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Activity of 2,4-Diaminoquinazoline Compounds against *Candida* species

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Forty recent clinical isolates of three different *Candida* sp. were tested in the microtiter system for susceptibility to two new 2,4-diaminoquinazoline (DAQ) compounds, amphotericin B and flucytosine. The two DAQ preparations showed activity similar to amphotericin B and flucytosine. The geometric mean minimal inhibitory concentrations for these four drugs were as follows: DAQ 1A, 0.64 μg/ml; DAQ 2A, 1.39 μg/ml; amphotericin B, 1.03 μg/ml; and flucytosine, 0.72 μg/ml. An additional seven DAQ compounds were tested but showed less or no activity against 17 *Candida* isolates. Forty-eight-hour viability studies with DAQ 2A alone or in combination with amphotericin B, flucytosine, or sulfamethoxazole were carried out with one isolate of intermediate susceptibility to each of these agents except sulfamethoxazole. For this isolate the combination of DAQ 2A and sulfamethoxazole was synergistic, and the combination of DAQ 2A and AMB was either synergistic or additive, whereas the combination of DAQ 2A and flucytosine was antagonistic. Although regrowth of cultures exposed to DAQ 2A was noted over a 48-h period, neither degradation of the drug nor development of resistance to the drug could be detected. Swiss white mice receiving DAQ 1A at a dose of 6 mg/kg for 5 days showed no obvious signs of toxicity, including weight loss.

Currently there are two agents available for the treatment of systemic *Candida* infections, amphotericin B (AMB) and flucytosine (FC) (5-7, 12). Because of limitations associated with each agent, efforts continue to develop additional drugs with activity against these species. Members of the 2,4-diaminoquinazoline (DAQ) class of compounds, which are antifolates, were shown by Hynes et al. (17) to be active in vitro against *Candida albicans* while displaying no toxicity in mice at potentially therapeutic levels. Hariri and Larsh (11) used a DAQ drug to cure mice of infection with *Cryptococcus neoformans*. The purpose of this study was to investigate the in vitro activity of some other DAQ (9; K. W. Ledwig, Chem. Abstr. **88**:105410d) drugs against several *Candida* sp. In addition, viability experiments were carried out with one of the more active DAQ compounds when combined with either AMB, FC, or sulfamethoxazole (SMX). Drug stability and resistance development were investigated, and limited data are also discussed concerning the toxicity of a new DAQ compound in the mouse.

MATERIALS AND METHODS

Isolates. Forty recent clinical isolates of *Candida* sp. were identified as to species by conventional techniques (2, 10). Thirty-two were identified as *C. albicans*, four were identified as *C. tropicalis*, and four were identified as *C. krusei*.

Media. Synthetic amino acid medium-fungal (SAAMF) was utilized and was kindly supplied by John R. Dixon (Grand Island Biological Co., Product Development Office, Lawrence, Mass.). This completely defined medium has been shown to have a number of advantages for the in vitro testing of antifungal compounds (14, 16).

Drugs. H. E. Alburn of Wyeth Laboratories, Philadelphia, Pa., provided five of the DAQ drugs tested (Fig. 1, compounds 1A to 5A), and the other four DAQ drugs (Fig. 1, compounds 1B to 4B) were obtained through the courtesy of E. F. Elslager of Parke-Davis Laboratories, Detroit, Mich. AMB (Calbiochem, San Diego, Calif.), FC (Hoffman-La Roche, Nutley, N.J.), and the antifolates methotrexate (Lederle, Pearl River, N.Y.), pyrimethamine (Burroughs Wellcome, Research Triangle Park, N.C.), and trimethoprim (Burroughs Wellcome) were used in susceptibility testing, but SMX (Burroughs Wellcome) was used only in the viability studies. Stock solutions of all drugs were prepared at a concentration of 10 mg/ml. The DAQs and methotrexate were stored in the dark at room temperature, whereas all other drug solutions were prepared just before use. Me_2SO was used to solubilize all the agents except FC (distilled water), trimethoprim (0.05 N HCl) and SMX (0.1 N NaOH).

Susceptibility testing. Minimal inhibitory concentrations (MICs) were determined with the microtiter system. The inoculum employed was 5,000 colony-forming units per ml, and the drug concentrations

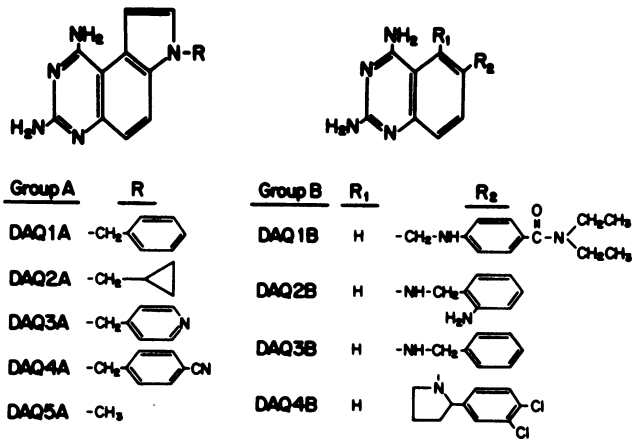

FIG. 1. *Shown are the structures of the nine DAQ compounds.*

ranged from 50 to 0.025 μg/ml. The final volume in the microtiter wells was 0.1 ml. The concentration of Me_2SO never exceeded 1%, and preliminary experiments showed that this did not have any inhibitory effect on any of the *Candida* isolates. After inoculation the microtiter plates were sealed with cellophane tape and incubated at 37°C for 24 h. Small pinholes were made in the plastic tape above each well allowing for aerobic conditions. Minimum fungicidal concentrations (MFCs) were obtained by subculturing, with a 0.001-ml bacteriological loop, on brain heart infusion agar, and the lowest concentration of antifungal agents which yielded no growth on subculture was considered to be the MFC. MIC and MFC determinations were performed in duplicate, and the maximum difference noted between duplicates was one dilution. The higher dilution was always utilized to determine the MIC and MFC. AMB, FC, and the two most active DAQs, DAQ 1A and DAQ 2A, were tested against 40 *Candida* isolates; the remaining compounds were tested against 17 isolates.

Viability studies. One of the more active compounds, DAQ 2A, was employed with one of three other compounds, FC, AMB, or SMX, to determine the effect of these agents individually and in combination against an isolate of *C. albicans*. This isolate had the following susceptibilities: AMB, MIC/MFC = 0.75/0.75 μg/ml; FC, MIC/MFC = 0.75/3.1 μg/ml; DAQ 2A, MIC/MFC = 0.75/0.75 μg/ml; and SMX, MIC/MFC > 50 μg/ml.

In these viability experiments the initial concentration of *Candida* organisms ranged between 10^4 and 10^5 colony-forming units per ml. Each of the drugs was employed at a concentration equal to its MIC, except for SMX which was inactive against the *Candida* isolate used at 50 μg/ml and was utilized at this concentration. DAQ 2A was also used at a 10-fold-higher concentration in experiments for studying its effect when combined with SMX. In these experiments the concentration of Me_2SO was also maintained at less than 1%, and the flasks were incubated in a shaking water bath at 37°C for 48 h, with a sample of 0.5 ml taken at 0, 3, 6, 9, 12, 24, 30 and 48 h. Serial dilutions were plated out of each sample to determine the number of viable organisms. Fungal growth without any drug was run 11 times in the course of these experiments, and a control curve was constructed from all of these so that these curves are identical for Fig. 2, 3, and 4. The effect of DAQ 2A at 0.75 μg/ml was repeated eight times, and a single curve was constructed from these data and is depicted in Fig. 3 and 4. All the other agents and combinations were run four times, except DAQ 2A at 7.5 μg/ml, SMX, and the combination (Fig. 2), which were run three times each.

Drug stability and resistance development. The stability of DAQ 2A (at 7.5 μg/ml) was studied by taking samples from times 0 and 48 h from two flasks treated exactly like those in the viability study except that only one was inoculated with *Candida*. The fungistatic activity of the supernatants, obtained by centrifugation, was then determined with the microtiter system. Also, an attempt was made to detect resistance development by repeating the MIC determination of the isolate used in the viability studies after 48 h of exposure to DAQ 2A at a concentration of 0.75 μg/ml.

Toxicity experiment. ICR Swiss female white mice weighing between 22 and 25 g were treated with 6 mg of DAQ 1A per kg per day administered intraperitoneally for 5 days. Six animals were given the drug and six animals were given Me_2SO, and their weights were determined daily.

RESULTS

Table 1 summarizes the susceptibility data for all compounds which showed any activity at a concentration of 50 μg/ml. AMB, FC, DAQ 1A, and DAQ 2A all showed comparable inhibitory activity against the 40 *Candida* isolates. The geometric mean MICs for these compounds were as follows: AMB, 1.03 μg/ml; FC, 0.72 μg/ml; DAQ 1A, 0.64 μg/ml; and DAQ 2A, 1.39 μg/ml. Only AMB showed fungicidal activity against the majority of the isolates. The eight isolates which were not *C. albicans* exhibited a similar susceptibility pattern to these agents except for FC; the two isolates resistant to FC were *C.*

TABLE 1. *Susceptibility of Candida isolates to AMB, FC, and seven DAQ compounds*

Agent	Determination	No. of isolates susceptible at concn of agent (μg/ml):										
		0.05	0.1	0.19	0.38	0.75	1.5	3.1	6.2	12.5	25	≥50
AMB	MIC				3	19	15	3	0	0		
	MFC				1	5	17	12	3	2		
FC	MIC		3	6	10	9	4	5	1	0	1	1
	MFC		0	0	2	2	4	8	7	6	0	11
DAQ 1A	MIC	2	1	2	9	13	12	1	0	0	0	0
	MFC	0	0	0	1	1	2	1	11	15	8	1
DAQ 2A	MIC				3	8	22	5	2	0	0	0
	MFC				0	2	0	1	12	4	3	18
DAQ 3A	MIC					1	1	3	2	3	5	2
	MFC					0	0	0	0	1	1	15
DAQ 4A	MIC						3	7	5	2	0	0
	MFC						0	0	1	1	3	12
DAQ 5A	MIC								1	1	2	13
	MFC								0	0	0	17
DAQ 3B	MIC						3	3	6	4	1	0
	MFC						0	0	0	1	5	11
DAQ 4B	MIC							1	6	4	4	2
	MFC							0	2	4	5	6

tropicalis and *C. krusei*. DAQ 3A, DAQ 4A, DAQ 5A, DAQ 3B, and DAQ 4B demonstrated less activity, whereas DAQ 1B, DAQ 2B, methotrexate, pyrimethamine, and trimethoprim all showed no activity at a concentration of 50 μg/ml.

Viability data are shown in Fig. 2, 3, and 4. Figure 2 shows the effect of DAQ 2A, SMX, and the combination of both agents on the growth of *Candida*. The combination resulted in more pronounced killing of the organism, and regrowth occurred later and to a lesser degree, indicating that an inactive concentration of SMX was sufficient to markedly augment the action of DAQ 2A. The regrowth of the organism after 24 h of exposure to DAQ 2A was marked, so that by 48 h the original concentration of organisms was slightly exceeded.

Figure 3 demonstrates the effect of DAQ 2A, AMB, and the combination of both agents on *Candida* growth. AMB resulted in more than a 10-fold reduction in numbers of *Candida* organisms when compared with the control at 12 h, although by 48 h considerable regrowth had occurred. The most striking effect of the combination of DAQ 2A and AMB was to delay regrowth of the organisms after 12 h.

The effects of DAQ 2A, FC and the combination of both agents on *Candida* growth are shown in Fig. 4. FC retards growth of the yeast, but when combined with DAQ 2A the growth curve is similar to the control curve. DAQ 2A alone results in slower growth initially, but by 30 h the concentration of organisms is equal to that of the control. It should be noted that these viability studies employed an inoculum 10 times greater than that used to determine MICs. This may explain why some of these drugs, despite being used at a concentration equal to the MIC of the *Candida* isolate, only minimally inhibited its growth.

Resistance development to DAQ 2A was not detected because the MIC of the isolate (0.75 μg/ml) used in the viability studies was not significantly different from that obtained after 48 h of exposure to the drug (1.5 μg/ml or one twofold dilution). In addition, 0- and 48-h samples of SAAMF, containing an initial DAQ 2A concentration of 7.5 μg/ml in the presence and absence of organisms, were both effective at a 1:8 dilution in inhibiting the organism used in the viability studies.

Finally, mice given 6 mg of DAQ 1A per kg per day for 5 days showed no weight loss or other signs of toxicity.

DISCUSSION

The data in Table 1 show that two new DAQ compounds, DAQ 1A and DAQ 2A, were as effective as AMB and FC in inhibiting the growth of 40 *Candida* isolates. However, AMB acted fungicidally, whereas the DAQ drugs were more similar to FC in that only occasional isolates were killed by lower drug concentrations. However, from the viability experiments it was evident that if the concentration of drug was increased 10-fold against the same isolate, killing of the organism occurred (compare Fig. 2 and 3).

The synergistic activity of the DAQ 2A-SMX combination has a rational basis. SMX antagonizes folate synthesis, and DAQ 2A acts to inhibit the enzyme dihydrofolate reductase,

thereby providing a sequential blockade of a single biochemical pathway. A similar phenomenon occurs when trimethoprim is combined with SMX (13). This potentiation of DAQ 2A's activity by SMX may be important in utilizing DAQ drugs for the treatment of fungal infection.

The enhanced activity of AMB by DAQ 2A, which is demonstrated by the slower regrowth of the organism but not by greater killing, is of considerable interest. To explain this enhanced activity one can propose that AMB acts on the fungal cell membrane to increase its permeability to other drugs (19). This interpretation may be the explanation for the enhancement, by AMB, of the activity of FC (20), clotrimazole (4) and antimicrobials such as rifampin (3) and some tetracyclines (1, 18) against fungi.

The antagonism of the activity of FC by DAQ 2A was not expected but is explainable. FC enters the fungal cell and is converted to fluorodeoxyuridine monophosphate (7). This com-

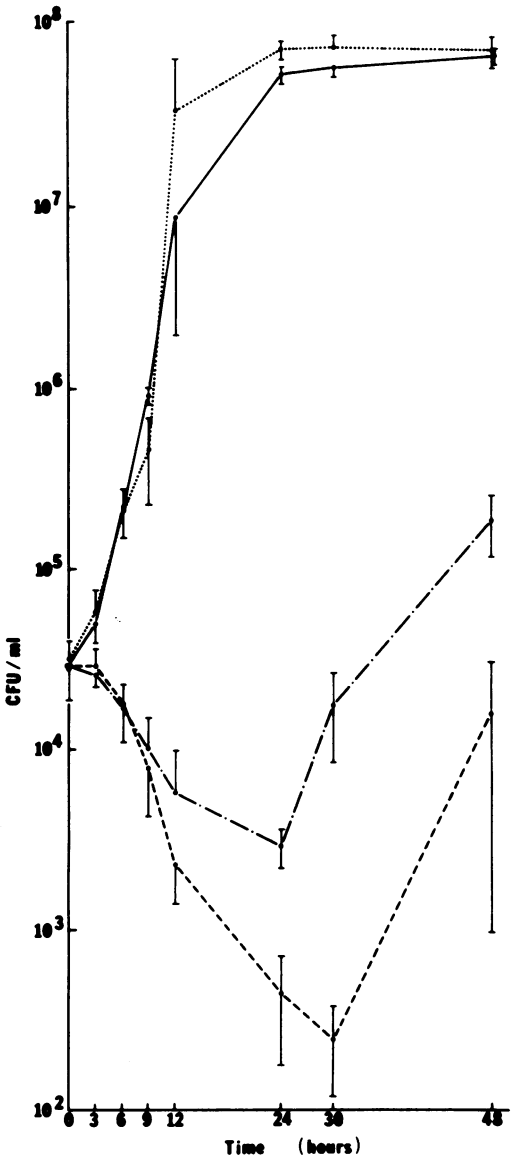

FIG. 2. *Effect of DAQ 2A and SMX, individually and in combination, on C. albicans in SAAMF broth. DAQ 2A was used at a concentration 10 times the MIC of the Candida isolate used. Symbols: ——, control with no drugs; ·····, 50 μg of SMX per ml; -·-·-, 7.5 μg of DAQ 2A per ml; -----, 50 μg of SMX plus 7.5 μg of DAQ 2A per ml.*

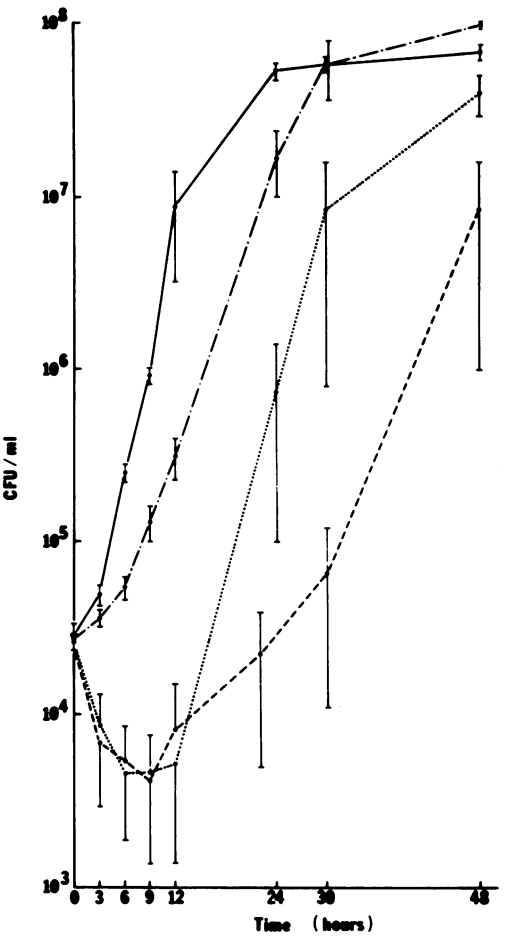

FIG. 3. *Effect of DAQ 2A and AMB, alone or combined on C. albicans in SAAMF. Shown are means plus and minus one standard error. AMB and DAQ 2A were used at a concentration equal to the MIC of the Candida isolate used. Symbols: ——, control with no drugs; ·····, AMB at 0.75 μg/ml; -·-·-, DAQ 2A at 0.75 μg/ml; -----, DAQ 2A plus AMB both at 0.75 μg/ml.*

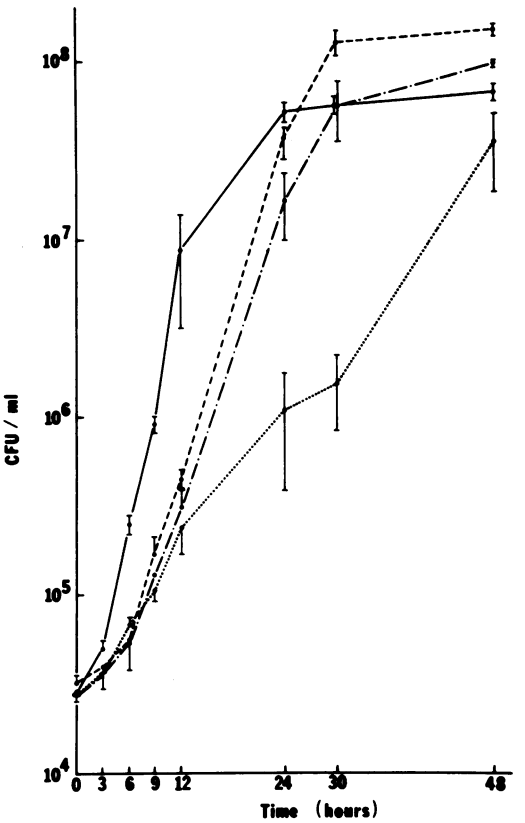

FIG. 4. *Effect of DAQ 2A and FC, alone and combined, on Candida albicans in SAAMF broth. Shown are means plus and minus one standard error. DAQ 2A and FC were used at a concentration equal to the MIC of the Candida isolate used. Symbols: ——, control with no drugs; ·····, FC at 0.75 μg/ml; -·-·-, DAQ 2A at 0.75 μg/ml; -----, DAQ 2A plus FC each at 0.75 μg/ml.*

pound, in the presence of 5,10-methylenetetrahydrofolate, is capable of irreversibly inhibiting the action of the enzyme thymidylate synthetase (8). However, DAQ 2A inhibits the formation of folate derivatives including 5,10-methylenetetrahydrofolate and could thereby interfere with FC's action.

The regrowth of *Candida* when exposed to 7.5 μg of DAQ 2A per ml is puzzling because no breakdown of the drug could be detected during the 48-h incubation period, nor did the isolate develop resistance to the drug during this time. We have no explanation for this phenomenon. The regrowth observed after 12 h of exposure to AMB is probably related to the breakdown of this material in SAAMF (15).

The in vivo activity of some DAQ compounds and the lack of obvious toxicity in the mouse of one of the most active of these compounds encourage further work on the use of these drugs as antifungal agents in animal models. Furthermore, if activity can be shown in an animal model, potentiation of activity should be sought by utilizing combinations of DAQ compounds with sulfonamides or AMB.

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