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Reversible Fluconazole Resistance in *Candida albicans*: a Potential In Vitro Model

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To study the development and potential mechanisms of antifungal resistance in relation to antifungal exposure, reversible fluconazole resistance was examined in vitro. *Candida albicans* ATCC 36082 blastospores were passed in liquid yeast nitrogen base medium containing either 4, 8, 16, or 128 μg of fluconazole per ml, and susceptibility testing was performed after each passage. High-level fluconazole resistance (50% inhibitory concentration, >256 μg/ml) developed in the isolates after serial passage in medium containing 8, 16, or 128 μg of fluconazole per ml, but not in isolates passed in 4 μg of fluconazole per ml. Reduced susceptibility was noted within four to seven passages, which was equivalent to 14 to 19 days of exposure to the drug. However, all isolates returned to the susceptible phenotype after 8 to 15 passages in medium lacking the drug; thus, fluconazole resistance was reversible in vitro. In vivo, organisms retained the resistant phenotype after a single passage in the rabbit model of infective endocarditis. Restriction digest profiles and karyotypic analysis of the parent strain and selected fluconazole-resistant and -susceptible isolates from each group were identical. Investigations into the molecular mechanisms of this reversible resistance failed to reveal increased accumulation of mRNA for 14α-demethylase, the target enzyme for fluconazole, or for the candidal multidrug transporters *CDR1* and *BEN*^{r}. This process of continuous in vitro exposure to antifungal drug may be useful as a model for studying the effects of different antifungal agents and dosing regimens on the development of resistance and for defining the mechanism(s) of reversible resistance.

Oropharyngeal candidiasis is a problem of increasing significance in the human immunodeficiency virus-infected population. An estimated 80 to 95% of patients infected with human immunodeficiency virus will experience at least one episode of oropharyngeal candidiasis during the course of their illness. Even though most respond well to a short course of azole therapy, up to 50% will experience a relapse within 1 month after the completion of therapy (4). Currently, the number of patients who experience multiple recurrences of mucosal candidal infections and eventually fail to respond to azole therapy is rising (2, 6, 20). A number of studies have estimated the incidence of clinical fluconazole resistance to be from 6 to 36%, depending on the patient group studied and the case definition used (2, 6, 13). Risk factors suggested to be of importance in the development of fluconazole-resistant mucosal candidal infection include duration of exposure to fluconazole (2, 5, 6, 13, 17) and degree of immunosuppression (6). One study found resistance to fluconazole to be more common in patients who had received a cumulative dose of fluconazole of more than 10 g, indicating that the total dosage of fluconazole may also influence the development of azole resistance (17). However, the relative influence of duration and level of fluconazole exposure on the development or stability of resistance is unknown.

There are many possible reasons for the failure of azole therapy, including inadequate patient compliance, decreased drug absorption or increased drug metabolism due to the use of concomitant medications, infection with an azole-resistant organism, or selection or induction of resistance in the infecting organism during therapy. Accumulating evidence indicates that failure of therapy due to selection or induction of resistance is becoming more common, and a number of investigators have demonstrated a correlation between in vitro fluconazole resistance in *Candida albicans* and clinical failure (2, 5, 6). Furthermore, several studies have used genotypic analysis of isolates to determine that such failures are often due to de novo resistance in a given organism, not selection of an organism with a genotype resulting in a lower level of susceptibility (8, 17).

There are several possible mechanisms of resistance to azole antifungal agents (18, 22). First, failure to accumulate drug intracellularly may result either from a lack of drug penetration due to a change in membrane lipids or sterols or, perhaps more commonly, by active efflux of drug. Recently discovered multidrug transporter genes, *CDR1* and *BEN*^{r}, have been shown to have increased expression in strains of *C. albicans* with high-level fluconazole resistance (21). Second, increased production of the target enzyme 14α-demethylase has been cited as a mechanism of fluconazole resistance in one *Candida glabrata* isolate (23), as well as several *C. albicans* isolates (25). However, transformation studies with *Saccharomyces cerevisiae* have shown that a 20-fold increase in expression of 14α-demethylase increases the MIC of fluconazole only fivefold (14). Thus, it is unlikely that high-level azole resistance develops by this mechanism alone. A third mechanism attributed to azole resistance is a point mutation of the 14α-demethylase gene, potentially leading to a diminished affinity of azoles for the enzyme (25, 26). Finally, alteration in membrane sterol and/or lipid content may also confer resistance (10). Several resistant isolates have been found to accumulate nontoxic 14α-methyl fecosterol instead of the toxic compound 14α-methyl-3,5-diol,

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suggesting that these organisms have a mutation in the sterol $\Delta_{5,6}$-desaturase (15). There is no consensus regarding the most frequent mechanism of resistance, and some isolates have exhibited multiple mechanisms of resistance simultaneously (23, 25).

Despite increasing reports of fluconazole resistance in *C. albicans*, the investigations into the molecular mechanisms of resistance cited above have been performed with relatively few isolates. All of these isolates exhibited stable azole resistance, and little is known about short-term or reversible resistance. Furthermore, it is unknown how different fluconazole dosing regimens affect the development and stability of resistance. To address some of these questions, we developed an in vitro model of fluconazole resistance in *C. albicans*. Using this model, we discovered that reversible high-level fluconazole resistance can rapidly develop after exposure to relatively low concentrations of fluconazole.

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MATERIALS AND METHODS

Materials. Fluconazole was supplied as a powder (Pfizer Inc., New York, N.Y.), reconstituted in distilled water to a concentration of 1 mg/ml, filter sterilized, and stored in aliquots at −70°C. Amphotericin B (Pharma-Tek, Huntington, N.Y.) was likewise reconstituted to a concentration of 3.33 mg/ml and stored. Yeast nitrogen base (YNB) broth (Difco, Detroit, Mich.) supplemented with 0.5% glucose and RPMI 1640 medium with L-glutamine buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS; American Bioorganics, Inc., Niagra Falls, N.Y.) were prepared according to the manufacturer's recommendations. Sabouraud dextrose agar (Difco) was also prepared according to the manufacturer's recommendations.

Organism. *C. albicans* ATCC 36082, originally a clinical isolate, was obtained from the American Type Culture Collection (Rockville, Md.). The organism was cultured in YNB broth with or without fluconazole as described below, and the numbers of cells per milliliter were estimated by spectrophotometry (600 nm).

Development of resistance. A single colony of *C. albicans* ATCC 36082 was used to inoculate 10 ml of YNB broth which was incubated overnight on a rotating drum at 27°C. An aliquot of this culture containing 10^6 cells was then transferred to 10 ml of YNB broth containing 4, 8, 16, or 128 μg of fluconazole per ml, and the cells were incubated as described above. When the cultures reached a density of approximately 10^8 organisms/ml, aliquots containing 10^6 cells were transferred into fresh YNB broth containing the same respective fluconazole concentration and incubated as described above. At each passage, a 1-ml aliquot of the culture suspension was mixed with 0.5 ml of 50% glycerol, and the mixture was frozen at −70°C for subsequent susceptibility testing.

Stability of resistance in vitro. Isolates found to exhibit fluconazole resistance were serially cultured as described above in YNB broth without fluconazole. At each passage, fluconazole susceptibility was determined as described below. Passages were continued until the fluconazole susceptibility of the organisms had returned to the baseline.

Susceptibility testing. The susceptibilities of organisms to fluconazole were determined by a modification of the broth microdilution method described previously (7). Briefly, organisms from frozen aliquots were cultured in YNB broth containing the respective concentration of fluconazole used previously. Next, 10^3 organisms were inoculated into successive wells of a 96-well microtiter plate containing serial twofold dilutions of fluconazole ranging from 0.5 to 256 μg/ml in YNB broth. Control wells contained drug-free YNB. The parental strain of *C. albicans* ATCC 36082 was also included in each assay. The concentration of fluconazole that inhibited growth of the organisms by 50% (IC_{50}) was determined after 48 h of incubation at 35°C by spectrophotometry (405 nm).

Selected isolates were also tested for susceptibility to amphotericin B and fluconazole by the previously reported M27-P microdilution method (19). One thousand *C. albicans* blastospores were inoculated into each well of a 96-well microtiter plate containing serial twofold dilutions of fluconazole (0.5 to 256 μg/ml) or amphotericin B (0.0313 to 16 μg/ml) in RPMI 1640 medium buffered to pH 7.0 with MOPS. The plates were incubated at 35°C for 48 h. Amphotericin B susceptibility was assessed by visual estimation of the concentration of drug that inhibited 100% of growth (IC_{100}) compared to the growth of the drug-free control after 48 h of incubation. Fluconazole IC_{80}s were determined spectrophotometrically (405 nm) after 48 h of incubation.

RNA extraction and Northern (RNA) blotting. Selected fluconazole-susceptible and -resistant *C. albicans* isolates from each drug concentration group (8, 16, and 128 μg of fluconazole per ml) were grown in 150 ml of fluconazole-free YNB broth in a shaking incubator at 30°C until the mid-logarithmic phase of growth. One hundred grams of ice was added to the suspension, and cells were harvested by centrifugation, washed once with 10 ml of cold distilled water, and flash frozen in an ethanol-dry ice bath. The candidal RNA was extracted with glass beads and phenol as described by Langford and Gallwitz (16). Ten micrograms of total RNA per lane was then electrophoresed in a 1% agarose formaldehyde gel and transferred to nylon membranes (Micron Separation, Inc., Westboro, Mass.). The membranes were hybridized with the *C. albicans* 14α-demethylase gene (generously provided by T. White, University of California, San Francisco) that was labeled with [^{32}P]dCTP by the random primer method (NEblot; New England Biolabs, Beverly, Mass.). Membranes were also probed with a [^{32}P]dCTP-labelled actin gene from *C. albicans* to correct for differences in RNA loading.

Expression of drug transporter genes. Membranes containing total RNA prepared in our laboratory were probed for expression of *CDR1* and *BEN*^{r} mRNA (kindly performed by D. Sanglard, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland) as described previously (21). Differences in the amounts of RNA loaded in these blots were corrected by measuring the constitutively expressed *TEF3* gene.

Phenotypic analyses of isolates. Growth curves were determined for the strains used for RNA isolation as described above. The biochemical fermentation patterns of the parent strain and a resistant isolate were determined with the API system (bioMerieux, Hazelwood, Mo.).

Karyotypic analyses of isolates. The parent strain (ATCC 36082) and one resistant and one susceptible isolate from each drug exposure group were analyzed for molecular relatedness by karyotype and restriction endonuclease digestion (kindly performed by M. Pfaller, University of Iowa Hospitals and Clinics, Iowa City) as described previously (3, 24). DNA samples isolated by standard techniques were analyzed on karyotype gels with Fast Lane Agarose (FMC, Rockland, Maine), with a switch time ramped from 120 to 280 s over 24 h. *S. cerevisiae* chromosomal DNA was included as a size standard. Restriction endonuclease digestion was performed with *Bss*HII and *Sfi*I (New England Biolabs), and the resulting digests were run with SeaKem GTG Agarose (FMC), with a switch time ramped from 10 to 90 s over 24 h. All electrophoretic analyses were performed at 13°C on a CHEF DR II (Bio-Rad, Hercules, Calif.) apparatus with 1% agarose.

Animal studies. Polypropylene catheters were placed across the aortic valves of New Zealand White rabbits (weight, 2.5 kg) as described previously (27). Two animals each were injected with 2×10^7 CFU of logarithmic-phase *C. albicans* ATCC 36082 (parent strain; IC_{50}, 1.0 μg of fluconazole per ml) or a resistant strain (strain 128-8, which had been passed in 128 μg of fluconazole per ml eight times and for which the fluconazole IC_{50} was >256 μg/ml). The animals were sacrificed 72 h after infection, and the cardiac vegetations, kidneys, and spleen were excised, weighed, homogenized, and quantitatively cultured in duplicate on Saboraud dextrose agar with or without fluconazole at 128 μg/ml.

RESULTS

Development of resistance. All isolates serially passed in 8, 16, or 128 μg of fluconazole per ml developed high-level fluconazole resistance (IC_{50}s, ≥256 μg/ml) (Fig. 1). Reduced susceptibility to fluconazole was detected after four to seven passages, corresponding to 14 to 19 days of exposure to the drug. Organisms grown in 128 μg of fluconazole per ml attained high-level fluconazole resistance the most rapidly (IC_{50}s, ≥256 μg/ml after only four passages, or 15 days of drug exposure). An equivalent level of resistance took the longest to develop in organisms exposed to 8 μg of fluconazole per ml, with IC_{50}s of ≥256 μg/ml occurring after eight passages, or 19 days of drug exposure. A rapid rise in IC_{50}s was found for organisms grown in 16 and 128 μg of fluconazole per ml, whereas for organisms grown in 8 μg/ml, IC_{50}s increased in a more stepwise fashion. For isolates grown in 4 μg of fluconazole per ml, no significant increase in the IC_{50}s was found after 10 passages (25 days), at which time that portion of the experiment was terminated (data not shown).

Susceptibility testing was performed in YNB because it was the medium used during the development of resistance. However, selected isolates representing both fluconazole-susceptible and -resistant organisms were tested by the microdilution method of the M27-P protocol of the National Committee for Clinical Laboratory Standards for comparison, since this method uses different medium (RPMI 1640) and a different growth inhibition cutoff (IC_{80}) from those used in our protocol. The IC_{80}s of fluconazole by the M27-P protocol were within 1 dilution of the IC_{50}s obtained by the YNB testing method

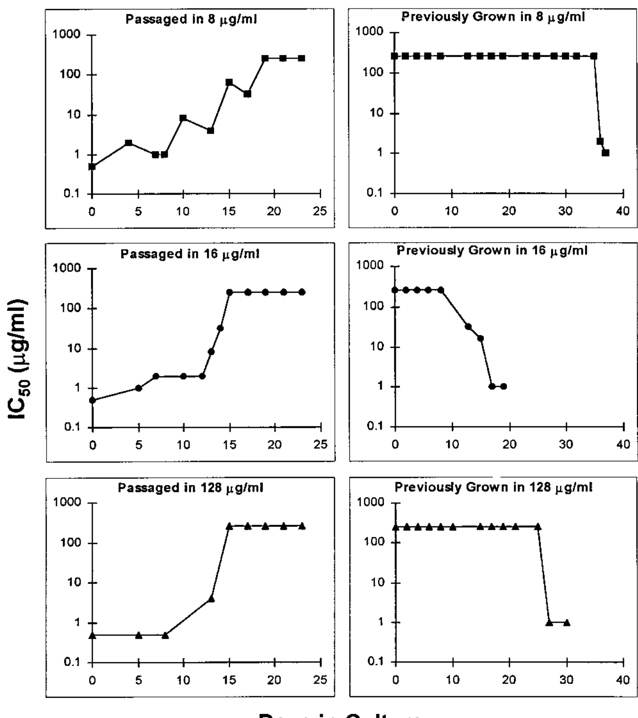

FIG. 1. Time of induction of fluconazole resistance for isolates grown in YNB containing 8, 16, or 128 μg of fluconazole per ml (left panels) and time until reversion of fluconazole-resistant isolates to susceptibility phenotype when passed in YNB broth without fluconazole (right panels). Each datum point represents one passage.

(Table 1). There was no increase in the IC_{100} of amphotericin B for the resistant isolates tested (data not shown).

Phenotypic and genotypic analyses. The metabolic profiles of the parent strain and a resistant isolate were similar. Growth curves for the fluconazole-sensitive and -resistant isolates used for extraction of total RNA revealed that doubling times did not differ significantly between the strains (Table 2). Genotypic analyses revealed that all strains examined had identical karyotypes and restriction enzyme profiles (Fig. 2).

Stability of resistance in vitro. After 23 days of exposure to fluconazole, fluconazole-resistant organisms were passed in YNB broth without fluconazole to assess the stability of resistance. All isolates eventually reverted to the susceptible phenotype of the parent strain (Fig. 1), but they required up to 38 days to revert. The isolate grown in 16 μg of fluconazole per ml maintained high-level resistance for the shortest duration, reverting to the susceptible phenotype after only seven passages (17 days) in drug-free medium. This time to reversion was reproducible in duplicate tests (data not shown).

Stability of resistance in vivo. The effect of in vivo passage on fluconazole resistance was examined by using the rabbit model of infective endocarditis. *C. albicans* isolates recovered from two animals 72 h after infection with a resistant isolate retained the resistant phenotype. Both the fluconazole-sensitive and -resistant strains caused endocarditis and disseminated infection in the spleens and kidneys of all rabbits tested. In addition, the mean cardiac fungal density was approximately the same (log 5 CFU/g of tissue) in all animals.

Expression of 14α-demethylase and multidrug transporters. To evaluate the potential mechanisms responsible for fluconazole resistance, we examined the level of mRNA accumulation of some candidal genes believed to be associated with azole resistance. Differences in the accumulation of 14α-demethylase mRNA between the strains (Fig. 3) were not correlated with fluconazole susceptibility. Likewise, all strains contained low to undetectable levels of mRNA for the multidrug transporter genes *CDR1* and *BEN*^{r} (data not shown).

TABLE 1. Comparison of susceptibilities by method of testing

Isolate[a]	IC_{80} (μg/ml) by M27-P method	IC_{50} (μg/ml) by YNB broth method
Parent	2	1
8-R	>256	>256
8-S	0.5	1
16-R	>256	256
16-S	1	1
128-R	>256	>256
128-S	1	1

[a] Isolate nomenclature: number, concentration of fluconazole in medium (in micrograms per milliliter); R, resistance phenotype; and S, reverted susceptibility phenotype.

TABLE 2. Growth rates of susceptible and resistant isolates in YNB broth at 30°C

Isolate[a]	Doubling time (h)
Parent	1.7
8-R	1.5
8-S	1.6
16-R	1.9
16-S	1.6
128-R	1.9
128-S	1.9

[a] Isolate nomenclature: number, concentration of fluconazole in medium (in micrograms per milliliter); R, resistance phenotype, and S, reverted susceptibility phenotype.

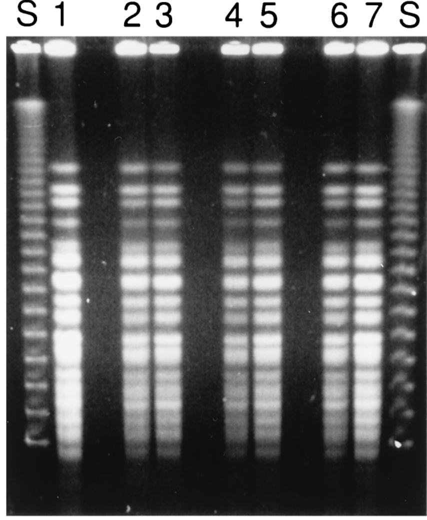

FIG. 2. Restriction endonuclease digestion with *Bss*HII of DNA from the isolates listed in Table 1. The isolates are as follows: lane 1, ATCC 36082 (parent strain); lanes 2 and 3, resistant isolate grown in YNB broth with 8 μg of fluconazole per ml and the reverted susceptible isolate; lanes 4 and 5, resistant isolate grown in YNB broth with 16 μg of fluconazole per ml and the reverted susceptible isolate; and lanes 6 and 7, resistant isolate grown in YNB broth with 128 μg of fluconazole per ml and the reverted susceptible isolate. S, *Bss*HII-digested *Saccharomyces* DNA, which was used as a control.

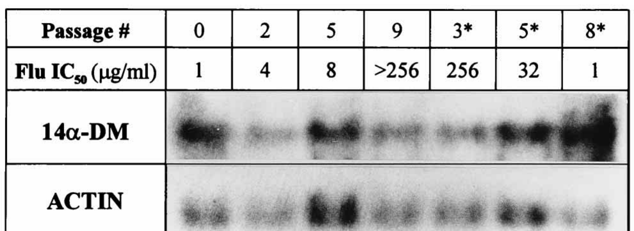

FIG. 3. Expression of 14α-demethylase (14α-DM) RNA in selected isolates grown in YNB broth with 16 μg of fluconazole (Flu) per ml. The asterisks indicate passages in drug-free medium.

DISCUSSION

Using in vitro conditions simulating chronic fluconazole exposure, we created high-level fluconazole resistance in *C. albicans*. This resistance developed rapidly (within 15 to 20 days) for all organisms exposed to concentrations of fluconazole of ≥8 μg/ml. Organisms exposed to 4 μg of fluconazole per ml remained susceptible to the drug even after 10 passages (25 days). Thus, it is possible that this concentration did not create a strong enough selective pressure or that a longer period of time was necessary to develop the resistance.

Although the fluconazole-resistant organisms eventually reverted to the susceptible phenotype with prolonged growth in drug-free medium, they remained resistant after a 3-day passage in the rabbit model of endocarditis. Whether longer growth in vivo would result in a loss of fluconazole resistance similar to that in the in vitro model remains to be determined. Also, it has been suggested that fluconazole-resistant *C. albicans* isolates are less virulent in vivo. We used too few animals in our study to be able to draw any conclusions regarding the relative pathogenicity of these organisms. However, the fluconazole-resistant strain retained at least some ability to cause endocarditis in vivo.

The increasing incidence of fungal infections and the widespread use of the newer oral triazoles have led to a resurgence of interest in antifungal resistance. Previous attempts to create azole resistance in vitro have provided limited information. Holt and Newman (11) attempted to induce clotrimazole resistance in *C. albicans* by passing organisms on increasing concentrations of drug in agar medium, but they found no increase in the MICs after 10 to 15 passages. In a recent review, Iwata (12) cited a number of other attempts to induce azole resistance, which yielded disappointing results. However, high-level resistance (a greater than 100-fold increase in the MIC) to amphotericin B has been successfully induced in *C. albicans* after 30 to 40 passages in drug-containing solid medium (1). Thus, it is possible that previous investigations provided organisms an insufficient duration of exposure to drug. It is also possible that passage in liquid medium is more efficient at inducing resistance, as has been our experience (unpublished data). Similarly, Hernandez et al. (9) were able to induce an increase in the fluconazole MIC by serially passaging *C. albicans* in liquid medium containing fluconazole. In that study, for two of four *C. albicans* strains assayed, an eightfold increase in the MIC was found after 16 to 17 passages.

In our study, the emergence of resistance may have resulted from one of several potential mechanisms: (i) selection of a subpopulation with an unstable, reversible mutation, (ii) selection of a subpopulation with altered metabolic activities conferring decreased fluconazole susceptibility, or (iii) up-regulation or induction of a latent resistance mechanism. Each of these possibilities could explain our present findings. The karyotypic and restriction enzyme analyses revealed no detectable genetic difference between the resistant and sensitive organisms. However, it should be noted that such analyses are generally not sensitive enough to detect minor chromosomal mutations (e.g., point mutation of a single base pair). Also, we observed no significant differences in the growth rates or carbohydrate fermentation profiles of the sensitive and resistant isolates, so it is unlikely that metabolic differences or differential growth rates accounted for resistance. On the other hand, minor metabolic changes that may not cause an alteration in the fermentation profile or growth rate could account for resistance. Finally, we found no consistent changes in the accumulation of mRNA of the 14α-demethylase gene, as has been reported by White (25) and others (14), or of two known multidrug transporters, *CDR1* and *BEN*^{r}, reported by Sanglard and coworkers (21). However, since that study was performed, new drug transporters have been described.

Possible resistance mechanisms that remain to be investigated include (i) reduced intracellular drug accumulation, which may be due to an alteration in the permeability of the cell to the drug or active drug efflux; (ii) bypass of the normal sterol biosynthetic pathway; or (iii) mutation of a key gene encoding a fluconazole target, such as 14α-demethylase. Finally, it is possible that these isolates may display a novel mechanism of resistance.

In conclusion, these studies demonstrate that with the ATCC 36082 strain of *C. albicans*, continuous exposure to relatively low concentrations of fluconazole is sufficient for the development of high-level resistance. This resistance was reversible and did not appear to be mediated by increased expression of 14α-demethylase or of two known multidrug transporters. This in vitro model may be useful for evaluating the effects of different dosing regimens on the development of fluconazole resistance, since longitudinal human studies of continuous fluconazole exposure support our findings of increasing levels of resistance with time (8, 13). Other parameters could also be addressed with this type of model, including examinations of whether other *C. albicans* isolates develop resistance as rapidly as strain ATCC 36082 and comparison of the ability of different azole antifungal drugs to select resistance phenotypes. Finally, a model such as this could enable future investigation into the molecular mechanism(s) of reversible resistance, which has received limited attention to date.

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