

## Drug Interaction Studies of a Glucan Synthase Inhibitor (LY 303366) and a Chitin Synthase Inhibitor (Nikkomycin Z) for Inhibition and Killing of Fungal Pathogens

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**The interaction between inhibitors of components of the fungal cell wall, glucan and chitin, was studied in vitro with the respective synthase enzyme inhibitors LY 303366 and nikkomycin Z. With *Aspergillus fumigatus* synergy was noted for inhibition and killing, and synergistic activity was also noted for some isolates of other species presently regarded as difficult to treat.**

Because of the paucity of current antifungal drugs and targets (15), the development of new agents directed at novel targets is welcome. Particularly appealing are agents directed at fungus-specific targets, such as the cell wall. At present, two classes of cell wall inhibitors, glucan and chitin synthase inhibitors, are in development. Their spectra of activity have limitations; in particular, fungicidal activity against filamentous pathogens and agents of the endemic mycoses may be a gap for one or both of these classes.

This is a preliminary survey of the interaction of these two classes against pathogens which represent problems in therapy, particularly *Aspergillus* spp. The rationale includes evidence that glucan and chitin are structurally linked in the cell wall (5, 9), so dual inhibition could produce an enhanced effect. Hydrolytic enzymes (e.g., chitinase and glucanase) inhibit fungi synergistically (2, 11). Moreover, there is evidence that fungi may adapt to inhibition of synthesis of one wall component by compensatory production of another (19); this again leads to the theoretical expectation that hits on two targets could produce an enhanced effect.

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LY 303366 (LY) (D. A. Stevens, M. Martinez, and M. J. Devine, Abstr. 36th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F46, 1996) was selected as a representative of the group of glucan synthase inhibitors, and nikkomycin Z (NZ) (6) was selected as the representative of chitin synthase inhibitors.

Susceptibility testing was performed by broth macrodilution (twofold dilution) in a checkerboard design. The methodology has been extensively reported, providing the largest data set for *Aspergillus* tested by one method in one laboratory (3). The methods for inoculum preparation and for the determination of MIC and minimum fungicidal concentration (MFC) have been described in detail (16) for the organisms (randomly selected from our culture collection) studied here, with the exception of the *Fusarium* species, for which we employed the same methods as for other filamentous organisms. In brief, the end point for the MIC is the first clear tube and  $\geq 96\%$  killing

is defined as the MFC end point. For *Candida albicans*, the National Committee for Clinical Laboratory Standards standard was used (12).

Checkerboard drug interaction methodology, including the calculation of a fractional inhibitory concentration index (FIC<sub>i</sub>) (4), has been detailed elsewhere (3, 17). In brief, an FIC<sub>i</sub> of 1 represents an additive effect, an FIC<sub>i</sub> of  $>2.0$  demonstrates antagonism, and an FIC<sub>i</sub> of  $<1.0$  demonstrates synergy. The upper end of concentrations tested was 50 to 100  $\mu\text{g/ml}$  for LY and 800 to 1,600  $\mu\text{g/ml}$  for NZ, as governed by considerations of maximum solubility and drug availability. The lower end was determined in part by MIC determinations prior to checkerboard testing (Stevens et al., 36th ICAAC); to have several rows of the checkerboard below the MIC available for determination of synergy necessitated some series descending to 0.002 and 0.00003  $\mu\text{g/ml}$  for NZ and LY, respectively. In instances where marked resistance to a drug resulted in failure to determine a precise MIC, a precise FIC cannot be calculated. Such instances are common with these agents, as they do not produce a clear tube with filamentous organisms (1). In such instances, the most conservative assumption was made, i.e., that the MIC was the next highest dilution above that tested; this assumption could thus result in underestimating the degree of positive drug interaction (i.e., presents the upper FIC<sub>i</sub> limit).

Analogous procedures were performed to examine the interaction for killing. Clear (and trace growth) tubes in the checkerboard matrix were subcultured, as in the determination of MFCs. This enables the calculation of a fractional fungicidal concentration index (FFC<sub>i</sub>), analogous to FIC<sub>i</sub>.

The results with five clinical isolates of *Aspergillus* (all *Aspergillus fumigatus*) are shown in Table 1. Although cell wall inhibitors produce deformed *Aspergillus* mycelial growth in vitro (1), neither drug alone was active using the classical MIC and MFC end points. All five isolates showed powerful synergy for both inhibition and killing (Table 1). For example, for isolate 10AF the MIC and MFC were 800 and  $>100 \mu\text{g/ml}$  for NZ and LY, respectively; the isolate was inhibited and killed by 25  $\mu\text{g}$  of NZ plus 3.1  $\mu\text{g}$  of LY/ml.

These findings led to a representative survey of other pathogens for which currently available therapy produces less than desirable results. For *Rhizopus* sp. isolates 94-2 and 94-69, both the MIC and MFC for NZ and LY were 50 and  $>50 \mu\text{g/ml}$ , respectively. For both isolates there was synergy for inhibition

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TABLE 1. Susceptibility of *A. fumigatus* to cell wall inhibitors and their drug interaction

<i>A. fumigatus</i> isolate	MIC, MFC ( $\mu\text{g/ml}$ ) of:		FIC <sub>i</sub>	FFC <sub>i</sub>
	NZ	LY		
10AF	800	>100	$\leq 0.045$	$\leq 0.045$
92-270	>1,600	>50	$\leq 0.075$	$\leq 0.075$
92-245	>1,600	>50	$\leq 0.125$	$\leq 0.125$
93-19	>1,600	>50	$\leq 0.090$	$\leq 0.090$
94-46	>1,600	>50	$\leq 0.038$	$\leq 0.038$

and for killing (FIC<sub>i</sub> and FFC<sub>i</sub> for both isolates were  $\leq 0.375$ , though two tubes in the matrix for 94-69 showed growth in the presence of  $\geq 50 \mu\text{g}$  of NZ/ml, which for those tubes would represent antagonism). The MICs and MFCs of NZ for *Fusarium* sp. isolates 96-1 and 93-198 were 1,600 and 800  $\mu\text{g/ml}$ , respectively, and those of LY were  $>50 \mu\text{g/ml}$ . For 96-1, the drugs were modestly synergistic for both inhibition (FIC<sub>i</sub>,  $\leq 0.5$ ) and killing (FFC<sub>i</sub>,  $\leq 0.5$ ), and for 93-198 they were modestly antagonistic (FIC<sub>i</sub> and FFC<sub>i</sub>,  $\geq 2.06$ ).

For *Coccidioides immitis* strain Silv., tested in the mycelial phase (16), the MIC and MFC of NZ were 800 and  $>800 \mu\text{g/ml}$ , respectively, and those of LY were 12.5  $\mu\text{g/ml}$ . There was powerful synergy for inhibition (FIC<sub>i</sub> = 0.008; as both MICs were on scale, a precise index can be computed) but no synergy for killing (no killing in any tube with  $<12.5 \mu\text{g}$  of LY/ml). This indicated the need to study *C. immitis* further, using the more clinically relevant parasitic phase (10). The MIC and MFC were both  $\geq 25$  and 0.78  $\mu\text{g/ml}$  for LY and NZ, respectively. It is noteworthy that this pathogen is thus 1,000-fold more susceptible to NZ inhibition in the parasitic phase and that NZ is  $>1,000$ -fold more active in killing. A related chitin synthase inhibitor has been shown to also be much more active against the parasitic phase of *C. immitis* (7). In contrast, LY is less active against the parasitic phase. In combination, there was again synergy for inhibition (FIC<sub>i</sub>  $\leq 0.129$ ) but now also powerful synergy for killing (FFC<sub>i</sub>  $\leq 0.129$ ).

For *C. albicans* isolate 94-93, LY was very inhibitory (MIC = 0.008  $\mu\text{g/ml}$ ) and fungicidal (MFC = 2  $\mu\text{g/ml}$ ), whereas NZ had no activity (MIC and MFC  $> 2,048 \mu\text{g/ml}$ ). NZ potentiated LY inhibition (FIC<sub>i</sub>  $\leq 0.13$ ), and there was a trend for improvement of killing, but this did not meet the cutoff for killing used. For *Histoplasma capsulatum* isolate G217B, yeast form, there was slight antagonism in inhibition (FIC<sub>i</sub> = 2.02) and indifference with respect to killing (neither drug killed alone or together at the concentrations studied).

Synergy between azole drugs and NZ has been described previously (8). This was confirmed with itraconazole and an *A. fumigatus* isolate (FIC<sub>i</sub>  $\leq 0.09$ , FFC<sub>i</sub>  $\leq 0.14$ ). LY acted less synergistically with itraconazole for inhibition (FIC<sub>i</sub>  $\leq 0.51$ ), and there was no synergy for killing. When all three drugs were combined by adding constant amounts of a third drug to a standard checkerboard, there was no further improvement in the two-drug synergistic interactions already described.

In summary, LY-NZ synergy was most impressive for *Aspergillus* and *Coccidioides* and less so for *Candida* and *Rhizopus*. *Fusarium* studies gave a mixed picture, and a *Histoplasma* study was not promising. Thus the results were genus and even isolate specific. Some synergistic combinations between a glu-

can synthase inhibitor studied earlier (now abandoned), ciclofungin, and NZ have similarly been reported (14). The findings reported here also suggest further exploration of combination therapy and point to where such inquiry is more likely to be productive. Important questions are raised, including whether the positive interactions can also be demonstrated using a recently developed, slightly different standardized methodology for testing filamentous fungi (13) and whether these observations apply to other inhibitors in each class and to other *Aspergillus* species. Although powerful synergy for inhibition, killing, or both between NZ and LY was seen, more isolates need testing and the relevance of the synergism for in vivo activity needs to be determined.

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