and humans and that the mTOR kinase domain can regulate rapamycin-sensitive cell cycle progression in yeast cells (3). Earlier studies with rapamycin implicated the translational regulators p70 S6 kinase and PHAS-I as components functioning downstream of TOR (196, 327). Recently, mTOR was shown to phosphorylate PHAS-I and thereby mediate its dissociation from eukaryotic initiation factor 4E (eIF-4E) (35, 39) (Fig. 4). Dissociation of PHAS-I from eIF-4E is a crucial step toward activating translational initiation of certain mRNAs (reviewed in reference 188).

Phosphorylation and activation of p70 S6 kinase is mitogen regulated and rapamycin sensitive (327). Interestingly, phosphorylation of p70 S6 kinase by mTOR in vitro at residues that are phosphorylated and rapamycin sensitive in vivo has been observed (39). Collectively, these observations support the view that in mammalian cells, TOR control of translational

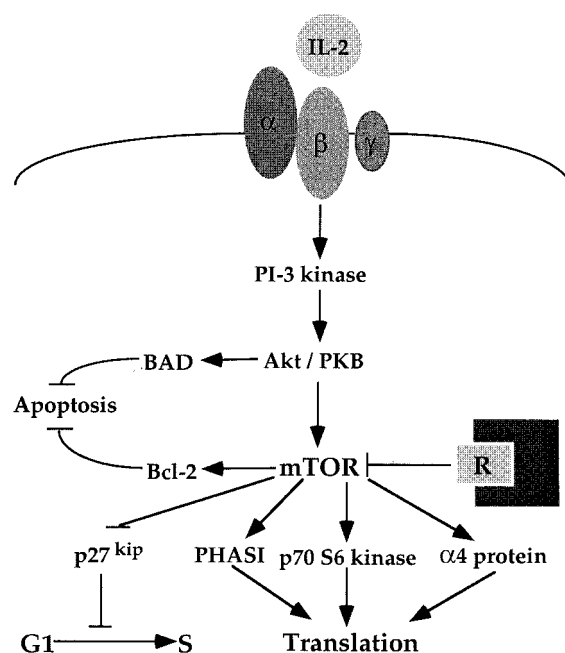


FIG. 4. Rapamycin mechanism of action. Rapamycin inhibits a conserved signaling cascade that drives cell proliferation in response to interleukin-2 (IL-2) and other cytokines. The target of rapamycin kinase (TOR) functions to regulate the activities of the translational regulators PHAS-I and p70 S6 kinase. Signaling via the Tor cascade provides a proliferative signal that can in part prevent apoptosis. As a consequence, rapamycin promotes apoptosis under certain conditions.

initiation occurs via phosphorylation of PHAS-I and p70 S6 kinase (Fig. 4). Although TOR action in control of translational initiation appears very similar in yeast and mammalian cells, the molecular TOR effectors do not seem to be conserved. No obvious p70 S6 kinase homologue is apparent in the completed yeast genome sequence. We have detected a robust kinase activity intrinsic to the yeast TOR1 protein by using PHAS-I as the substrate (4). Similar to the mTOR kinase, TOR1 protein kinase activity is blocked by active-site mutations and is inhibited by FKBP12-rapamycin and wortmannin (4).

Another possible target of the TOR kinase signaling cascade is the phosphatase regulator TAP42 in *S. cerevisiae* and its homologue, the $\alpha 4$ protein in mammals (Fig. 4). Overexpression of TAP42 suppresses mutations in the protein phosphatase 2A (PP2A) subunits SIT4, PPH21, and PPH22 (75). Interestingly, the *TAP42* gene is essential, and studies of a conditional mutant reveal that TAP42, like TOR, is required for translation initiation (75). TAP42 forms a stable complex with SIT4 that is disrupted by either rapamycin or nutrient deprivation, and certain conditional alleles of *TAP42* confer modest dominant rapamycin resistance in yeast, possibly by stabilizing the TAP42-PP2A complex initiation (75). Recent studies suggest that the TOR signaling pathway in yeast regulates the activity of a nutrient-regulated kinase, Npr1, that regulates the stability of nutrient permeases on the plasma membrane in response to nutrient availability, and that TAP42 may also participate in this regulatory cascade (292). Finally, the TAP42 homologue in mammalian cells, $\alpha 4$, is a known phosphoprotein that associates with PP2A, and the stability of the $\alpha 4$ -PP2A complex is disrupted by rapamycin (150, 236). Further studies are required to define the role of TOR kinase activity in regulating PP2A signaling cascades that are conserved from *S. cerevisiae* to humans.

In both yeast and mammalian cells, the FKBP12-rapamycin complex inhibits G₁-to-S cell cycle progression, but the mechanisms of cell cycle arrest are as yet unknown. In mammalian cells, rapamycin inhibits several cell cycle-linked events, including mitogen-induced activation of p70 S6 kinase (55, 184, 273), cyclin-dependent kinase activation, retinoblastoma protein phosphorylation (208), and downregulation of the cdk/cyclin inhibitor p27 kip1 (246) (Fig. 4). However, cells derived from mice in which the p27 gene has been disrupted by homologous recombination are only partially rapamycin resistant, indicating that rapamycin can inhibit cell cycle progression by p27-independent mechanisms (208). On the other hand, mutations in the yeast PP2A phosphatase subunit SIT4 cause a G₁ cell cycle arrest similar to that due to rapamycin (88, 313), and this may result from inhibition of TOR-dependent TAP42-SIT4 activity by the FKBP12-rapamycin complex.

Growth factors such as cytokines and insulin trigger both mitogenic and antiapoptotic signals, providing a homeostatic balance between proliferation and cell death (276). In addition to its antiproliferative effects, rapamycin enhances apoptosis in mammalian cells in response to growth factor or serum deprivation, Fas activation (228), dexamethasone (151), and cisplatin (299), indicating that mTOR is a component of a signal transduction pathway dedicated to survival. One of the immediate events upon stimulation by growth factors, cytokines, or insulin is the activation of PI3-kinase (reviewed in reference 47). A prominent downstream target of PI3-kinase is the activation of the serine/threonine kinase Akt (also known as protein kinase B) (99). Recently, overexpression and activation of Akt was shown to rescue cells from Myc- or Fas-mediated apoptosis (136, 163). Among other responses, stimulation by interleukin-2 and epidermal growth factor results in the transcriptional induction of the transcription factors c-Fos, c-Jun, c-Myc, and the antiapoptotic protein bcl-2. Interestingly, rapamycin blocks the induction of bcl-2 but not of c-Fos, c-Jun, or c-Myc (231). Furthermore, evidence has been provided that mTOR kinase activation by insulin is mediated by the PI3-kinase Akt pathway (298). While there are multiple relevant targets for the survival signal of Akt, including direct phosphorylation and thereby inactivation of the apoptotic factor BAD, it is possible that the antiapoptotic effects of Akt are mediated at least in part by mTOR. Finally, rapamycin has recently been discovered to markedly inhibit the growth of several pediatric brain tumors, notably medulloblastomas; thus, rapamycin may find additional clinical use as an antitumour drug, possibly in combination with current chemotherapy regimens. Collectively, these observations suggest that the TOR proteins play important roles in cell cycle control and proliferation by pathways that have been conserved from yeast and fungal pathogens to humans.

WORTMANNIN AS AN INHIBITOR OF PI3, PI4, AND PI-RELATED KINASES

Wortmannin is a hydrophobic estrogen-related fungal metabolite of the fungus *Talaromyces wortmanni* (Fig. 2). The in vivo anti-inflammatory and immunosuppressive effects shown by wortmannin first suggested that it was a potent inhibitor of signal transduction pathways (reviewed in reference 321). Wortmannin blocks cellular responses emanating from stimulation of G-protein-coupled receptors. For example, wortmannin inhibits neutrophil activation by a variety of ligands (252) and histamine secretion by basophilic leukemia cells (354). In addition, wortmannin blocks insulin stimulation of glucose uptake in adipocytes (251).

Several lines of evidence suggested that the relevant molec-

ular target of wortmannin in these effects is the lipid kinase that phosphorylates the 3 position of the phosphatidylinositol ring, PI3-kinase. Wortmannin blocks antigen- or insulin-dependent stimulation of PI3-kinase activity in rat basophilic and adipocytes, respectively (251, 252). In mammalian cells, PI3-kinase is a heterodimer composed of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit that interacts with other signal transduction elements via SH2 domains (160). The activity of PI3-kinase is potently inhibited in vivo and in vitro by wortmannin (251, 252, 311). Furthermore, by using anti-wortmannin antibodies and protease digestion, it was shown that wortmannin forms a covalent complex with an active-site residue of the 110-kDa PI3-kinase catalytic subunit, lysine 802 (351).

Although wortmannin potently inhibits the PI3-kinase with a 50% inhibitory concentration (IC₅₀) of 5 nM, more recent studies have shown that it also inhibits PI4-kinases. A wortmannin-sensitive membrane-associated PI4-kinase was identified and cloned in mammalian cells (227, 239). Demethoxyviridin, a structural analog of wortmannin, inhibits an unidentified membrane-associated PI4-kinase from the fission yeast *Schizosaccharomyces pombe* (IC₅₀ = 100 nM) (348). Interestingly, wortmannin is also toxic to the budding yeast *S. cerevisiae* (60). However, although wortmannin can inhibit the yeast PI3-kinase VPS34 in vitro at concentrations higher (IC₅₀ = 3 μM) than those required to inhibit the mammalian PI3-kinase (308), mutant yeast cells lacking VPS34 are viable and remain wortmannin sensitive (60). This result suggests that wortmannin toxicity in yeast is mediated via another target. These observations led to the identification of a wortmannin target in yeast as the PI4-kinase STT4 (60). Thus, overexpression of STT4 in yeast rescues cells from wortmannin toxicity. Moreover, STT4 PI4-kinase activity in vitro is sensitive to 10 nM wortmannin. The second PI4-kinase in yeast, PIK1, is resistant to wortmannin inhibition (60).

The inhibitory activity of wortmannin is not restricted to PI3- and PI4-kinases, and at higher concentrations wortmannin also inhibits several members of a novel family of PI-related protein kinases. These wortmannin-sensitive enzymes include the mammalian target of rapamycin mTOR (IC₅₀ ≈ 200 nM [36]) and yeast TOR1 (IC₅₀ ≈ 100 to 200 nM [4]), and also DNA damage control proteins including the human DNA-dependent protein kinase (DNA-PK) (IC₅₀ = 200 nM [a more recent study reported 16 nM] [287]), the ataxia-telangiectasia (AT) mutated (ATM) protein (IC₅₀ = 150 nM), and the ATM- and Rad3-related protein ATR (IC₅₀ = 1.8 μM) (132, 287).

Unreplicated and damaged DNAs are detected by checkpoints, which in turn trigger a cascade of cellular events including cell cycle arrest, DNA repair, and cell death or apoptosis (162, 233). The ATM, ATR, and DNA-PK proteins are components of the molecular machinery that senses and responds to DNA damage. Because DNA-PK is activated by binding to DNA double-strand ends, this kinase may play a proximal sensing role in initiating the checkpoint that responds to DNA damage. Consistent with this hypothesis, murine Scid (severe combined immunodeficiency) cells and the mutant Chinese hamster ovary cell line V-3, which are radiosensitive and defective in double-strand DNA break repair and VDJ recombination, also lack the DNA-PK catalytic subunit, whereas the murine XRCC5 mutant cell lines lack the Ku-80 component of DNA-PK (20, 26, 107, 169, 264, 278, 315). Moreover, the radiosensitive human cell line MO59J, which is defective in DNA double-strand break repair, also lacks the DNA-PK catalytic subunit (190). Interestingly, the human XRCC7 gene, to which the DNA-PK catalytic subunit maps, complements the defects in DNA double-strand break repair

and VDJ recombination in murine Scid cell lines (305). Substrates and possible effectors of DNA-PK include transcription factors such as the tumor suppressor p53, SP1, Fos, Jun, Myc, serum response factor, and the C-terminal domain of RNA polymerase II (reviewed in reference 7).

The clinical disorders displayed by AT patients, who carry a mutated ATM gene, underscore the crucial role of DNA damage checkpoints in the maintenance of genomic stability. AT individuals suffer a progressive cerebellar degeneration, immunodeficiency, radiation hypersensitivity, a higher incidence of certain forms of cancer, premature aging, and death during the second or third decade of life (38). Cells bearing a mutated ATM gene are extremely sensitive to ionizing radiation (158) and are defective in the G₁, S, and G₂ cell cycle checkpoints as well as in the p53 DNA damage response pathway (161, 165).

Wortmannin radiosensitizes CHO-K1 cells at concentrations of 1 to 20 μ M, which are not cytotoxic (27). Moreover, human tumor cell lines are sensitive to wortmannin treatment. The most wortmannin-sensitive cell lines are GC3 colon carcinoma, IGROV1 ovarian carcinoma, and CCRF-CEM leukemia cells, for which the IC₅₀ is in the range of 0.3 to 2.1 μ M (297). Other cancer cell lines, particularly breast carcinomas that overexpress the Her/neu or epidermal growth factor receptor, were refractory to wortmannin treatment (IC₅₀ > 50 μ M). The degree of sensitivity to wortmannin shown by these tumor cell lines may be a reflection of the oncogenic mechanisms operating in these different forms of cancer as well as of the different affinities with which wortmannin inhibits these mechanisms. Thus, enhanced proliferation resulting from perturbations in the response to extracellular signals such as growth factors or cytokines in which the PI3-kinase plays a role are likely to be more susceptible to wortmannin inhibition than perturbations in the mechanisms emanating from DNA damage responses mediated by the DNA-PK, ATM, and ATR kinases.

The resistance of cancer cells to DNA-damaging agents such as radiation is largely mediated by DNA repair processes. The ability of wortmannin to inhibit key DNA repair enzymes makes it a potential candidate for use in combination with ionizing radiation. In accord with this prediction, recent studies found that wortmannin potently inhibits DNA double-strand break repair and sensitizes cells to ionizing radiation (27). Furthermore, the concentration of drug required to render cells radiosensitive, 10 to 20 μ M for 10% survival, is in the range at which ATM and DNA-PK are inhibited by wortmannin (27, 287). In summary, the cytotoxic and radiosensitizing activities of wortmannin highlight its potential as an antineoplastic drug. However, the multiple molecular targets inhibited by wortmannin raise caution about its immediate use in human subjects. Thus, use of wortmannin as an antineoplastic or antifungal agent awaits the development of wortmannin derivatives with higher specificity for individual targets in both eukaryotic cell types. Crystallographic studies of wortmannin bound to its target, as well as more knowledge of the oncogenic mechanisms operating in different types of cancer and pathogenic fungal species, should prove useful in developing wortmannin-based strategies for anticancer and antifungal chemotherapy treatments.

CALCINEURIN IS THE CONSERVED TARGET OF CYCLOSPORIN A AND FK506

FK506 (tacrolimus) and cyclosporin A (CsA) are natural products of soil microorganisms that have potent immunosuppressive and antifungal activity (Fig. 2). CsA and FK506 were discovered to be potent inhibitors of mixed lymphocyte re-

sponses (24, 168) and have been widely used to treat and prevent graft rejection and graft-versus-host disease in solid-organ and bone marrow transplant recipients. Furthermore, both drugs recently have been shown to play a role in reversing multidrug resistance in several types of cancer by inhibiting the efflux of anticancer drugs. The mechanisms of action of these immunosuppressants have been extensively studied in the ascomycetous yeast *S. cerevisiae* as well as in T cells (for reviews, see references 33, 45, 46, 139, and 296). FK506 and CsA diffuse into the cell and bind intracellular receptors known as immunophilins, which catalyze *cis-trans*-peptidylprolyl isomerization, a rate-limiting step in protein folding (for reviews, see references 76, 139, and 291). FK506 binds to the immunophilin FKBP12, whereas CsA binds to the protein cyclophilin A. Although FK506 binds to the FKBP12 active site and inhibits prolyl isomerase activity, this is not the mechanism of immunosuppression in T cells or the toxic action in *S. cerevisiae*. Instead, the FKBP12-FK506 protein-drug complexes target proteins required for signal transduction and cell growth. The target of the FKBP12-FK506 and cyclophilin A-CsA complexes in *S. cerevisiae* and in T cells is calcineurin, a Ca²⁺-calmodulin-regulated serine/threonine-specific protein phosphatase (29, 94, 199).

Calcineurin, also known as PP2B, is a heterodimer protein composed of catalytic (CnA) and regulatory (CnB) subunits (172). The catalytic subunit has a molecular mass of 60 kDa and contains a calmodulin binding domain. The regulatory subunit is a 19-kDa member of the EF-hand family and contains four Ca²⁺ binding loops (2). Calcineurin activation requires both Ca²⁺ and calmodulin binding. Calcineurin has been characterized from several different organisms including *S. cerevisiae* (62, 63, 201, 356), *C. neoformans* (249), *Dictyostelium* (64), *Drosophila* (124), mouse (167), and human (122, 123, 235).

The X-ray crystal structures of the calcineurin AB holoenzyme and of the inhibited calcineurin AB-FK506-FKBP12 ternary complex have been solved (119, 171) (Fig. 5). These studies reveal that the FKBP12-FK506 complex binds to a hydrophobic groove that lies between the CnA catalytic subunit and the CnB regulatory subunit, making contacts with both subunits of the heterodimeric enzyme (Fig. 5). These observations are in accord with previous genetic and biochemical studies which had revealed the importance of both the CnA (42) and CnB (191, 229) subunits in inhibition of calcineurin by FKBP12-FK506 and cyclophilin-CsA (Fig. 5).

In T cells, calcineurin is activated in response to an increase in the level of intracellular Ca²⁺ that occurs upon stimulation of the antigen receptor pathway (reviewed in reference 44). Calcineurin is known to mediate T-cell activation by dephosphorylation of the NF-AT transcription factor, which unmasks its nuclear localization signal and thus promotes NF-AT translocation into the nucleus, where it induces a number of genes in response to cytokine activation (154, 245). Other proposed functions of calcineurin in mammalian cells include roles in apoptosis (355), neutrophil migration (144, 189), and other pathways regulated by Ca²⁺, such as neuronal growth (50, 90; for a review, see reference 143).

Why are CsA and FK506 specific for calcineurin, and why are calcineurin inhibitors relatively specific for T cells in a complex organism? First, while the CnA catalytic subunit is homologous to other protein phosphatases, the CnB subunit is unique to calcineurin. Because FKBP12-FK506 and cyclophilin A-CsA binding to calcineurin is mediated in part by the CnB subunit, this renders these inhibitors highly specific for calcineurin and not for other related phosphatases. Second, FK506 and CsA are relatively specific for T cells because T

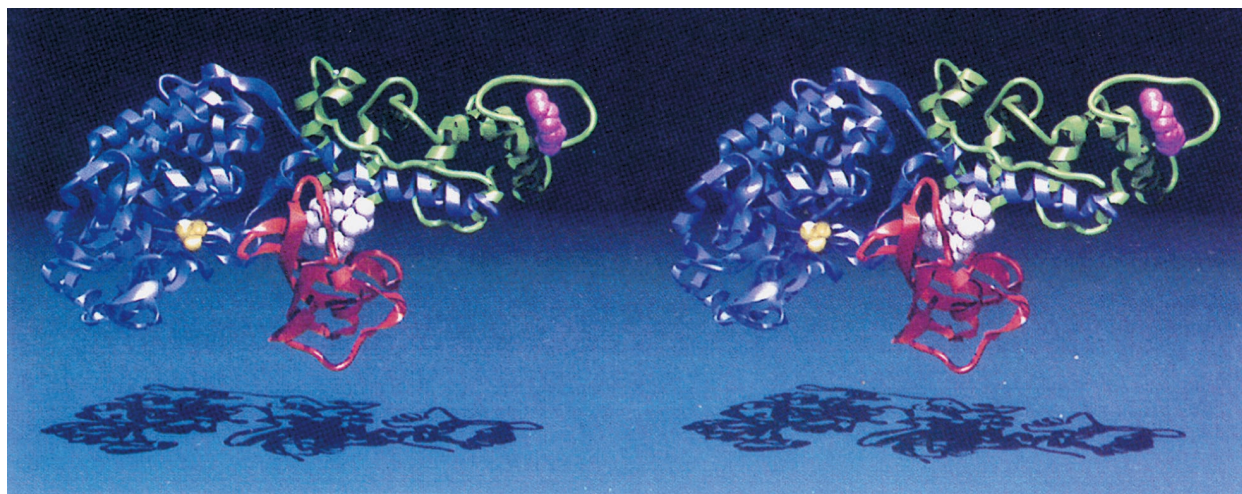


FIG. 5. Structure of the FKBP12-FK506-calcineurin complex. The ternary complex between CnAB, FK506, and FKBP12 is depicted. Calcineurin is a heterodimer, composed of the CnA catalytic subunit (blue) and the CnB regulatory subunit (green). A molecule of phosphate bound in the calcineurin active site is shown in yellow, and the N-terminal myristoyl group on CnB is shown in purple. FKBP12 (red) bound to FK506 (grey) binds in a hydrophobic groove composed of an extended α -helical arm of CnA and regions of CnB. Note that the FKBP12-FK506 inhibitor complex does not bind in the active site of calcineurin but probably inhibits by occluding the docking of large protein substrates to the phosphatase. Modified from reference 119 with permission of the publisher.

cells have lower levels of calcineurin than do other tissues, and thus lower concentrations of inhibitor are required to block T-cell function. In addition, some isoforms of the calcineurin target NF-AT are relatively T-cell specific.

The mechanism of action of CsA and FK506 has been conserved from T cells to the yeast *S. cerevisiae*. In a similar way to T cells, CsA and FK506 bind to their respective yeast immunophilins and target the yeast homolog of calcineurin. Two genes encoding the catalytic and one the regulatory subunit have been characterized (62, 63, 182, 201, 356). Vegetative growth of the majority of *S. cerevisiae* strains is not sensitive to CsA or FK506. Even though calcineurin is not required for vegetative growth of yeast, it is required for yeast survival after prolonged exposure to pheromone (62, 63, 342). Furthermore, calcineurin mutation or inhibition confers hypersensitivity to Li^+ , Na^+ , Mn^{2+} , and other ions (86, 222, 240, 271). Calcineurin mediates the regulation of ion homeostasis and cell wall synthesis by activating the transcription factor *Crz1/Tcn1*, which in turn regulates the transcription of several genes that encode ion pumps or cell wall biosynthetic enzymes (including *FKS2*, *PMR2*, *PMCL1*, and *PMR1*) (218, 309). Finally, a variety of mutations that render calcineurin essential for viability have been identified, and these strains are sensitive to growth inhibition by CsA and FK506 (29, 142, 259, 318).

CALCINEURIN IS THE TARGET OF CYCLOSPORIN A AND FK506 IN PATHOGENIC AND OTHER FUNGI

The immunosuppressants FK506 and CsA are toxic to several fungi, including *Aspergillus fumigatus* (135), *Cryptococcus neoformans* (249), *Neurospora crassa* (318), and *Coccidioides immitis* (170). The action of CsA and FK506 in these organisms is mediated via conserved complexes with cyclophilin A and FKBP12 homologs. Calcineurin appears to be the conserved target, and its inhibition mediates the toxic activity of these compounds. The genes encoding calcineurin have been cloned from several fungi. In the opportunistic pathogen *C. neoformans*, calcineurin is essential for growth at 37°C, in 5% CO_2 , or at alkaline pH, conditions which mimic the human host environment. As a consequence, calcineurin is required for *C. neoformans* virulence in an animal model of cryptococcal

meningitis (249). As in *S. cerevisiae*, calcineurin controls Na^+ and Li^+ homeostasis in *C. neoformans*. On the other hand, calcineurin plays a role in the regulation of cell cycle in the pathogenic fungus *Aspergillus nidulans*, where it is essential for cell cycle progression at the G_1 -to-S-phase transition (277). Furthermore, calcineurin is involved in hyphal growth, and the regulatory subunit is required for normal vegetative growth in the ascomycete *Neurospora crassa* (177, 275). Calcineurin mutants of the fission yeast *Schizosaccharomyces pombe* are viable but have defects in cytokinesis, cell polarity, mating, spindle body positioning, and growth at 22°C (358). In summary, calcineurin plays an important role in normal growth, morphology, mating, and virulence in fungi.

Even though CsA and FK506 are toxic to pathogenic fungi such as *C. neoformans*, their immunosuppressive activity outweighs their antifungal potential because immunosuppression exacerbates fungal infection in animal models (263) and humans. However, nonimmunosuppressive CsA and FK506 analogs that retain some level of antifungal activity have been identified (249, 250). One such analog is the C-18-hydroxy C-21-ethyl derivative of FK506 (L-685,818), which is toxic to *C. neoformans* as a result of inhibition of calcineurin. Thus, drug analogs can capitalize on subtle structural differences between host and fungal targets to spare host immune function yet impair fungal growth. Because CsA and FK506 have been approved for use in transplant recipients, the development and implementation of analogs with a novel indication should be more rapid, given clinical and toxicity experience and approval of the parent compounds.

CYCLOSPORIN A AND FK506 ACTION ON MULTIDRUG RESISTANCE MECHANISMS

Multidrug resistance (MDR) is a generalized phenomenon in which cells develop resistance to chemically dissimilar compounds. One of the best-characterized mechanisms of MDR involves overexpression of ATP binding cassette (ABC) transporters. These conserved proteins function as drug efflux pumps and have been implicated in MDR in bacteria (8, 105, 268), fungi (12, 285), helminths (30), and human cancer cells (110, 112, 148, 193). In human cancer cells, P-glycoprotein

(Pgp) is the best characterized ABC transporter and is responsible for resistance to standard cancer chemotherapy regimens (for reviews, see references 16, 106, 113, and 197). Pgp is a 170-kDa membrane protein that consists of two similar parts, each with an ATP binding site and six membrane binding domains (159). In vitro, MDR mediated by Pgp can be reversed by the binding of several pharmacological compounds to Pgp, thereby blocking efflux of anticancer drugs. CsA and FK506 are known to bind to and inhibit mammalian Pgp (9, 69, 98, 153, 232, 269, 283). Furthermore, CsA has been used as an MDR reversal agent for acute leukemia and has shown promising preliminary results (198, 215).

Since the onset of the AIDS epidemic, the frequency of fungal infections has increased dramatically and the isolation of azole-resistant yeast strains has become more common (5, 335). Thus, there is an ongoing need for more effective antifungal therapies with reduced toxicity and reduced emergence of antifungal drug resistance. Antifungal drug resistance is mediated partially through an MDR mechanism involving several ABC transporters, including the CDR1 and CDR2 proteins characterized from *C. albicans* (272, 284, 285). CsA, FK506, or analogs may inhibit these fungal ABC transporters involved in antifungal resistance, since FK506 inhibits the *S. cerevisiae* ABC transporter PDR5 (82). In addition, two non-immunosuppressive CsA analogs that are potent inhibitors of mammalian MDR seem to have similar effects on lower eukaryotes, such as *Toxoplasma gondii* and *Plasmodium falciparum*; however, the binding of these analogs to mammalian or parasite Pgp has not yet been demonstrated (15, 302).

ANGIOGENESIS INHIBITORS: THE FUMAGILLIN TARGET METHIONINE AMINOPEPTIDASE 2 IS CONSERVED FROM YEAST TO HUMANS

The development of clinically important tumors involves an initial neoplastic transformation of a mammalian cell and also requires the recruitment of new blood vessels (angiogenesis) to supply the tumor with oxygen and nutrients. Interestingly, angiogenesis occurs mainly at sites of new tissue growth, and tumors actively secrete growth factors that trigger angiogenesis. Over 20 years ago, Judah Folkman suggested that novel chemotherapy agents might target angiogenesis to restrain tumor growth (92, 93). Recently, several natural products have been shown to inhibit angiogenesis in in vitro culture and animal models, and they show promise in animal studies as chemotherapy agents (21, 149, 258). These include fumagillin, a natural product of the pathogenic fungus *A. fumigatus*, the fumagillin analog TNP-470 which is in clinical trials, and ovalicin (Fig. 6).

Although fumagillin potently inhibits angiogenesis, the target of fumagillin was only recently identified. Early studies revealed that fumagillin and its derivative TNP-470 inhibited vascularization and caused endothelial cells to arrest in the G₁ phase of the cell cycle (1). Moreover, fumagillin inhibits early cell cycle events, including phosphorylation of the retinoblastoma protein, expression of cyclin E, and activity of the cyclin-dependent kinases cdk2 and cdk4. Recently, two groups took similar approaches to identify the target of fumagillin as a highly conserved enzyme, methionine aminopeptidase 2 (MetAP2) (117, 303). Both groups synthesized fumagillin affinity matrices or radiolabelled photo-cross-linking derivatives. These modified compounds were shown to retain the antiangiogenic activity of the parent fumagillin molecule against human vascular endothelial cells. Next, these affinity reagents were used to identify a 67-kDa fumagillin binding protein that was present in bovine brain or mouse embryo extracts. Binding

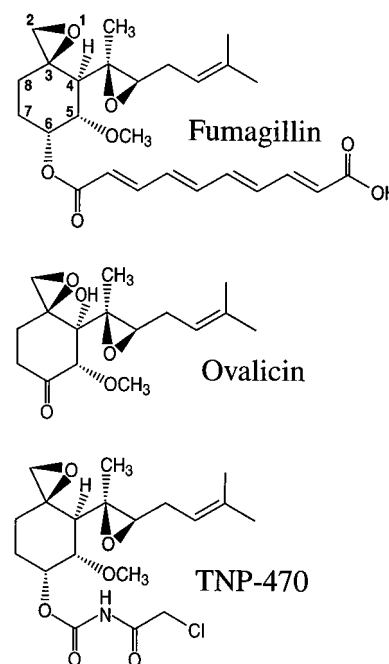


FIG. 6. Structures of angiogenesis inhibitors. The structures of the natural-product angiogenesis inhibitors fumagillin and ovalicin and of the derivative TNP-470, currently in clinical trials, are depicted.

to this 67-kDa protein was specific and was competed by unlabelled fumagillin. Amino acid sequence analysis by two approaches revealed sequences derived from a known protein, the enzyme MetAP2. Antisera to MetAP2 specifically recognized the 67-kDa fumagillin binding protein. Moreover, fumagillin derivatives and ovalicin bind covalently to MetAP2 and inhibit its activity with a tetrapeptide substrate in vitro. The abilities of a series of fumagillin and ovalicin analogs to inhibit MetAP2 enzymatic activity and endothelial cell proliferation were well correlated, providing additional evidence that MetAP2 is the relevant in vivo target (117). MetAP2 has a second activity, namely, the ability to inhibit phosphorylation of eIF-2 α by the heme-regulated inhibitor kinase. This activity is not inhibited by fumagillin, indicating that it is the aminopeptidase activity of MetAP2 that is the target for inhibition by fumagillin (117). Finally, the immunosuppressive and antiangiogenic activities of fumagillin analogs are strongly correlated, which suggests a unique role of MetAP2 in lymphocytes (319).

Remarkably, genetic studies with *S. cerevisiae* demonstrated that the relevant in vivo target of fumagillin and ovalicin is MetAP2 (117, 303). In previous studies, two MetAP enzymes were identified in *S. cerevisiae* (192). The two enzymes are encoded by the *MAP1* and *MAP2* genes. Individual *map1* or *map2* mutant cells are viable, albeit slow growing, whereas *map1 map2* double mutants are inviable, indicating that at least one isoform of the enzyme is essential for viability and that the two enzymes are at least partially redundant for an essential function (192). Most interestingly, *MAP1 MAP2* wild-type yeast strains expressing both MetAP1 and MetAP2 are not at all sensitive to fumagillin whereas *map1* mutants that lack the MetAP1 enzyme and express only the MetAP2 enzyme are sensitive to 50 nM fumagillin or ovalicin (117, 303). These studies demonstrate that fumagillin and ovalicin inhibit the MetAP2 enzyme in vivo and that MetAP2 is the relevant in vivo target for the cytotoxic effects of these angiogenesis in-

hibitors. Moreover, these genetic studies confirm that fumagillin and its derivatives do not inhibit the MetAP1 enzyme *in vivo*, because wild-type cells expressing both MetAP1 and MetAP2, or the *map2* mutant cells expressing only MetAP1, were resistant to fumagillin. While the target(s) of MetAP2 in yeast or mammalian cells required for viability is not yet known, these studies provide a genetic approach to define important cellular targets of MetAP2 *in vivo*.

Fumagillin binds covalently to the MetAP2 enzyme, and recent biochemical studies and the X-ray crystal structure of the fumagillin-MetAP2 complex (Fig. 7) reveal that the ring epoxide of fumagillin becomes covalently cross-linked to an active-site histidine residue (118, 200, 207). Lowther et al. found that fumagillin covalently modifies a conserved active-site histidine residue in the distantly related *Escherichia coli* MetAP enzyme (207). Recent studies revealed that His231 in the human MetAP2 enzyme is covalently modified by reaction with the ring epoxide of fumagillin (118). Finally, the X-ray structure of the fumagillin-human MetAP2 complex has recently been solved at 1.8-Å resolution (200). This structure reveals that the inhibitor is covalently linked to His231 in the active site and via a rich network of interactions with the ring portion of fumagillin but has essentially no contacts to the extended chain of the inhibitor (Fig. 7). Modeling provides insights into the specificity of fumagillin as an inhibitor of MetAP2 but not of the closely related MetAP1 enzyme, in which the target active-site histidine residue is not close enough to covalently cross-link to the drug and in which the drug binding pocket is narrower than in MetAP2 (200). These studies provide a structural basis for further design of MetAP2 inhibitors with antiangiogenic and immunosuppressive activities.

In previous sections we described the use of yeast as a model system to analyze the functions of drugs that specifically target T lymphocytes of the mammalian immune system. Now these studies reveal that yeast can also be used to identify and study the cellular targets of inhibitors of angiogenesis, a process that is highly specific to mammalian systems but is also linked to pathogenic fungi through an understanding in yeast.

What is the function of the MetAP2 enzyme in vascular endothelial cell proliferation and angiogenesis? While the specific function of MetAP2 required for angiogenesis has not yet been elucidated, there are several very interesting possible models. First, the primary function of this enzyme is to remove the initiator methionine at the amino terminus of proteins. This posttranslational processing event is required for myristoylation, a lipid modification to glycine residues that functions in protein targeting and stability. Proteins that are known to be N-myristoylated include the catalytic subunit of cyclic AMP-dependent protein kinase, the G α subunits of heterotrimeric G proteins, the regulatory B subunit of the protein phosphatase calcineurin, oncogenic protein kinases such as src, and the ADP-ribosylation factor that regulates protein synthesis. N-myristoyltransferase is known to be essential in *S. cerevisiae* and *Cryptococcus neoformans* (203), and differences between the substrate specificity of the *C. neoformans* NMT1 enzyme and the mammalian enzyme have been demonstrated, which could be exploited in the development of novel antifungal agents. Another function of N-terminal processing is to reveal the adjacent amino acid which then regulates protein stability by the N-end rule, in which following cleavage of the N-terminal methionine, the now penultimate amino acid residue regulates protein stability (323). Inhibition of MetAP2 would be predicted to stabilize proteins that are normally short-lived because of N-end rule-mediated degradation, and this might perturb endothelial-cell proliferation and angiogenesis. Third,

it is possible that one or more target proteins are nonfunctional when the amino-terminal methionine is simply not removed by MetAP1 or MetAP2. Finally, it is worth noting that the tissue distributions of MetAP1 and MetAP2 have not yet been determined, and endothelial cells might have relatively low levels of MetAP2 compared to other tissues, rendering them more uniquely susceptible to inhibition by fumagillin. Similar arguments have been proposed to explain, in part, the marked specificity of CsA and FK506 for T cells that have relatively lower levels of their common target protein, calcineurin (see above).

Fumagillin does not exhibit broad-spectrum antimicrobial activity, possibly because many organisms express both the MetAP2 fumagillin-sensitive target and the fumagillin-resistant MetAP1 enzyme. However, one early report revealed that fumagillin is toxic to a fungal disease of honeybees that is due to *Nosema* (164). More recently, the fumagillin analog TNP-470, which is in clinical trials as an angiogenesis inhibitor, has been shown to have potent activity against microsporidia, which are obligate intracellular parasites that cause diarrhea and wasting syndrome in AIDS and other immunocompromised patients (58). These findings suggest that further analysis of fumagillin and its analogs as novel antimicrobial agents, possibly with a highly selective spectrum of action, would be warranted.

HSP90-DEPENDENT STEROID RECEPTORS AND ONCOGENIC KINASES STUDIED IN YEAST: MECHANISMS OF ACTION OF GELDANAMYCIN AND ESTROGEN RECEPTOR ANTAGONISTS

The chaperone complex associated with the 90-kDa heat shock protein (HSP90) serves a dual function in the cell. First, HSP90 and associated partner proteins interact with heat-damaged proteins following heat shock and either promote refolding by inhibiting aggregation or target severely damaged proteins for degradation (295). Second, under normal cellular conditions, the HSP90 chaperone complex interacts with several proteins that require HSP90 for activity, including steroid receptors and oncogenic kinases such as src. For example, HSP90 binding maintains steroid receptors in a state competent to bind hormone. The HSP90 complexes consist of several proteins, including HSP70, p60, p48, p23, and a large immunophilin, which can be either cyclophilin 40, FKBP52, or FKBP54. Remarkably, the HSP90 chaperone complex has been conserved from the yeast *S. cerevisiae* to humans, and thus yeast genetic approaches can be used to elucidate the functions of the HSP90 chaperone complex with target proteins. Importantly, the HSP90-target complex is targeted by several different drugs with antineoplastic or antifungal activity, including geldanamycin (HSP90), CsA (cyclophilin 40), FK506 and rapamycin (FKBP52/54), and steroid receptor agonists and antagonists such as tamoxifen (estrogen receptor). For recent reviews of HSP90 functions and structure, see references 41 and 289.

Previous studies have focused on the heterologous expression of mammalian steroid receptors and the oncogenic kinase src in *S. cerevisiae* (49, 91, 209, 225, 266). Such studies have demonstrated that a variety of different steroid receptors can be expressed in a functional form and that ligand binding results in transcriptional activation of corresponding target reporter genes. These systems provide a powerful assay of steroid receptor function. Mutations that reduce the cellular levels of Hsp90 compromise steroid receptor function, providing direct evidence that Hsp90 is required for steroid receptor function (266). Moreover, Hsp90-associated proteins, includ-

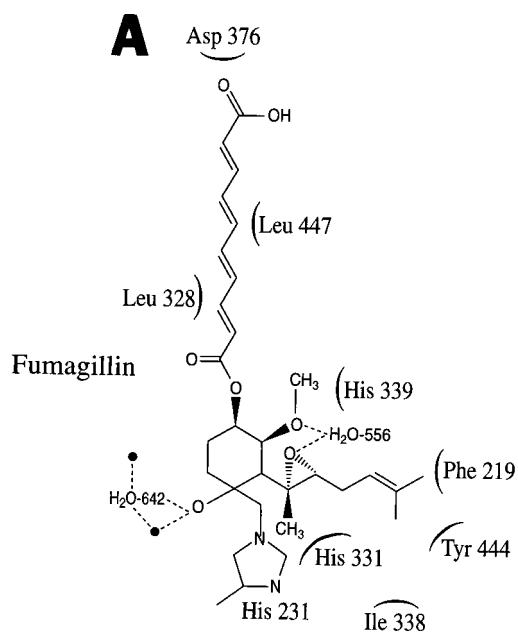
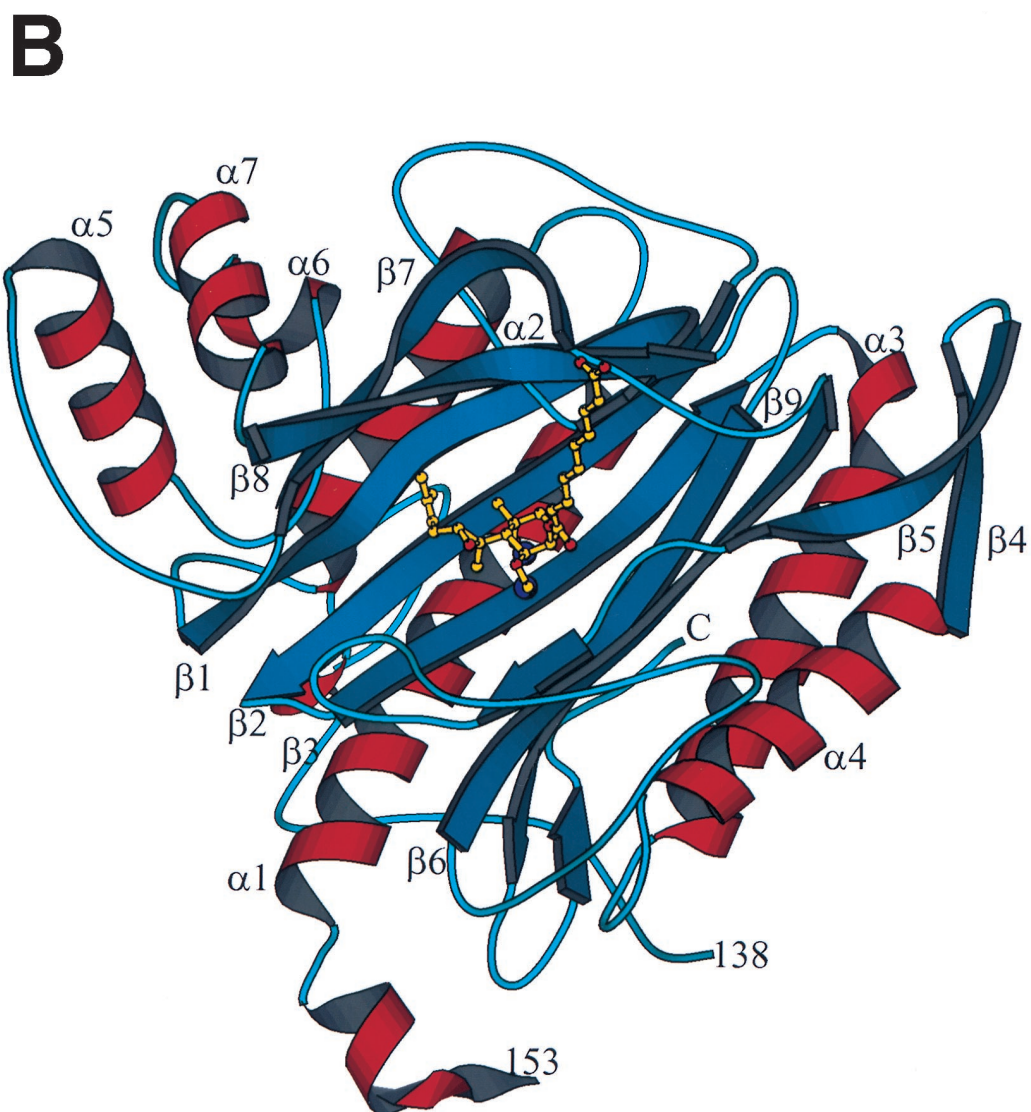


FIG. 7. Structure of the fumagillin-MetAP2 complex. (A) Schematic view of fumagillin bound to the active site of MetAP2. (B) Structure of the human MetAP2 enzyme (blue and red) and the bound molecule of fumagillin (gold). Notably, the active-site residue His231 is covalently linked to a reactive ring epoxide on fumagillin. This figure is based on the structure of the aminopeptidase-fumagillin complex solved by Liu et al. (200) and was generously provided by Maria C. Nonato and Jon Clardy.



ing Hsp70, p60/Sti1, and cyclophilin 40, are conserved in yeast (48, 78, 241) and are required both for steroid receptor function and for the stability of oncogenic kinases (49, 79).

Recent studies revealed that the novel antiproliferative agent geldanamycin inhibits HSP90. Geldanamycin is a benzoquinone ansamycin natural product of *Streptomyces hygroscopicus* subsp. *geldanus* that inhibits the proliferation of a wide variety of tumor cell lines. Initial findings revealed that geldanamycin inhibits the activity of several different protein kinases, but subsequent studies revealed this to be an indirect effect of HSP90 inhibition (336). The X-ray crystal structure of an HSP90-geldanamycin complex reveals that the drug binds in the N-terminal ATP binding site that has recently been defined on HSP90 (Fig. 8) (116, 274, 310). Geldanamycin binding inhibits HSP90-dependent ATPase activity (256). Geldanamycin also prevents binding of the p23 cochaperone to HSP90 and inhibits the release of substrates (22, 85). Yeast mutants lacking the yeast p23 homolog SBA1 are viable, and steroid receptor signaling is still sensitive to geldanamycin in *sba1* mutant cells, indicating that inhibition of p23 binding is not the only mechanism by which geldanamycin inhibits HSP90 functions (22, 85). Yeast mutants lacking the cyclophilin 40 homolog CPR7 are uniquely sensitive to reductions in HSP90 levels (79). We recently discovered that yeast *cpr7* mutants are hypersensitive to growth inhibition by geldanamycin (78). These findings suggest that further studies with yeast will provide a facile route to analyze inhibition of HSP90 by geldanamycin in vivo.

The use of the estrogen receptor antagonists tamoxifen and raloxifene in breast cancer treatment and prevention has generated considerable enthusiasm (253). Further studies of their novel antagonist and partial agonist actions on the estrogen receptors in different tissues are of paramount importance. A central question in the field is how a single agent, such as tamoxifen, can have beneficial antiestrogenic activities in the breast yet undesired estrogenic action in the endometrium. On the other hand, raloxifene exhibits antiestrogenic action in both tissues. There are several possible explanations. First, a second estrogen receptor has been recently discovered (181), and differing actions on the α and β forms of the estrogen receptor could provide an explanation for these disparate actions (255). Second, the estrogen receptors are associated with different adapter proteins in different tissues, or at different estrogen response elements in the genome (353), and these accessory proteins or sites of action could be responsible for differences in the actions of estrogen receptor ligands.

Studies with yeast provide an important model for steroid receptor functions and interactions with antagonists such as antiestrogens. For example, a variety of different steroid hormone receptors, including the α form of the estrogen receptor, have been reconstituted in yeast cells (221). These types of yeast model systems have been used to (i) define atypical estrogen response elements from the genome (65), (ii) study the actions of agonists and antagonists of the receptor (300), (iii) analyze the structure and function of the hormone binding domain of the human estrogen receptor (84, 349), (iv) demonstrate that the antiestrogen agent ICI 164,384 has no estrogen agonist activity with the α estrogen receptor expressed in yeast (19), and (v) isolate and analyze the functional status of the wild-type and mutant human estrogen receptors present in breast carcinoma cells, revealing that receptor variants with altered activity may be responsible for resistance to tamoxifen (322). These studies underscore the potential of yeast as a model system for further study of novel estrogen receptor ligands and promise to revolutionize the treatment and prevention of breast cancer with reduced undesirable side effects

in other tissues. Finally, it is worth noting that with prolonged therapy, many breast carcinoma cells either develop resistance to or become dependent upon tamoxifen for proliferation. Additional antagonists, such as raloxifene, may be particularly useful in treating tamoxifen-resistant or -dependent tumors.

Studies have also examined the possible effects of FK506 on the activity of steroid receptors. In this case, the power of yeast as a model system is revealed by studies that exclude, rather than demonstrate, interesting models of drug action. Previous studies revealed that FK506 could potentiate gene activation by the progesterone receptor expressed in *S. cerevisiae* (316). An FK506 analog that poorly inhibits calcineurin (15-*O*-desmethyl-FK520) failed to enhance progesterone signaling in yeast, leading the authors to suggest that calcineurin might be required to dephosphorylate and activate the progesterone receptor (316). However, FK506 continued to enhance signaling even in yeast mutant strains lacking FKBP12 (316), in which FK506 has no effects on calcineurin activity or function (29). The authors also suggested that FK506 might potentiate progesterone signaling by acting on a yeast homolog of the HSP90-associated FKBP52 or FKBP54 protein (316), but now that the yeast genome has been sequenced, it is clear that there are no FKBP52 or FKBP54 homologs in yeast. What, then, is the target for FK506 action in this system? Studies by Yamamoto and colleagues have identified an ABC transporter MDR homolog in yeast, LEM1, which pumps steroid hormones out of the cell. FK506 is known to inhibit mammalian MDR pumps and also turns out to inhibit the yeast LEM1 steroid pump (178). Hence, FK506 effectively increases the intracellular concentrations of exogenously added steroids by inhibiting steroid export by LEM1. Thus, the effects of FK506 on progesterone receptor function in yeast are indirect and result from a relative increase in intracellular steroid levels, an effect mediated by LEM1 and not by calcineurin, FKBP12, or an FKBP52-FKBP54 homolog.

N-MYRISTOYLTRANSFERASE: NOVEL TARGET FOR CHEMOTHERAPEUTIC AND ANTIFUNGAL DRUGS

The enzyme *N*-myristoyltransferase (NMT) modifies proteins at the N-terminal end with lipophilic myristic acid groups, a modification that is involved in both membrane targeting and protein stability. The enzyme is conserved from microorganisms to humans and is known to be essential for viability in *S. cerevisiae*, *C. albicans*, and *Cryptococcus neoformans* (81, 203). Several oncoproteins are known to be myristoylated in mammalian cells, including the src and yes oncogenic tyrosine kinases; however, despite the potential for clinical benefit, inhibitors of NMT as antineoplastic agents remain to be fully explored (for a review, see reference 87).

On the other hand, great progress has been made recently in the development of novel NMT inhibitors with potential as antifungal agents. Pioneering studies by Gordon and coworkers identified the NMT genes in *S. cerevisiae*, *C. albicans*, and *Cryptococcus neoformans* and demonstrated that these enzymes are essential for viability (81, 157, 203, 204). Hence, inhibitors would be expected to have fungicidal activity, which may be important in the development of newer broad-spectrum antifungal agents in severely immunosuppressed patients. An important recent advance is the solution of a high-resolution X-ray crystal structure of *C. albicans* NMT (333).

Lodge, Gordon, Sikorski and coworkers identified important differences in the substrate specificity of the fungal and mammalian NMT, suggesting that inhibitors with relative specificity for the fungal enzyme could be obtained (204). Starting with the octapeptide inhibitor ALYASKLS, a series of imidazole-

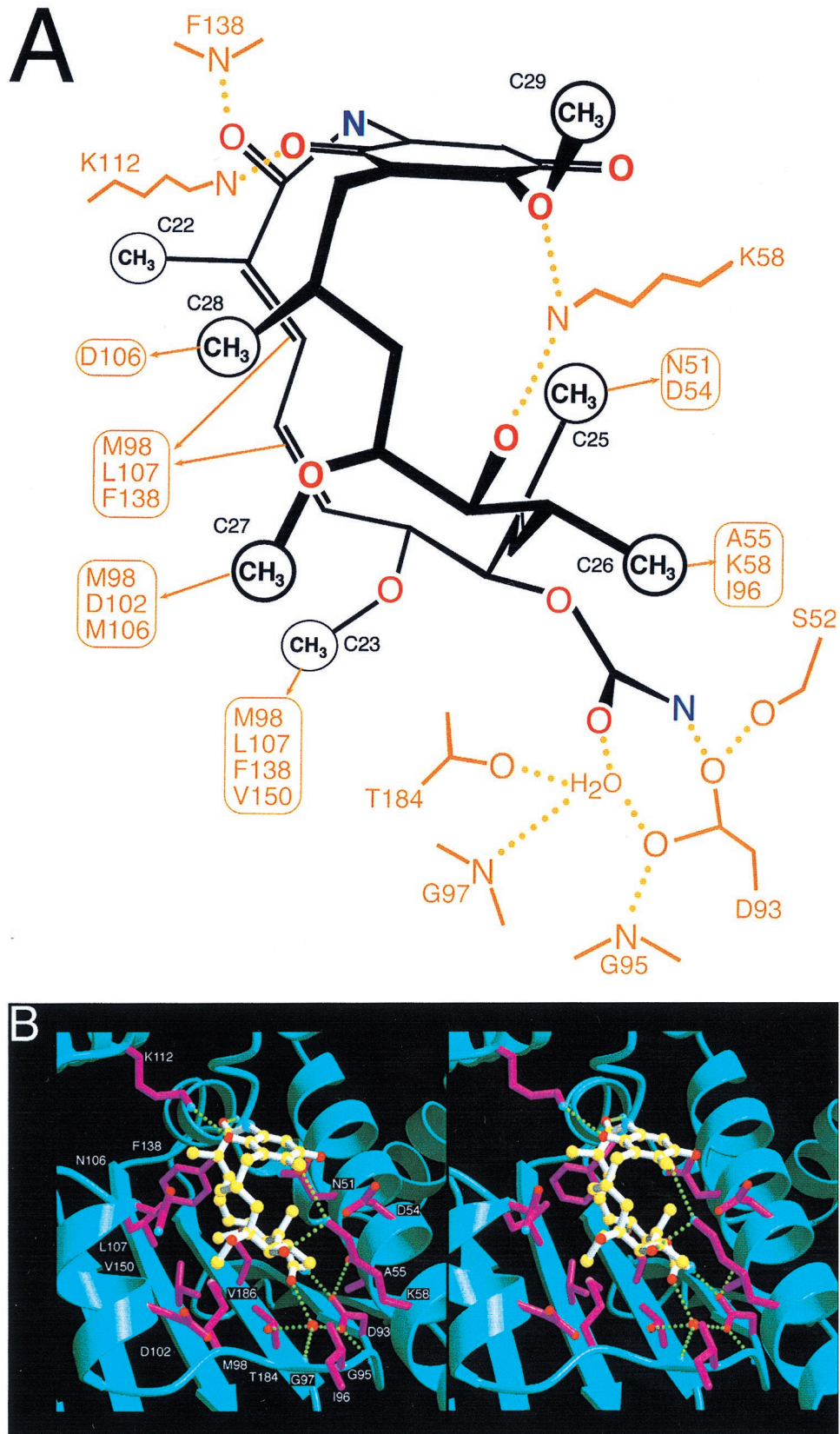


FIG. 8. The HSP90 chaperone complex is targeted by geldanamycin. (A) Schematic view of geldanamycin bound in the ATPase active site of HSP90. (B) Stereoview of the geldanamycin-HSP90 active-site complex, with HSP90 shown in blue, side chains shown in purple, and geldanamycin shown in white and yellow. Interactions between geldanamycin and HSP90 are indicated by green dotted lines. (C) Space-filling model of the complex between geldanamycin, which snugly docks into the ATPase binding site on the surface of HSP90 (blue). Modified from reference 310 with permission of the publisher and kindly provided by Nikola Pavletich.

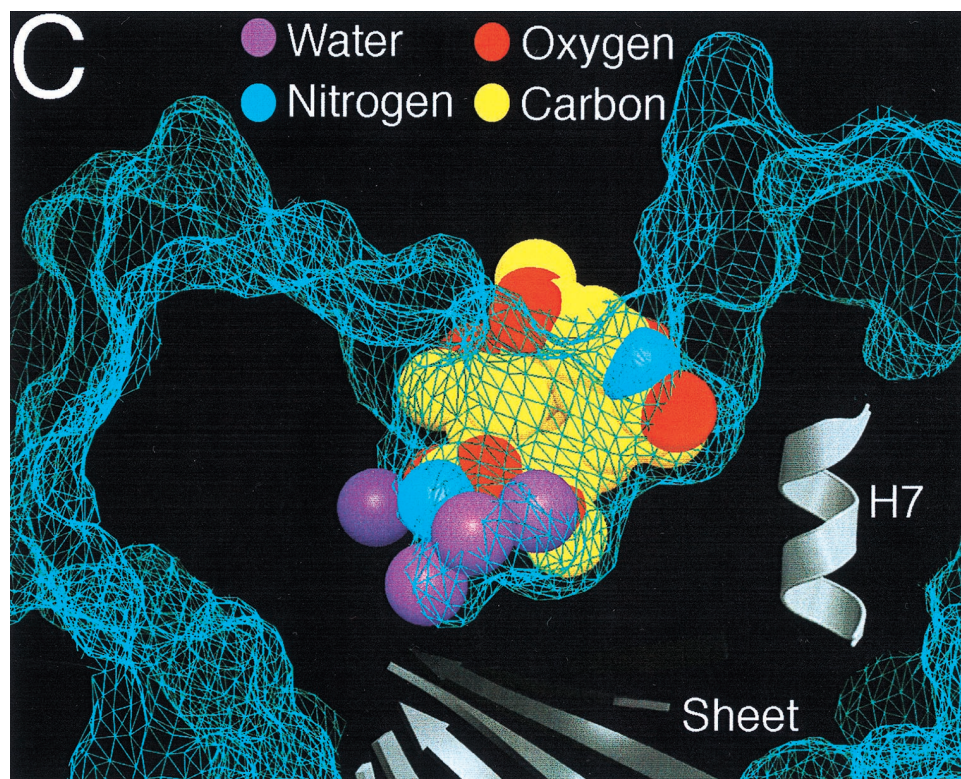


FIG. 8—Continued.

substituted serine-lysine dipeptide amides were designed as candidate specific inhibitors of *C. albicans* NMT (71). Further structure-function optimization led to the synthesis of a series of tripeptide-based inhibitors that are markedly selective for the fungal rather than the human NMT (560- to 2,200-fold) and which have antifungal activity against *C. albicans* at $\sim 50 \mu\text{M}$ (71). Recently, nonpeptide derivatized versions of these inhibitors that retain antifungal activity and selectivity for the fungal rather than the human NMT have been synthesized (202), and trials of these compounds in animal models of fungal infection should be forthcoming. Finally, other investigators have been exploring the potential of myristic acid analogs as inhibitors of NMT as novel antifungal agents (257).

These studies on the design of selective inhibitors of fungal but not human NMT raise an important question in antifungal drug discovery, namely, when is there enough difference between the fungal and mammalian enzymes that such selective activity can be achieved? This is now a central question in the development of novel antifungal agents, and one bias has been to target only fungus-specific enzymes, such as 1,3- β -glucan synthase. In our opinion, this disregards a wealth of excellent antifungal targets that differ significantly from their mammalian homologs. Indeed, recent studies on the identification of novel and quite specific inhibitors of the mammalian enzyme cyclooxygenase 2 (COX2) are an important case in point. Aspirin and other nonsteroidal anti-inflammatory drugs inhibit both COX2 to yield their therapeutic benefits (analgesia, antipyresis, antithrombosis, and anti-inflammation), but they also inhibit cyclooxygenase 1 (COX1) in the gastric mucosa to cause undesired ulcerogenic side effects. However, a number of nonsteroidal anti-inflammatory drugs differ in their relative potency against COX1 and COX2, and this initially suggested that isoform-specific inhibitors could be obtained. This is in

fact the case; the COX2-specific inhibitor celecoxib has just received Food and Drug Administration approval, and a number of other compounds are nearing approval. What is important about this example is that COX1 and COX2 differ by only a single amino acid in the active site, and this sequence divergence is apparently sufficient to achieve selective inhibition. A second case in point is the HIV-1 protease, which is sensitive to a variety of different protease inhibitors, yet single-amino-acid substitutions in the active site/drug binding pocket permit it to retain its enzyme activity but render the strains drug resistant. Clearly, a quite modest sequence difference should be fully sufficient for specific targeting of fungal rather than host enzymes. These differences can be further exploited and modeled since crystal structures are available for a series of these potential targets (Fig. 1, 3, 5, 7, and 8).

INHIBITORS OF SPHINGOLIPID METABOLISM AS ANTIFUNGAL AGENTS

Sphingolipids are ubiquitous and essential components of eukaryotic plasma membranes (127, 131). The basic structure and metabolism of these lipids are conserved in fungal and human cells, with some differences which could be exploited for the development of antifungal drugs. Correspondingly, compounds such as ceramide trigger apoptosis, and thus agonists of sphingolipid signaling may find use as anticancer chemotherapeutic agents. Enzymes in the sphingolipid metabolic pathways are targets of an array of natural compounds produced by many microorganisms (103, 223). We review sphingolipid metabolism to illustrate how sphingolipid metabolism in fungi is similar to and yet different from that in animals, and we suggest a strategy to use the inhibitors of the enzymes in sphingolipid metabolism as novel antifungal drugs.

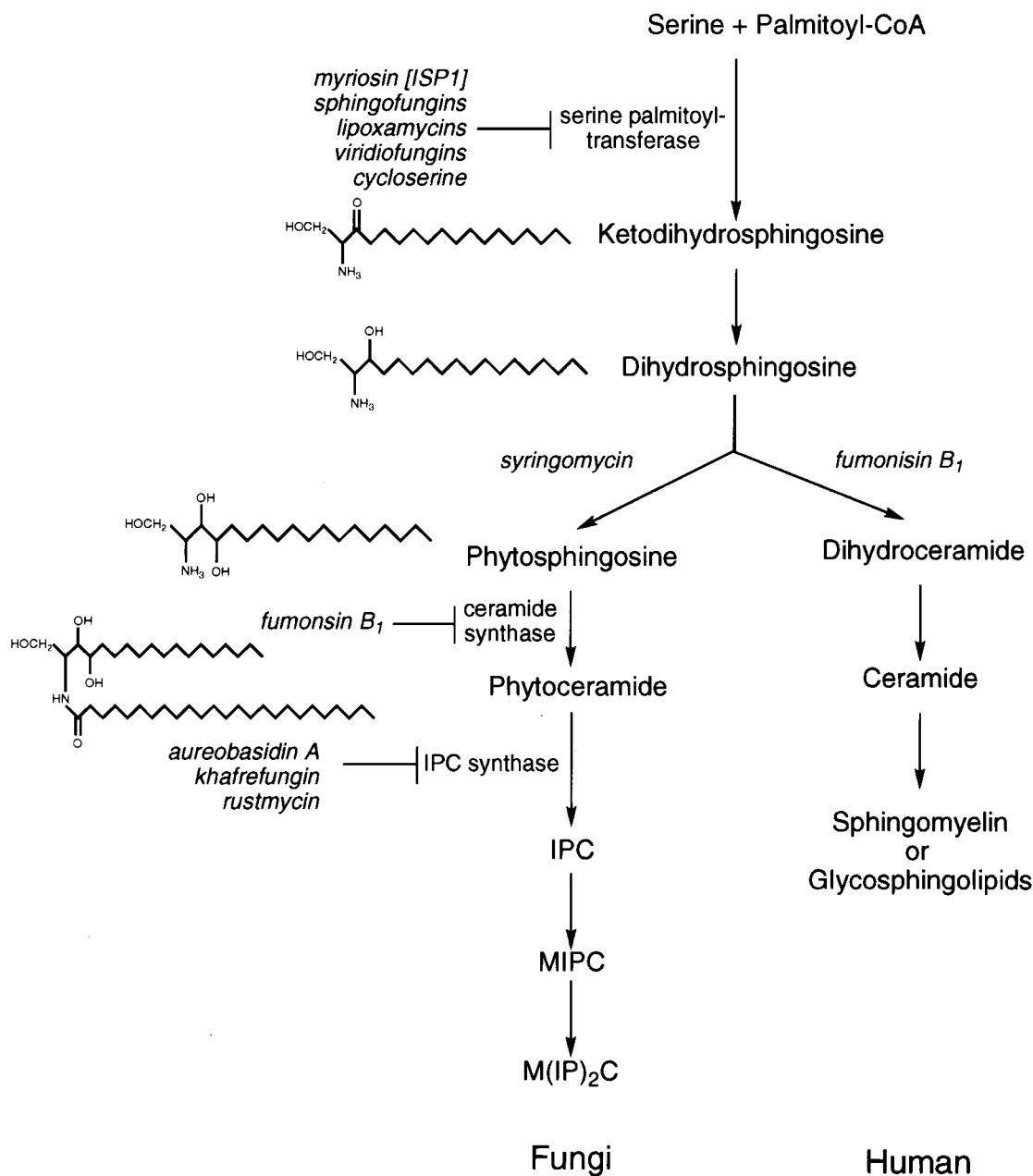


FIG. 9. Sphingolipid metabolic cascades are conserved between yeast and humans. Differences in the de novo synthesis of sphingolipids between human and fungi are shown. Inhibitors of specific enzymes are shown in italics.

The first half of the de novo synthesis pathway producing sphingoid bases and ceramides is essentially conserved among fungi, plants, and animals, while the second half of the pathway involves the more complex sphingolipids and is dissimilar among fungi, plants, and animals (73) (Fig. 9). The conserved first half starts with the rate-limiting step in de novo synthesis involving condensation of L-serine and palmitoyl coenzyme A to produce 3-ketodihydrosphingosine (3-ketosphinganine), which is subsequently converted to dihydrosphingosine (sphinganine). In animal cells, dihydrosphingosine is N acylated to form dihydroceramide (N-acyl sphinganine) and then desaturated to ceramide (N-acyl sphingosine), while in fungi and plants, it is either hydroxylated to phytosphingosine (5-hydroxysphinganine) and then N acylated to phytoceramide (N-acyl-5-hydroxysphinganine) or first N acylated

to dihydroceramide and then hydroxylated to phytoceramide. Phytoceramide in fungi and ceramide in humans are the basic backbones upon which the more complex sphingolipids are built.

In animals, the phosphocholine moiety of phosphatidylcholine is transferred to ceramide to yield sphingomyelin (a phosphosphingolipid) or added to a variety of sugar moieties to become one of more than a few hundred different types of glycosphingolipids. In fungi, phytoceramide is sequentially added to phosphorylated sugar moieties to become inositolphosphoceramide (IPC), mannose inositolphosphoceramide (MIPC), and mannose diinositolphosphoceramide [M(IP)₂C]. Analysis of sphingolipids from *S. cerevisiae*, *C. albicans*, and *Cryptococcus neoformans* suggests that sphingolipid metabolism is conserved among many fungi (325, 332).

Sphingolipids make up only a small portion of membrane phospholipids and yet are structurally diverse and essential. This suggests that sphingolipids may be involved in biological regulation or signal transduction rather than as structural components of the plasma membrane. Biological phenomena involving sphingolipids or metabolites, such as ceramide, sphingosine, and sphingosine 1-phosphate, are numerous and include apoptosis, cell cycle regulation, cellular senescence, differentiation, calcium homeostasis, and tumor progression (130). As an example, extracellular stimuli such as Fas, tumor necrosis factor alpha, ionizing radiation, and chemotherapeutic agents activate sphingomyelinase, which hydrolyzes sphingomyelin to ceramide and phosphocholine. Ceramide induces pleiotropic biological responses, including apoptosis, which could be clinically useful in cancer treatment strategies (130, 248). The proapoptotic function of ceramide was suggested to be counteracted by antiapoptotic function of sphingosine 1-phosphate, a further metabolite of ceramide (61, 307).

S. cerevisiae has been the target of extensive studies of sphingolipid metabolism and signal transduction (73). In *S. cerevisiae*, heat stress activates serine palmitoyltransferase, a key sphingolipid biosynthetic enzyme, leading to a marked increase in sphingoid bases including dihydrosphingosine and phytosphingosine (74, 155). Recently, it was shown that the serine palmitoyltransferase is also activated by diverse stresses including nutrient starvation and DNA damage, resulting in increased levels of phytosphingosine (56). This, in turn, leads to the degradation of nutrient permeases necessary for growth (56).

Serine palmitoyltransferase is the enzyme catalyzing the committed step in sphingolipid biosynthesis (Fig. 9). In *S. cerevisiae*, serine palmitoyltransferase is composed of the *LCB1* and the *LCB2* gene products (37, 237). Inhibitors of this enzyme block the production of all sphingolipids, which, because these are essential lipids, is lethal. Many inhibitors of this pathway, including myriocin (223), sphingofungins (361), lipoxamycins (210, 334), viridifungins (212), and cycloserine (337), have been reported. Except for cycloserine, all of these are natural compounds and show very potent antifungal activity in the nano- to picomolar concentration range. However, since the serine palmitoyltransferase is conserved in both function and amino acid sequence from fungi to humans, these agents also inhibit the mammalian enzyme to various degrees.

Dihydrosphingosine desaturase converts dihydrosphingosine to dihydroceramide in humans and dihydrosphingosine to phytosphingosine in fungi (Fig. 9). In *S. cerevisiae*, this enzyme is encoded by the *SYR2* gene (120, 126), and a mutation in this gene renders yeast resistant to syringomycin, a cyclic lipodepsipeptide produced by *Pseudomonas syringae* pv. *syringae* (57, 306). At present, it is not known if syringomycin has specific inhibitory effects on the fungal enzyme compared to the mammalian enzyme.

Ceramide synthase acylates dihydrosphingosine and phytosphingosine to produce dihydroceramide and phytoceramide, respectively (Fig. 9). Fumonisin B₁ and australifungins inhibit this enzyme activity. Fumonisin B₁ was shown to be active against both fungal and mammalian enzymes (223, 224), but it has relatively low cytotoxicity against *S. cerevisiae* (350). Australifungin is isolated from the fermentation extracts of *Sporomniella australis* and shows antifungal activity against *Candida* spp., *Cryptococcus neoformans*, and *Aspergillus* spp. (211); it has not yet been determined if australifungin also inhibits the mammalian enzyme.

IPC synthase catalyzes the addition of inositol phosphate to phytoceramide, which is the first fungus-specific step in sphingolipid biosynthesis (238). Therefore, this enzyme is an ideal

target of antifungal drugs. There are several natural compounds that inhibit the activity of this enzyme, including aureobasidin A, khafrefungin, and rustmycin (Fig. 10). These are potent inhibitors of the enzyme, with IC₅₀s in the subnanomolar range. Aureobasidin A is a cyclic depsipeptide isolated from *Aureobasidium pullulans* and has been reported to show antifungal activity against *C. albicans*, *Aspergillus* spp., and *S. cerevisiae* (83, 134, 137, 238). A number of novel aureobasidin analogs with enhanced antifungal activity against *C. albicans* have been recently reported (185). In addition, aureobasidin is exported from fungal and mammalian cells by MDR pumps, and some novel aureobasidin analogs with increased activity against mammalian MDR pumps have been described and may prove useful in treating MDR tumors (186). Khafrefungin is an aldonic acid ester linked to a C₂₂ modified alkyl chain and shows fungicidal activity against *C. albicans* and *Cryptococcus neoformans* (214). Notably, khafrefungin does not inhibit the synthesis of mammalian sphingolipids including sphingomyelin. Rustmycin is a 14-member macrolide from an actinomycete (Fig. 10); it has especially potent fungicidal activity against *Cryptococcus neoformans* and was used in a mouse model, where it effectively reduced infection with this yeast (213).

That fungal sphingolipids are essential and differ in some respects from human sphingolipid metabolism makes these an attractive target for antifungal drugs. Because some parts of the metabolic pathways are more highly conserved than others, the enzymes which are more dissimilar may represent better targets. In this regard, the IPC synthase is the enzyme catalyzing the first step of fungus-specific sphingolipid synthesis, and therefore it may be an ideal target for antifungal drug development. Several antifungal drugs have been isolated from microbial extracts which were screened for inhibitory effects on IPC synthesis, and more extensive screening could identify additional inhibitors for preclinical testing in animal models of fungal infection.

YEAST AS A MODEL TO IDENTIFY NOVEL TOXINS WITH CHEMOTHERAPEUTIC POTENTIAL AGAINST FUNGI AND TUMORS

Recent advances in genomic sequencing, analysis of gene expression with genome arrays, combinatorial chemistry, and novel screening approaches promise to revolutionize drug discovery. Lee Hartwell and Steve Friend have recently pioneered a novel approach in which *S. cerevisiae* is used to discover compounds with novel chemotherapeutic potential (133, 331). The strategy involves the construction of a panel of isogenic yeast strains with mutations in genes encoding proteins and enzymes involved in DNA replication, recombination, repair, and cell cycle checkpoint function. Importantly, these mutants are viable but are more sensitive to DNA damage or agents that perturb the cell cycle than are the isogenic wild-type cells. Next, a panel of natural and synthetic compounds in the National Cancer Institute collection of compounds was screened for increased toxicity against one or more mutant strains compared to wild-type cells. A number of especially promising compounds have been identified by this approach. Next, these compounds were screened for activity against a panel of 60 human tumor cell lines. In several cases, compounds with activity against a restricted subset of the cell lines were identified. The hypothesis is that tumor cells, like the mutant yeast strains, may have mutations in DNA repair or cell cycle checkpoint functions that render them uniquely sensitive to a particular toxin compared to wild-type cells. Thus, the very mutations that originally contribute to the development of neoplastic potential can be used to target the tumor cell for destruction by

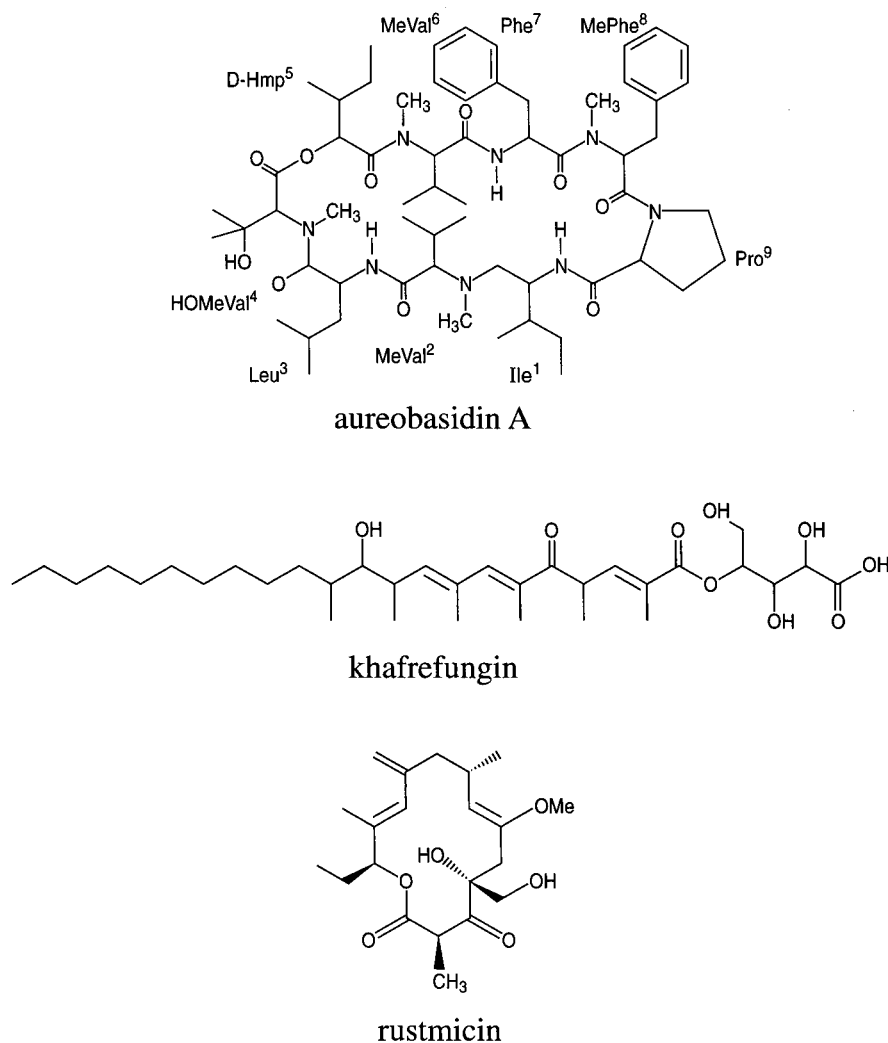


FIG. 10. Inhibitors of sphingolipid biosynthesis. The structures of three inhibitors of IPC synthase, aureobasidin A (a cyclic peptide), khafrefungin, and rustmicin, are shown.

novel agents. The great facility with which the yeast genome can be manipulated by homologous recombination and the speed of simple growth assays suggest that this type of approach could be quite robust. An additional adaptation might be to include combinatorial chemistry approaches on microbeads that can be used to assay for yeast growth inhibitors in simple plating assays (23, 147).

Sequencing of the *S. cerevisiae* genome has been recently completed, and two different whole-genome expression analysis approaches have been developed. The first involves binding of complete open reading frames for the ~6,000 yeast genes as an ordered array on the surface of a glass slide (70, 187, 290). The array is then hybridized to fluorescently labelled cDNA derived from mRNA of cells grown under different conditions. The second approach involves the generation of high-density whole-genome arrays by chemical synthesis of short oligonucleotides on a scaffold matrix (146, 341, 343).

Genome arrays to monitor genome-wide expression patterns are now being exploited for drug discovery. As an example, Marton et al. recently analyzed the changes in gene expression in yeast cells that had been treated with the calcineurin inhibitor FK506 and compared these cells to mutant cells in which

the genes encoding the calcineurin catalytic subunits had been deleted (216). While many of the changes in gene expression pattern were similar in cells lacking calcineurin and in cells with inhibited calcineurin, interestingly FK506 had additional effects on gene expression that were not attributable to calcineurin or FKBP12 inhibition. Importantly, many genes involved in amino acid biosynthesis were induced by FK506, and this induction required the transcription factor GCN4, which is known to be involved in activating the expression of amino acid biosynthetic genes in response to amino acid starvation (216). Earlier findings had revealed that FK506 inhibits amino acid transport in *S. cerevisiae* and that this drug effect is independent of calcineurin and the four known FKBP proteins in yeast (77, 138, 206).

A second recent example of the potential of combinatorial chemistry and whole-genome expression analysis is the development of a novel inhibitor of the human cdk2 cyclin-dependent kinase and the related *S. cerevisiae* Cdc28 kinase (114). Starting with novel trisubstituted purine analogs of the inhibitors olomoucine and roscovitine, Gray et al. (114) used combinatorial chemistry to develop more specific and potent inhibitors of the human cdk2 kinase. Subsequently, the X-ray

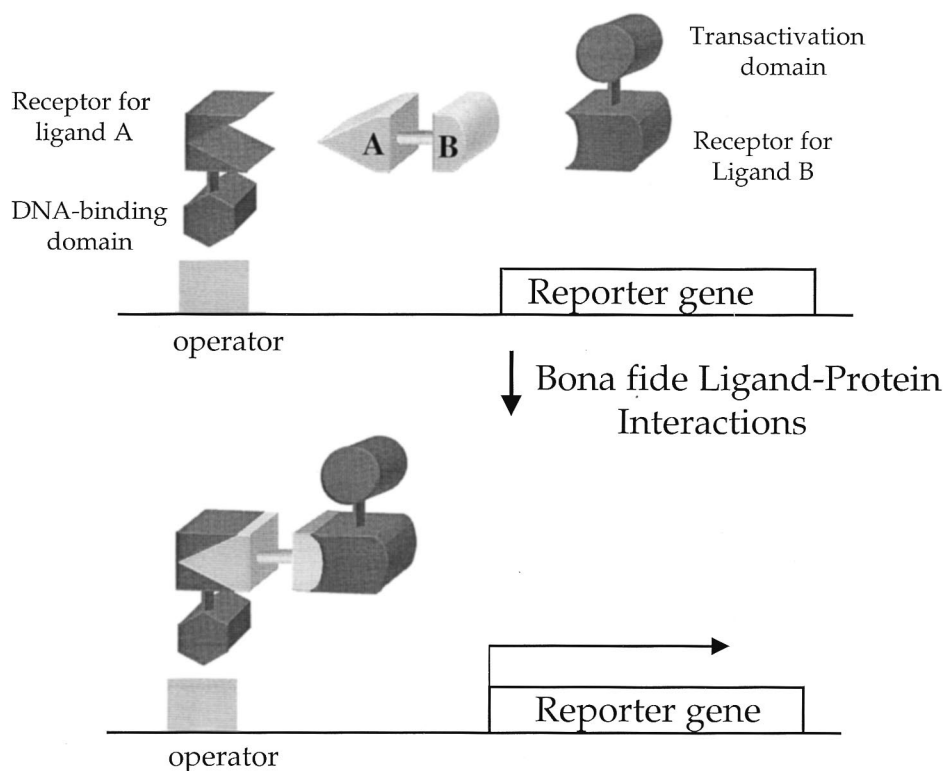


FIG. 11. Schematic view of the yeast three-hybrid assay for drug target discovery. The two-hybrid system for detecting protein-protein interactions was modified to develop a genetic method to identify drug targets. The GAL4 DNA binding domain was fused to a steroid receptor ligand binding domain. The GAL4 activation domain was fused to a library. The third hybrid is a conjugate drug in which dexamethasone (A) is conjugated to a second ligand (B). This hybrid drug is added to cells, and reporter gene expression is monitored to identify clones from the library that encode proteins that interact with the ligand of interest.

structure of the complex between one analog, purvalanol B, and cdk2 was solved, revealing the molecular details of inhibitor binding to the cdk2 ATP binding site. Interestingly, this inhibitor also has potent activity against the *S. cerevisiae* CDC28 kinase and the related PHO85 kinase. Using a drug-sensitized yeast strain with mutations in the *ERG6* gene, which is required for the final biosynthetic step leading to the membrane sterol ergosterol and two genes encoding MDR pump homologs (*PDR5* and *SNQ2*), Gray et al. observed inhibition of yeast growth with an IC_{50} of 30 μ M (114). Examination of the pattern of gene expression by whole-genome arrays revealed similar, but not identical, alterations in gene expression by inhibitor compared to *cdc28* or *pho85* mutations (114).

One final novel approach that has been developed has great promise to exploit *S. cerevisiae* for use in the discovery of novel drug targets. A novel variation on the yeast two-hybrid system, called the three-hybrid assay, has been developed to identify novel drug targets (194). The three-hybrid assay has several components. First, the DNA binding domain of the GAL4 transcription factor is fused to the glucocorticoid receptor (the first hybrid). Next, the transcriptional activation domain of the GAL4 protein is fused to a random library of cDNA sequences from a human cell line or organism of interest (the second hybrid). Finally, the third hybrid molecule is a hybrid drug in which glucocorticoid has been fused to a drug of interest, such as FK506. In this three-hybrid system, the ability of the hybrid steroid-drug conjugate to mediate the binding of the two isolated halves of the GAL4 transcription factor is then detected by monitoring the expression of reporter genes fused to a promoter regulated by the GAL4 transcription factor (Fig. 11). When the hybrid drug can bind to both halves of the GAL4

transcription factor fusion proteins, reporter gene expression is induced. For example, when the steroid-FK506 conjugate was added to yeast cells expressing the GAL4-DNA binding domain-steroid receptor fusion protein and transformed with a library of human T-cell cDNA clones fused to the GAL4 activation domain, library isolates in which the GAL4 activation domain had been fused to the human FK506 binding protein FKBP12 were identified. While the full promise of this three-hybrid system has yet to be realized in the identification of a novel, unknown drug target, clearly there are a multitude of possible applications in the identification of proteins which interact with small molecules that can be tethered to a steroid and can penetrate yeast cells.

Finally, with the use of the yeast genome sequence and functional genomics, it is possible to identify all genes in *S. cerevisiae* essential for viability. These essential genes could then become targets for candidate chemical library screens. In fact, with human and yeast databases, unique essential genes could be identified by an electronic Southern analysis and those unique to fungi or mammalian cells could be used as a focus for antifungal or anticancer drug discovery.

CONCLUSION

In contrast to bacterial infections, cancer and viral and fungal infections are particularly difficult to treat and target with novel drugs because they all result from maladies caused by eukaryotic cells similar to or modified from the host cells. However, many of the same strategies that are used in the design of novel cancer chemotherapy agents can be brought to bear on the development of antiviral and antifungal agents.

This is most apparent in the recent development of several novel antiviral agents (zidovudine, didanosine, and zalcitabine) by using nucleotide analogs that had been originally developed as candidate anticancer chemotherapy agents. Similar approaches can be used in the development of novel antifungal drugs. Although mammals have diverged from yeasts and fungi over approximately one billion years of evolution, recent studies underscore that much of the basic cellular machinery and signaling pathways are remarkably highly conserved and that yeasts and fungi are excellent model systems to understand the mechanisms of action and identify the targets of antineoplastic drugs. Finally, there is a rich collection of natural products produced by microorganisms that remains to be explored for novel chemotherapeutic potential. Microorganisms probably evolved to produce these natural products as antimicrobial agents, yet these compounds often have quite unique and useful actions in mammals via conserved target proteins, and these activities remain to be exploited for therapeutic benefit.

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