MINIREVIEW

Impact of Melanin on Microbial Virulence and Clinical Resistance to Antimicrobial Compounds

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Melanins are negatively charged, hydrophobic pigments of high molecular weight (54, 88, 95, 139) that are composed of polymerized phenolic and/or indolic compounds (Fig. 1) (45, 128). Melanins are produced by organisms in all biological kingdoms, including a wide variety of pathogenic bacteria, fungi, and helminths (reviewed in reference 90). Remarkably little is known about the structures of melanins, despite their abundance in the global biomass. This is due to the inability of current biochemical and biophysical techniques to provide a definitive chemical structure, because these complex polymers are amorphous, insoluble, and not amenable to either solution or crystallographic structural studies. Consequently, our information on the structure of melanin is derived from the analysis of their degradation products and spectroscopic analysis of the melanin polymer (128). Characteristically, melanins are dark in color, insoluble in aqueous or organic fluids, resistant to concentrated acid, and susceptible to bleaching by oxidizing agents (17, 87, 103). Methods for partial chemical degradation of melanin followed by high-pressure liquid chromatographic microanalysis have been developed and are useful for the characterization of specific types of melanin (128, 129). An operational definition for a pigment as a melanin can be provided by electron spin resonance characteristics, since these pigments uniquely are stable organic free radicals (29).

Many diverse functions have been attributed to melanins. Melanins can serve as energy transducers and affect cellular integrity (reviewed in reference 48). Melanin is also used for sexual display and camouflage. For instance, the coloration in black and red hair arises from melanin (18). An example in which melanin is used for camouflage is the release of ink, a suspension of melanin particles, by the cuttlefish (*Sepia officinalis*) in response to danger (34). Melanin plays a major role in the innate immune system of insects, which synthesize the polymer to damage and entomb microbial intruders (85, 104). In insects, invading microbes activate a prophenoloxidase in the hemolymph, resulting in the encasement of the bacterial, protozoal, or fungal pathogen in melanin (78). Melanins in melanocytes in skin provide protection against sunlight and are also believed to contribute to the resistance of melanoma to therapeutic radiation (47). The role of melanin in other cir-

cumstances is uncertain, such as melanin in the neurons of the substantia nigra in the human brain (147, 148).

In mammals, melanin synthesis is catalyzed by a tyrosinase (114). In contrast, microbes generally synthesize melanin via various phenoloxidases (such as tyrosinases, laccases, or catacholases) and/or the polyketide synthase pathway (reviewed in reference 138). Melanins generated from 3,4-dihydroxyphenylalanine (DOPA) by phenoloxidases are referred to as eumelanins, which are generally black or brown. Yellow or reddish melanins are called pheomelanins and incorporate cysteine with DOPA. Brownish melanins derived from homogentisic acid by tyrosinases are called pyomelanins (144). Melanins formed from acetate via the polyketide synthase pathway are typically black or brown and are referred to as dihydroxynaphthalene melanins.

Melanin synthesis has been associated with virulence for a variety of pathogenic microbes. Melanin is believed to contribute to microbial virulence by reducing a pathogen's susceptibility to killing by host antimicrobial mechanisms and by influencing the host immune response to infection. Consequently, melanin and melanin synthesis pathways are potential targets for antimicrobial drug discovery. Interestingly, the drug-binding properties of both host and microbial melanins could influence the outcome of antimicrobial therapy.

This review discusses the impact of melanin production on microbial survival in the environment and during infection, on host immune responses, and on the efficacies of antimicrobial compounds. The capacity for melanin to bind to diverse compounds can affect the testing of antimicrobial drugs and reduce the activity of antimicrobial therapy.

MELANINS CONFER A SURVIVAL ADVANTAGE TO ENVIRONMENTAL MICROBES

Melanin synthesis in free-living microbes is likely to provide a survival advantage in the environment (Table 1) (117). This hypothesis is based on the fact that many fungi constitutively synthesize melanin, and even facultative melanotic microbes like *Cryptococcus neoformans* are melanized in soils (94). Melanin production in *C. neoformans* is associated with increased survival after ingestion by environmental amoeboid (118) or nematode (84) predators. Environmental predators often produce hydrolytic enzymes to digest microbes, and melanized *C. neoformans* cells are significantly less susceptible to cell wall-degrading enzymes than nonmelanized cells (109). Melanin

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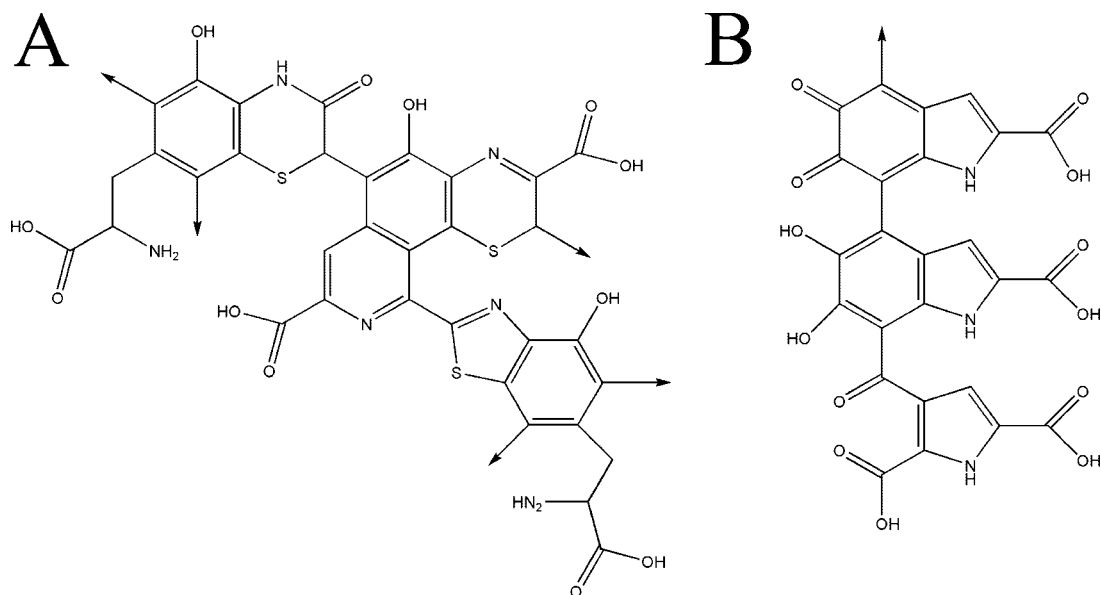
FIG. 1. Chemical structures of pheomelanin (A) and eumelanin (B) oligomers.

production in diverse environmental melanotic molds has been associated with reduced cellular susceptibility to enzymatic degradation (reviewed in reference 90). The mechanism of action for resistance to enzymatic hydrolysis is unclear but may involve sequestration of the enzymes on melanin or may occur by steric hindrance (54). Additional evidence supporting a protective role for melanin is provided by the fact that addition of synthetic melanin to suspensions of *Aspergillus nidulans* results in significant inhibition of the hydrolytic activity of glucanase-chitinase on the fungus (67).

Melanins confer resistance to UV light by absorbing a broad range of the electromagnetic spectrum and preventing photo-induced damage (48). Consequently, melanins are used commercially in photoprotective creams and eye glasses. Melanin protects several fungal and bacterial species from UV, solar, or gamma radiation (reviewed in reference 90). Increased melanin production is associated with the greater resistance of pigmented fungi to radiation (127, 149, 150). The protective properties of melanin against radiation injury could account

for the growth of black fungi in the highly contaminated Chernobyl Reactor No. 4 (83). The pigment significantly contributes to the ability of *C. neoformans* to withstand extremes in heat and cold (108). In fungal plant pathogens, melanization of appressorium allows a cell to maintain integrity while generating pressures in excess of 80 bar to facilitate the penetration peg's entry into a plant cell (reviewed in reference 90).

Melanins are able to bind to the heavy metals that are routinely found in the environment (35, 105, 153). The carboxyl, phenolic, hydroxyl, and amine groups on melanin provide numerous potential binding/biosorption sites for metal ions (reviewed in reference 35). Melanized *C. neoformans* cells are more resistant to killing by silver nitrate, a compound highly toxic to bacteria and fungi, than nonmelanized cells (42). That study demonstrated that melanin chelated the silver compound (42). Although other fungal melanins bind to metals (reviewed in reference 35), a protective role for metal binding has not been demonstrated in other microbes. However, the evidence from *C. neoformans* suggests that the utility of metals as antimicrobial drugs against melanin-producing organisms may be lower than that against non-melanin-producing microbes.

MELANIN PROTECTS MICROBES FROM HOST DEFENSES

The ability of melanin to protect microbes from host defenses is relevant to antimicrobial therapy because the clinical efficacies of some antimicrobial drugs are complemented by host immune defenses. Melanin has been shown to interfere with numerous host defense mechanisms (Table 2). Melanized *C. neoformans* yeast cells manifest increased resistance to phagocytosis in vitro and in vivo (82, 130). In macrophage-like cell lines, the phagocytic index for melanized *Paracoccidioides brasiliensis* yeast cells was half that for the nonmelanized cells (21). Since melanins are charged polymers (139), their pres-

TABLE 1. Melanization protects pathogenic fungi from environmental stressors

Stressor	Organism	Reference(s)
Enzymatic degradation	*C. neoformans*	109
	Aspergillus spp.	9, 15, 67
	E. dermatitidis	25
Radiation (UV, solar, gamma)	*C. neoformans*	108, 132
	S. schenckii	107
	E. dermatitidis	25
	Alternaria alternata	64, 83
	Cladosporium spp.	113, 149
	Pneumocystis spp.	51
Heavy metals	*C. neoformans*	42
Thermotolerance (heat and cold)	*C. neoformans*	108, 145

TABLE 2. Melanin production protects pathogenic fungi against mammalian host defenses

Effect	Microbe	Magnitude (%)[a]	Reference(s)
Phagocytosis	*C. neoformans*	7 (in vivo)	82
	C. neoformans	27 (in vitro)	8, 76, 130
	P. brasiliensis	9	21
	S. schenckii	50	107
	F. pedrosoi	36[b]	20
Killing by host cell	*C. neoformans*	31	8, 130
	Aspergillus fumigatus	18[c]	60
	E. dermatitidis	>36	33, 99, 115
	P. brasiliensis	>30	21
	S. schenckii	27	107
	F. pedrosoi	22	20
Oxidants	*C. neoformans*	22	8, 28, 55, 56, 58, 59, 134
	Aspergillus spp.	75	60, 77
	S. schenckii	20	107
Microbicidal peptides	*C. neoformans*	22	26

[a] Magnitude indicates the maximal percent increase in protection afforded to the organism by the production of melanin compared to that afforded to cells deficient in melanin.

[b] Represents attachment rather than ingestion of fungal cells.

[c] As measured by mitochondrial damage rather than numbers of CFU.

ence in the cell wall of *C. neoformans* can alter the fungal cell surface charge (88), and this may contribute to inhibition of phagocytosis. Melanization increased the cellular negative charge by 3 to 33% in nine different encapsulated strains and by 86% in an acapsular strain (88). In addition to reducing ingestion, melanization protects *C. neoformans* against killing by macrophages (130). Similarly, melanin production in *Fonsecaea pedrosoi* (20), *Sporothrix schenckii* (107), and *Exophiala* spp. (33, 99, 115) enhances resistance to killing by phagocytic cells. Melanin in pigmented *C. neoformans* yeast cells can produce complex immunomodulatory effects that contribute to virulence by eliciting changes in the host cytokine/chemokine response (50, 82). In particular, melanized yeast resulted in higher levels of interleukin-4 and monocyte chemoattractant protein 1 and increased the numbers of pulmonary leukocytes early after infection (82). *F. pedrosoi* melanin also activates humoral and cellular responses (2).

Melanization protects fungi, such as *C. neoformans*, *Aspergillus* spp., and *S. schenckii*, and bacteria, such as *Proteus mirabilis* and *Burkholderia cepacia*, against injury secondary to nitrogen- or oxygen-derived radical attack (reviewed in reference 90). *F. pedrosoi* melanin significantly inhibits nitric oxide production by macrophages, which affects the pathogenesis of chronic chromoblastomycosis (10). The melanized *F. pedrosoi* cells reduced the production of nitric oxide by approximately 50% compared to the amount produced by nonstimulated murine peritoneal macrophages and, similarly, suppressed nitric oxide production in macrophages stimulated by interferon gamma and lipopolysaccharide. Interestingly, melanin is not the only pigment that can modify the effects of oxidative attacks by host cells, since carotenoids in *Staphylococcus aureus* similarly function as antioxidants (75). However, carotenoids in *Exophiala*

dermatitidis do not reduce phagocytosis or protect against oxidative burst or killing by neutrophils (115).

Melanins are highly effective scavengers of free radicals (116) and have electron transfer properties (41). Electron transfer from free radical species generated in solution to melanin derived from *C. neoformans* has been demonstrated by electron spin resonance spectroscopy (131); and similar spectra have been generated with melanins from *Histoplasma capsulatum*, *S. schenckii*, *P. brasiliensis*, and *Pneumocystis* spp. (reviewed in reference 90). Also, *C. neoformans* melanin is involved in the reduction of Fe^{3+} to Fe^{2+} (97) and can facilitate redox cycling through the exportation of electrons to form extracellular Fe^{2+}, which maintains the reducing capacity of extracellular redox buffers (56). Melanized *C. neoformans* cells are also less susceptible to the toxic effects of microbicidal peptides than nonmelanized cells (26). The mechanism of action in this case appears to be adsorption of the microbicidal peptide such that it interferes with the peptide reaching its target.

EVIDENCE THAT MELANINS BIND TO DRUGS IN VITRO

Isotherm analysis of adsorption of drugs by melanin. Melanins bind to chemically diverse compounds (62, 70). The binding of gentamicin, methotrexate, and chlorpromazine to melanins has recently been revisited by using isotherm binding equations to characterize the adsorption of the drugs to synthetic and *Sepia officinalis* melanins (14). Although there were significant variations in adsorption, each drug bound to melanin. More gentamicin than the other drugs was bound by synthetic melanin. By the best-fit Freundlich equation for gentamicin [$q = q_o(KC)^{1/n}$ dm$^3 \cdot$ g^{-1}, where q is the amount absorbed [mmol $\cdot$ g^{-1}], q_o is the adsorption capacity, K is the energy of absorption, C is the equilibrium solution concentration of solute, and the heterogeneity index $1/n$ is between 0 and 1], the quantity of gentamicin absorbed with synthetic melanin was 0.49 dm$^3 \cdot$ g^{-1}, whereas 0.061 dm$^3 \cdot$ g^{-1} of methotrexate was bound. Equilibrium isotherms for the interaction of gentamicin with melanin revealed diverse interactions between the drug and melanin. In contrast, methotrexate appeared to bind to specific homogeneous sites. The data from that study (14) demonstrated the significant capacity of melanin to interact with drugs, since its adsorption capacity was comparable to those of other absorbers, like medicinal activated charcoal.

Scatchard plot analysis of drug binding by melanin. A Scatchard plot-type analysis of drug binding to melanin by the use of radiolabeled compounds has also demonstrated the presence of heterologous binding sites. There are at least two classes of binding sites on synthetic DOPA melanin for the aminoglycoside antibiotics gentamicin (141) and kanamycin (142). For kanamycin, the association constants for the strong and weak binding sites were 3×10^5 M^{-1} and 4×10^3 M^{-1}, respectively, and 0.64 μM kanamycin was required to saturate the binding sites in 1 mg melanin (142). Scatchard plot-type analyses with melanins have also revealed high- and low-affinity binding sites for cocaine (61, 102); amphetamines (6, 43); donarubicin (120); and the antiarrhythmics quinidine, disopyramide, and metoprolol (16). More recently, high-resolution magic angle spinning nuclear magnetic resonance spectroscopy

revealed highly specific melanin-binding sites for iodobenz-amides (11), which can be exploited to diagnose and stage melanoma by using radiolabeled drug.

Absorption studies with antifungals. Two methods have been used to establish that melanin binds to amphotericin B and caspofungin. First, the ability of melanin produced by *C. neoformans* and synthetic melanin to bind to these antifungal drugs was inferred from experiments whereby melanins were incubated with various compounds and then the antifungal activity of the solution was determined (52, 124, 125). For those studies, melanin particles were removed by centrifugation prior to the testing of the antifungal drug solutions in MIC and time-kill studies. Incubation of amphotericin B and caspofungin with melanin significantly reduced their antifungal activities for *C. neoformans*. In contrast, incubation of itraconazole, fluconazole, or flucytosine with melanin had no effect on their antifungal activities. One study showed a 16-fold increase in the MIC of amphotericin B after adsorption of the drug with 1×10^7 melanin particles derived from pigmented *C. neoformans* prior to MIC testing (52). Furthermore, the increase in MIC correlated with the amount of melanin particles added to the antifungal drug solution. Time-kill assays also demonstrated that the addition of melanin particles to amphotericin B or caspofungin significantly reduced their toxicities for *C. neoformans* (124). For example, 71% of yeast cells survived exposure to 2× the MIC of amphotericin B preincubated with synthetic melanin, whereas the rate of survival was 8% for the cells exposed to amphotericin B not incubated with melanin. Similarly, 79% of yeast cells exposed to caspofungin preincubated with melanin survived exposure to drug, whereas the rate of survival was 11% for cells incubated with native caspofungin. In contrast, incubation of azoles or flucytosine with melanin did not affect their MICs for *C. neoformans* or the abilities of the drugs to kill *C. neoformans*.

Antifungal drug binding to fungal melanin was also inferred by the finding that the elemental composition of melanin was changed after incubation with antifungal drugs. Incubation of amphotericin B or caspofungin with melanin altered the melanin C:N:O ratio, consistent with drug absorption (124). In contrast, no change in the melanin elemental composition was observed following the incubation of melanin with flucytosine, voriconazole, fluconazole, or itraconazole (124, 125). Since melanin is located in the cell wall, these data suggest and are consistent with a mechanism of acquired resistance, whereby fungal melanin binds to amphotericin B and caspofungin and prevents them from reaching their target sites.

Effect of melanin on the porosity of the microbial cell wall. Analysis of the microstructure of cell wall-associated melanin in *C. neoformans* has provided new insights into the potential of this polymer to interfere with antifungal drug activity (27). Cell wall-associated melanin is composed of discrete granules of roughly uniform dimensions (Fig. 2). This is significant, because a granular arrangement would significantly increase the surface area available for binding to certain types of drugs. Atomic force microscopy and transmission electron microscopy revealed that the melanin particles range in size from 40 to >100 nm, with an average particle diameter of 76 nm, which is similar to the results for mammalian melanin and melanin from *S. officinalis* (27). Transmission electron microscopy revealed that cell wall melanin is composed of two to five layers
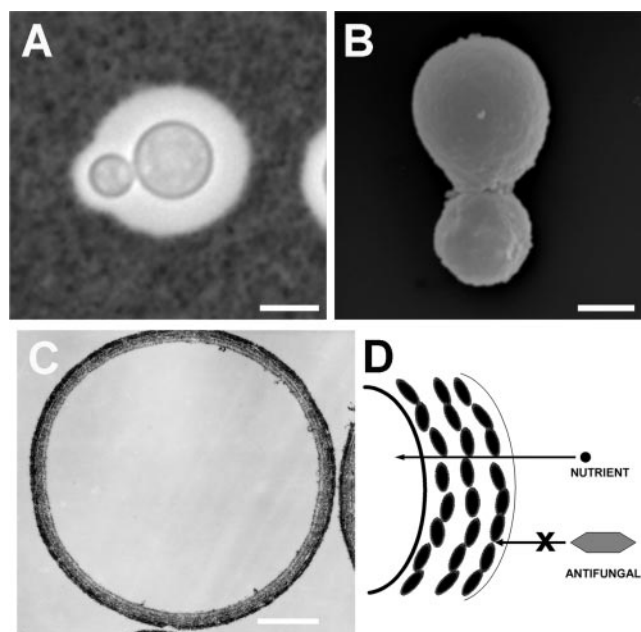

FIG. 2. The pathogenic yeast *Cryptococcus neoformans*. (A) India ink preparation showing a budding *C. neoformans* yeast cell with a large polysaccharide capsule surrounding the cell bodies. Bar, 5 μm. (B) A melanin "ghost," a melanin particle isolated from *C. neoformans* grown for 10 days in the presence of L-dopa by serial treatment of the yeast with enzymes, denaturant, chloroform, and hot acid. Bar, 2 μm. (C) Transmission electron micrograph of a cross-section of a *C. neoformans* "ghost" showing that the particle is formed of concentric layers of melanin. Bar, 1 μm. (D) Depiction of the melanin granules comprising the melanin layers, demonstrating how the packing of the granules results in pores that obstruct the passage of large molecules, such as amphotericin B or caspofungin. Obstruction of antifungal molecules can occur by virtue of a reduced melanin pore size or melanin binding. Panel D is based on data from reference 27.

of melanin particles arranged in a concentric manner. The thickness of the layer appears to depend on cell age, such that older cells may be significantly less susceptible to melanin-binding antifungal drugs than younger cells. In order for the fungal cells to survive, the polymer layers composed of granules cannot completely exclude nutrients. Nuclear magnetic resonance cryoporometry revealed that melanin ghosts contain pores with diameters between 1 and 4 nm, in addition to a small number of pores with diameters nearly 30 nm. The pore size decreases with the age of the yeast cell. Importantly, cryoporometry studies with melanin-binding antibody show that the larger pores appear to be internal to the smaller pores. In another study, the porosity of melanized cryptococcal cell walls was evaluated by elution of graded dextrans, and similar results were described (57). These findings suggest that the tight spaces between melanin granules may prevent or slow the entry of large drugs, such as amphotericin B (molecular mass, 924 g/mol) and caspofungin (molecular mass, 1,093.5 g/mol), into pigmented cells. This may be particularly significant for amphotericin B, since this drug tends to form large aggregates in solution (68). In contrast, azoles and flucytosine have significantly smaller molecular masses, and for these compounds, melanization does not reduce fungal cell susceptibility. Hence, melanin in the cell wall may also reduce the susceptibilities to

certain drugs by inhibiting their diffusion into the cell body and provide greater opportunity for the binding of drug by melanin.

BINDING OF COMPOUNDS BY MELANIN IN HUMANS IN VIVO

The binding of drugs to host melanin can damage certain tissues, and drug-melanin interactions have been implicated in the pathogenesis of several diseases, such as Parkinson's disease. In Parkinson's disease there is a loss of pigment in the melanotic dopaminergic neurons in the substantia nigra of the brain. A provocative connection between the drug-binding properties of melanin and the etiology of Parkinson's disease came from the observation that heroin contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) caused a similar neurological disease in drug users, possibly because melanin concentrated this compound in substantia nigra neurons (46). Chlorpromazine also accumulates in the substantia nigra (73), and the side effects of chlorpromazine and other phenothiazines include extrapyramidal disorders, such as tardive dyskinesia and parkinsonism. In contrast to MPTP, the parkinsonian symptoms secondary to phenothiazines are usually reversible. The specific retention of other drugs in pigmented tissues can damage cells in the skin, eye, and inner ear. These interactions are complex and depend on diverse factors, such as cysteine content, pH, and ionic interactions (79). As described above for synthetic DOPA melanin, Scatchard plot analysis has revealed the heterogeneity of binding sites on melanin for single compounds (69, 102, 122, 123). The conformation of the compounds may also influence these interactions. Binding is typically reversible, but the retention times can be protracted. For example, chloroquine can be detected in the melanin of the eye for a year after receipt of a single dose (74), and chloroquine therapy is associated with retinopathies (49) that can occur long after treatment (151). In addition to chloroquine, severe retinopathies can occur following melanin binding by chlorpromazine. Chloroquine also accumulates in dermal melanocytes and hair follicles (79), where it can occasionally cause irreversible hearing loss, tinnitus, and dizziness (44). Whereas hearing loss due to chloroquine is thought to be a result of effects on the eighth cranial nerve, quinine can accumulate in melanin in the stria vascularis of the cochlea and cause cellular degeneration (74).

Thioureylenes are selectively incorporated into melanin (71); and certain compounds, such as propylthiouracil, may cause a loss or the depigmentation of hair (70). The carcinogenic effects of polycyclic aromatic hydrocarbons may be compounded by the presence of melanin, since these compounds have a prolonged retention time in pigmented tissues (106). Herbicides, such as paraquat (which is structurally related to 1-methyl-4-phenylpyridinium [MPP$^+$], the neurotoxic metabolite of MPTP), avidly bind to melanin and cause parkinsonian symptoms in experimental animals (3). Cocaine and amphetamines are known to bind to melanin (12, 119), and testing of hair for these compounds is used for medical and legal purposes. The cytotoxic effects of anthracycline chemotherapeutics (such as doxorubicin and donorubicin) can be inhibited by melanin. For example, the 50% inhibitory concentration of donorubicin in an in vitro cell-based assay increased from 0.04 to 0.08 μM in the presence of melanin from *S. officinalis* (121).

The interaction of these chemotherapeutics with melanin may be responsible for decreased wound healing and for an unsatisfactory response of the tumor to the medication. However, many melanin-binding drugs commonly used in clinical practice, such as beta-blockers, benzodiazepines, and rifamycins, bind to melanin in vitro, without apparent adverse effects in vivo (53).

Aminoglycosides are positively charged at physiological pH and have a relatively high molecular weight, which limits their penetration into tissues (66). Nevertheless, aminoglycoside therapy causes significantly more toxicity in albino animals than in their pigmented counterparts. The administration of aminoglycosides can result in permanent vestibular and auditory ototoxicity (7). The cochlear melanin content has been correlated to the pigmentation of the host (65). The ototoxic effects of aminoglycosides have been shown by electrophysiological and morphological methods (19, 136, 143). However, no difference in outer hair cell degeneration in the organs of Corti was observed between albino and pigmented guinea pigs exposed to various concentrations of kanamycin (137). Aminoglycosides can also cause functional and morphological changes in the retina, particularly when they are administered by intravitreal injection. Studies with albino and pigmented rabbits have shown that ocular pigmentation can partially protect the retina from damage due to aminoglycosides (30). Although it is of unclear significance, the binding of aminoglycoside by melanin can augment the antibiotic's inhibitory effects on collagen synthesis in human fibroblasts in vitro (141).

The capacity of melanin to bind to aminoglycosides and other antibiotics may have important implications when these drugs are used in intraocular injections (4, 63). The in vitro efficacies of aminoglycosides, tertracyclines, and vancomyin were significantly reduced following incubation with melanin (5, 38). In fact, the mixing of 100 μg/ml of tobramycin with 1,000 μg/ml of melanin resulted in an immediate decrease in antibiotic activity of 80% (5). The efficacies of fluoroquinolones may also be affected, as these drugs are bound by melanin within the eye (37, 39, 40) and even in hair (140). Although melanin can bind to fluoroquinolones, penicillins, and cephalosporins, no reduction in antibacterial efficacy has been reported after these drugs have been incubated with melanin (38).

MELANIN AND THE EFFICACY OF ANTIMICROBIAL THERAPY

Melanin has been called an "an antifungal resistance factor," given its ability to reduce the susceptibilities of melanized cells to antifungal drugs (52). Notably, there is no evidence for the involvement of melanin in drug efflux pumps or in alterations in the synthesis of ergosterols or glucans in fungal cell wall/cell membrane structures.

Antifungal susceptibility testing. In vitro susceptibility measures the activity of a drug against a microbe, whereas clinical resistance is a lack of efficacy of a drug in vivo. Although in vitro resistance often correlates with clinical treatment failure, in vitro susceptibility does not necessarily predict clinical success. Standard MIC broth macrodilution testing by use of the M27A protocol for yeasts of the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee of

TABLE 3. Melanin production protects pathogenic fungi
against antimicrobial drugs

Antimicrobial drug	Microbe	Magnitude (%)[a]	Reference(s)
Amphotericin B	*C. neoformans*	55	52, 80, 124, 125, 133
	H. capsulatum	32	124
	B. dermatitidis	37	96
	P. brasiliensis	55	21
Caspofungin	*C. neoformans*	7	80, 124
	H. capsulatum	47	124
Azoles	*C. neoformans*	30	52, 124, 125
	P. brasiliensis	24	21
Sulfamethoxazole	*P. brasiliensis*	10	21

[a] Magnitude indicates the maximal percent increase in protection afforded to the organism by the production of melanin compared to that afforded to cells deficient in melanin.

Clinical Laboratory Standards) revealed no differences in susceptibility between melanized and nonmelanized *C. neoformans* cells (52, 124). Similarly, no differences in susceptibility to antifungals by MIC assays were measured between albino and pigmented cells of *E. dermatitidis* (101), *H. capsulatum* (124), or *Blastomyces dermatitidis* (96). However, the growth of melanized *C. neoformans* yeast cells in medium without a phenolic substrate resulted in large defects in the melanin layer of the parent cells after budding and an absence of melanin in the daughter cells (89). Hence, even if melanized cells are used initially, the daughter cells lack melanin, and the CLSI methodology does not compare the susceptibilities of melanized and nonmelanized cells. Incorporation of phenolic substrates into the testing medium for a microdilution or a macrodilution assay has not been possible because these either precipitate or autopolymerize into melanin (124). Hence, the CLSI protocol is not suitable for distinguishing differences in susceptibility between melanized and nonmelanized cells.

In contrast to the broth dilution method, time-kill studies with CFU determinations have revealed differences in the susceptibilities of melanized and albino cells to certain antifungal drugs (52, 124, 125) (Table 3). A major difference between these assays is that in time-kill studies the microbes are incubated with the antimicrobial drugs for hours rather than days. Thus, the melanin layer of the fungus remains largely intact. *C. neoformans* is significantly less susceptible to amphotericin B when the fungus is grown in the presence of L-dopa (133). This result was recently confirmed by time-kill studies with amphotericin and caspofungin, which revealed that the activities of these drugs against melanized cells were reduced by 55 and 7%, respectively, relative to their activities against nonmelanized cells (52, 124). In contrast, no differences in activity against melanized and nonmelanized *C. neoformans* cells was observed for voriconazole, fluconazole, itraconazole, or flucytosine (124, 125). However, melanized *C. neoformans* strains exhibited reduced susceptibilities to higher concentrations of fluconazole (52).

Time-kill assays similarly revealed that melanized *H. capsulatum* yeast cells were less susceptible to amphotericin B and caspofungin than nonmelanized *H. capsulatum* yeast cells

(124). Melanization was also associated with reduced susceptibility to amphotericin B in melanized versus nonmelanized *B. dermatitidis* cells (96). Melanized *P. brasiliensis* cells were also more resistant to amphotericin B, fluconazole, ketoconazole, itraconazole, and sulfamethoxazole than nonmelanized cells (21). However, melanization can also increase susceptibility to certain drugs. The antipsychotic drug trifluoperazine had greater fungicidal activity against melanized cryptococcal cells than it did against nonmelanized cryptococcal cells when activity was measured by both CFU determination and flow cytometry with propidium iodide staining (135). In contrast, chloroquine, which is highly bound by melanin, had no fungicidal effect on either melanized or nonmelanized *C. neoformans* cells (135). Hence, an association between the capacity of melanin to bind to a drug and reduced susceptibility to that drug by melanized cells is not apparent for all agents. This implies the existence of mechanisms other than simple absorption by melanin as an explanation for the differences in the activities of certain classes of drugs against melanized and nonmelanized cells.

The efficacies of antifungals to melanized cells can also be evaluated by a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) reduction assay (80). Specifically, XTT was used to show that melanization protected *C. neoformans* from amphotericin B and caspofungin in biofilms. For example, the metabolic activity of melanized *C. neoformans* cells in a biofilm exposed to 32 μg/ml of amphotericin B was 40%, whereas it was 20% for nonpigmented cells.

Impact of melanin binding on antifungal drugs. The finding that melanin can bind to amphotericin B and caspofungin, in combination with observations of the microstructure of melanin in *C. neoformans*, suggests a potential explanation for the difficulty in eradicating *C. neoformans* with these drugs. Melanization of *C. neoformans* yeast cells occurs in vivo (93), and the amount of melanin produced increases with time after infection (31, 95). Although amphotericin B is fungicidal to nonmelanized *C. neoformans* cells in vitro, amphotericin B therapy often fails to eradicate the fungus from patients (100, 152). Given the relative resistance of melanized cells to amphotericin B, the efficacy of this drug may be due to its activity against nonmelanized buds and to its facilitation of tissue clearance by host defenses through its powerful immunomodulating effects. Caspofungin is active in vitro against nonmelanized cells (36), but it is ineffective against experimental infections with *C. neoformans* (1). The inefficacy of caspofungin for *C. neoformans* in animal studies cannot be explained by its inability to inhibit either 1-3-β-D- or 1-6-β-D-glucan synthase (32). Since acapsular strains of *C. neoformans* are also resistant to caspofungin, the large polysaccharide capsules that can occur in vivo are not inhibiting the drug from engaging the fungus. Hence, for *C. neoformans*, the clinical resistance to caspofungin could be attributed in part to in vivo yeast cell melanization. Caspofungin is clinically effective against *Aspergillus* spp., a group of fungi that can produce melanin. However, its efficacy may be due to the fact that hyphae, the tissue-invasive form of this fungus, are not melanized (146).

Dematiaceous fungi are darkly pigmented molds that constitutively produce melanin during infection and are extremely difficult to treat with antifungal drugs (13). Although the clin-

ical relevance is not clear, antifungal susceptibility testing for filamentous fungi has recently been standardized (86). Amphotericin B has good activity against most clinically important dematiaceous fungi in vitro, but clinical resistance is not uncommon. *Scedosporium prolificans* and *Scopulariopsis brumptii* are consistently resistant to amphotericin B in vitro; and occasional resistance to this drug is reported in several other species, including *Chaetomium* spp., *Curvularia* spp., *Phialemonium* spp., and *Exophiala* spp. (81). Echinocandins are not clinically useful against these fungi. The inefficacies of the echinocandins against these fungi and the relative resistance of these fungi to amphotericin B may be associated with the dense production of melanin in these fungi. The broadest in vitro activity against dematiaceous fungi is achieved with azoles (81). In this regard, we note that azoles are not bound by melanin.

Impact of melanin binding on antibacterial agents. The role of melanin in the protection of bacteria from antimicrobial drugs is largely unexplored. Recently, an *Escherichia coli* strain expressing a recombinant plasmid containing a tyrosinase gene was constructed, and the *E. coli* strain produced melanin in medium supplemented with tyrosine (72). In contrast, *Pseudomonas aeruginosa* melanin does not appear to serve a protective role against antibacterial agents in vitro (111, 112). However, melanin in *Bacillus thuringiensis* can protect the bacteria against pesticides (98). Since little is known about the location of the pigment in bacteria that produce melanin, the protective effects may be limited. For example, if melanin is intracellular, antibiotics such as penicillins that target the cell wall would not be expected to interact with the polymer before they reach their molecular target.

CONCLUSIONS

Melanin production provides survival advantages to myriad microbes in the environment and during infection of diverse hosts. There is conclusive evidence that many types of drugs, including antimicrobial drugs, bind to melanin. In particular, the melanization of certain fungi is associated with reduced susceptibilities to polyene and echinocandin-type drugs in vitro. In contrast, melanization has not been associated with reduced susceptibilities to azole-type drugs, except at high concentrations. Current standard methods for antifungal drug susceptibility testing are not adequate to discern a melanin effect on antifungal drug activity, and measurement of this effect requires the use of time-kill assays or an assessment of metabolic activity. In vivo data demonstrate that compounds that inhibit melanization can reduce the virulence of *C. neoformans* and other fungi. The administration of monoclonal antibodies to melanin or glyphosate (which inhibits the melanization of *C. neoformans*) prolongs the survival of mice lethally infected with *C. neoformans* (92, 110). Similarly, several studies have demonstrated that melanin-deficient *E. dermatitidis* strains are less virulent than melanized strains (22–24, 33). The development of drugs that interfere with melanin polymerization or rearrangement may be useful therapeutic compounds for the treatment of these melanotic fungi and other pathogens that produce melanin (91). Also, it is possible that the use of agents that inhibit melanization may render melanotic fungi susceptible to drugs that bind to melanin. An interesting finding is the fact that voriconazole at 0.125 to 0.5 mg/liter can inhibit conidiation in diverse *Aspergillus* spp., resulting in white colonies (126). Ravuconazole, which is structurally similar to voriconazole, had similar effects only against *Aspergillus fumigatus* and *Aspergillus flavus*. It is possible that the inhibition of melanin formation in vivo may contribute to the therapeutic potencies of these triazoles by increasing the susceptibility to host defense mechanisms. The possibility that certain antifungal agents are less effective against melanotic molds should especially be considered when clinicians make choices for empirical therapy in patients with presumed mycotic diseases.

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