SUPPLEMENT ARTICLE

Combination and Sequential Antifungal Therapy for Invasive Aspergillosis: Review of Published In Vitro and In Vivo Interactions and 6281 Clinical Cases from 1966 to 2001

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The development of newer antifungal drugs is creating new potential combination therapies to combat the dismal mortality rate associated with invasive aspergillosis (IA). The efficacy of combination therapy for IA has not been established; sparse data on combination or sequential antifungal therapy depict interactions ranging from synergy to antagonism. We reviewed data from all published in vitro studies, animal model studies, and clinical reports and recent abstracts on combination and sequential antifungal therapy for IA from 1966–2001. Among cases of IA during 1966–2001, 249 were treated with 23 different antifungal combinations. Amphotericin B plus 5-fluorocytosine was the most commonly used (49% of cases), followed by amphotericin B plus itraconazole (16%) or plus rifampin (11%). Combination therapy resulted in improvement in 63% of patients, generally with amphotericin B plus 5-fluorocytosine or rifampin and indifference with amphotericin B plus itraconazole. In 27 in vitro reports, we found synergy (in 36% of reports), additivity (in 24%), indifference (in 28%), and antagonism (in 11%). Amphotericin B plus 5-fluorocytosine and amphotericin B plus rifampin showed generally positive interactions and amphotericin B plus itraconazole showed results that were largely indifferent. Eighteen animal model reports demonstrated synergy (in 14% of reports), additivity (in 20%), indifference (in 51%), and antagonism (in 14%). In general, amphotericin B plus 5-fluorocytosine, amphotericin B plus rifampin, and amphotericin B plus itraconazole showed indifferent results, whereas amphotericin B plus micafungin showed positive interactions. Thirty-four cases treated during 1990–2001 with sequential therapy, excluding amphotericin B followed by itraconazole, showed improvement in 68% of cases. Improvement was noted with amphotericin B or itraconazole followed by voriconazole but not with itraconazole followed by amphotericin B.

The rising incidence of invasive aspergillosis (IA) [1] has paralleled the marked increase in immunocompromised patients in the last several decades [2]. The overall survival rate among patients treated with amphotericin B is 34%–42% [3–5], and until recently there were only 2 antifungals with inherent activity against *Aspergillus*, amphotericin B deoxycholate and itraconazole. A recent practice survey of 595 cases of IA from the United States, Canada, and western Europe found

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that most clinicians used amphotericin B monotherapy for their most immunosuppressed patients (31% of patients treated), whereas for less immunosuppressed patients, they used itraconazole (10%) or amphotericin B followed by itraconazole (16%). Combination regimens were seldom used (amphotericin B plus 5-fluorocytosine, 2% of patients treated; amphotericin B plus rifampin, 2%; and amphotericin B plus itraconazole, 3% [2]). A similar European survey of 120 treated cases of IA revealed use of combination antifungal therapy for 91 patients (76% of patients treated), although therapy was usually sequential rather than concurrent [3].

There has been a recent surge in the development of newer antifungals to treat IA, including entirely new classes of drugs with novel targets [4], creating hope for treatment and increasing the permutations of new potential combination therapies. On the basis of treatment of other infectious diseases, such as HIV infection, tuberculosis, and cryptococcal meningitis [5], combination therapy seems logical. However, laboratory data on combination or sequential therapy for IA are sparse, and clinical data consist largely of individual case reports or subsets of patients in a series.

Although there are several recent reviews of combination therapy to treat systemic mycoses [6, 7], these generally focus on *Candida* and *Cryptococcus.* A few retrospective clinical reviews of combination therapy for IA exist [8, 9], but the only prospective trial ever published found no increased efficacy for combination amphotericin B plus 5-fluorocytosine [10]. Although no controlled clinical trial supports its use and the efficacy of combination therapy for IA has not been conclusively established [11], the range of data from synergy to antagonism parallels the wide range of unproven treatment practices used by clinicians. This review is the most comprehensive synthesis of the available data on combination and sequential antifungal therapy for IA, reviewing all in vitro, in vivo, and clinical reports and recent abstracts.

METHODS

We undertook a MEDLINE search with use of the keywords *"Aspergillus,"* "aspergillosis," "treatment," and "therapy," as well as text word searching. We scrutinized all English-language articles and their additional references published from 1966 to 31 December 2001 as well as abstracts from recent scientific meetings. Our search sought to discover all in vitro, in vivo (animal models), and clinical reports of combination and sequential systemic antifungal therapy for IA. We used combination reports available on MEDLINE from 1966 until 1 January 1990 from a previous review of 2121 published cases of IA [9]. We supplemented these with an additional review of all published combination and sequential therapy reports from 1990 through 31 December 2001. We therefore reviewed all in vitro, in vivo, and clinical reports of combination therapy for IA from 1966 to 2001 and sequential therapy from 1990 to 2001.

Combination therapy was defined as the use of concurrent systemic antifungal therapy for IA with $\geqslant 2$ agents. Sequential therapy was defined as the use of 1 systemic antifungal, followed by its discontinuation and replacement with another systemic antifungal. Although a frequent sequential antifungal approach is amphotericin B followed by itraconazole, we chose to focus on other sequential regimens because this is a common and well-tolerated regimen, considerable data about it have been published, and guidelines have previously recommended it [2, 11].

All in vitro and in vivo combination reports were included, but clinical reports reviewed were placed into 3 categories (table 1). The first category is those reports "reviewed." In the 1990 review, this included all articles concerned with the clinical aspects of aspergillosis; however, for the additional 1990–2001 review, this survey was limited to the clinical treatment of aspergillosis, eliminating articles pertaining only to diagnostic assays or radiologic studies without reported antifungal therapy.

We also included only cases of IA, excluding all reports of therapy for aspergilloma, allergic bronchopulmonary aspergillosis, chronic necrotizing pulmonary aspergillosis, or noninvasive cutaneous disease. As previously defined [9], each report required details of the underlying disease and a definite or probable diagnosis of IA. Most cases involved histologically proven specimens, but probable diagnosis was defined by the National Institute of Allergy and Infectious Diseases (NIAID)–sponsored Mycoses Study Group [12]. We excluded IA diagnosed at autopsy.

In addition, we included only those cases in which systemic antifungal therapy was used for IA, excluding all reports in which only topical, inhaled, or instilled antifungal therapy was

Table 1. Results of a MEDLINE search for reports of invasive aspergillosis treatment from 1966 to 2001.

	No. of reports or cases		
Variable	1966–1990[a]	1990–2001[b]	Total
Cases reviewed	2121	4160	6281
Reports reviewed	497	898	1395
Cases analyzed	446	3283	3729
Reports analyzed	210	491	701
Combination cases	89	386	475
Combination reports	54	236	290
Sequential cases	0	56	56
Sequential reports	0	41	41
Combination cases analyzed	78	171	249
Combination reports analyzed	46	82	128
Sequential cases analyzed	0	34	34
Sequential reports analyzed	0	27	27

[a] All reports detailing clinical aspects of invasive aspergillosis. In [9].
[b] Only cases of antifungal treatment of invasive aspergillosis.

used. Only immediate responses to therapy were required, because long-term follow-up was often not given and we focused on response rates and not cure rates. We excluded reports of empirical treatment, because they generally did not focus on the specific treatment of IA. Only the individual patients in a series who met all inclusion criteria were included.

The next category was those reports "analyzed." Here we included only patients treated for ⩾14 days to allow an adequate trial of therapy. Although specific doses of antifungals were often included in each report, to exclude all reports of prophylactic use we also included only cases in which antifungal treatment dosages were used, for example, ⩾0.5 mg/kg/day for amphotericin B, ⩾100 mg/kg/day for 5-fluorocytosine, and ⩾200 mg/day for itraconazole. When the dose or duration of therapy was not given, those reports were excluded from the "analyzed" section. For sequential reports, we included only those reports in which both drugs were given at treatment doses; that is, if a patient received low-dose prophylactic itraconazole and then treatment doses of amphotericin B, that would not constitute sequential therapy by our criteria. Patients receiving sequential therapy were required to have been treated with the first antifungal for a minimum of 7 days before switching.

Such strict inclusion criteria leads to several biases. The most encompassing and unavoidable bias in any detailed literature review is the published literature itself, which may favor successful reports. Requiring ⩾14 days of antifungal therapy also creates a bias toward success by selecting patients who lived long enough to tolerate therapy. Requiring disease and treatment details forced exclusion of numerous reports in which medical therapy was simply reported as "antifungals" or "amphotericin B" with no further specifics outlined. One difficulty in use of inclusion criteria was that some reports used nondescript terms such as "also" in detailing double antifungal therapy, which for some cases clearly indicated concurrent therapy and others implied sequential therapy. If this fact was not clear, the report was excluded from analysis.

LITERATURE REVIEW RESULTS

Denning and Stevens [9] previously reviewed a total of 2121 cases in 497 articles concerning clinical aspects of IA from 1966 to 1990. Further review of publications in the last 12 years, with a focus on those mentioning antifungal treatment, resulted in the discovery of an additional 4160 cases of IA in 898 articles. After exclusion on the basis of above criteria, Denning and Stevens analyzed 446 treatment courses in 379 patients from 210 articles. We analyzed an additional 3283 IA cases in 491 articles that met inclusion criteria. Therefore, we reviewed a total of 6281 cases of IA in 1395 published articles and analyzed 3729 cases in 701 articles from 1966 to 2001 (table 1 and table 2).

Our focus on combination and sequential antifungal therapy further narrowed the field of published reports. The previous 1990 review [9] revealed 89 clinical cases of combination therapy in 54 articles: amphotericin B plus rifampin (26 articles) and amphotericin B plus 5-fluorocytosine (63). The additional 12 years added 386 clinical cases of combination antifungal therapy in 236 articles. We excluded 11 cases in 7 articles from the 1990 review and 215 cases in 154 articles during 1990–2001 that did not meet inclusion criteria. Those cases excluded generally lacked adequate documentation of the amphotericin B dose used (143 of 226 cases, 63% of cases), but many did not meet the 14 days of therapy required (59 of 226, 26%) or both (25 of 226, 11%). Of the 226 cases excluded, ~53% were treated with amphotericin B plus 5-fluorocytosine and 34% with amphotericin B plus itraconazole. After exclusion we analyzed a total of 249 clinical combination cases in 128 articles, or 52% of the published combination reports (Appendix A, table A1). The distribution of IA disease location among the 249 analyzed cases of combination therapy shows the generally accepted predominance of pulmonary (51% of cases), cerebral (17%), and sinus disease (14%) (table 2).

Three combination regimens constituted the majority (table 3) of reported clinical experience: many (49% of cases) involved amphotericin B plus 5-fluorocytosine, whereas amphotericin B plus itraconazole (16%) and amphotericin B plus rifampin (11%) were less common. However, if the lipid formulations of amphotericin B were included, that is, amphotericin B lipid complex, amphotericin B colloidal dispersion, or liposomal amphotericin B, the frequency of those 3 combinations increased from 76% to 89% of the total number of combinations analyzed. Finally, inclusion of those 9 patients who were treated with 1 combination regimen and then changed to another increased the contribution of these 3 regimens to 91% of combination strategies ever reported.

The 249 cases yielded 27 different antifungal combinations, including 16 unique double-antifungal and 7 triple-antifungal regimens (table 4). Clinical outcomes of combination antifungal cases were stratified according to the reported results into several categories: patient improvement, no improvement, worsening of IA, or death from IA. If a patient's IA improved during antifungal treatment, yet the patient later died of relapse of the underlying malignancy or myocardial infarction, the combination report was scored as "improvement." A total of 63% of patients showed improvement, with mortality from IA at 34%. The combination with the greatest percentage of patient improvement was amphotericin B plus rifampin plus 5-fluorocytosine (5:1, improvement to death), followed by amphotericin B plus 5-fluorocytosine (2.3:1, improvement to death).

Twenty-seven reports of in vitro combination antifungal therapy for *Aspergillus* species were published during 1974–2001, analyzing 34 different combinations (Appendix A, table

Table 2. Results of MEDLINE search regarding invasive aspergillosis (IA) disease distribution.

Type of IA	No. of cases			
	1966–1990	1990–2001	Total	Treated with combination therapy[a]
Pulmonary	156	2755	2911	128
Sinusitis	102	262	364	35
Cerebral	33	118	151	43
Osteomyelitis	38	21	59	13
Invasive cutaneous	29	10	39	2
Renal	20	14	34	3
Endocarditis	15	13	28	9
Endophthalmitis/chorioretinitis	10	16	26	10
Keratitis	0	24	24	0
Spondylodiscitis/epidural abscess	16	7	23	13
Pleural/mediastinitis	16	0	16	1
Pericarditis/myocardial abscess	11	2	13	5
Orbital	0	9	9	4
Peritonitis/hepatic	0	9	9	3
Laryngeal	0	7	7	1
Mastoiditis	0	5	5	1
Scleritis/uveitis	0	4	4	4
Graft	0	4	4	1
Invasive otitis externa	0	2	2	1
Pelvic	0	1	1	0
Total analyzed	446	3283	3729	249

[a] Some patients had >1 form of IA.

A2). Although many clinical case reports also mentioned MIC data and some included in vitro combination studies, we reviewed only those studies devoted to in vitro combination analysis. The results were stratified into interactions leading to synergy, additivity, indifference, or antagonism (table 5). Analysis revealed 36% of reports demonstrated synergy, 24% demonstrated additivity, 28% demonstrated indifference, and 11% demonstrated antagonism. The most frequently tested combinations were, in descending order, amphotericin B plus 5-fluorocytosine, amphotericin B plus itraconazole, and amphotericin B plus rifampin.

There were 18 reports of in vivo combination antifungal therapy published during 1975–2001, analyzing 15 different combinations and their outcomes (Appendix A, table A3). The most frequently tested combination was amphotericin B plus 5-fluorocytosine, followed by amphotericin B plus itraconazole (table 6). As was the case in the analyses of the in vitro studies, we required all reports to be dedicated in vivo experiments. Only 2 reports also evaluated in vitro data [13, 14]. The in vivo results were less frequently positive: 14% of reports demonstrated synergy, 20% demonstrated additivity, 51% demonstrated indifference, and 14% demonstrated antagonism.

Clinical sequential therapy was not analyzed in the 1990 review, so data include only 1990–2001. As mentioned, we chose to exclude the guideline-approved [11] and well-tolerated [2] sequence of amphotericin B followed by itraconazole. We reviewed 56 cases in 41 reports and analyzed 34 clinical cases in 27 reports of sequential therapy (Appendix A, table A4). This revealed a similar pattern in which 1 therapeutic choice clearly predominated: almost 30% (10 of 34 cases) of the cases analyzed were treated with itraconazole followed by ampho-

Table 3. Most frequent clinical antifungal combinations used for 249 cases of invasive aspergillosis identified in a MEDLINE search.

Antifungal combination	No. (%) of cases
AmB + 5-FC	123 (49)
AmB + Rif	27 (11)
AmB + Itr	41 (16)

NOTE. AmB, amphotericin B; Itr, itraconazole; Rif, rifampin; 5-FC, 5-fluorocytosine.

Table 4. Outcomes of clinical treatment with antifungal combinations for 249 cases of invasive aspergillosis identified in a MEDLINE search.

	No. of cases identified			Treatment outcome, no. of cases			
Antifungal combination	1966–1990	1990–2001	Total	Improvement	Death	No improvement	Worse
AmB + 5-FC	55	68	123	84	36	3	…
AmB + Rif	23	4	27	18	9	…	…
AmB + Itr	…	41	41	20	21	…	…
Itr + 5-FC	…	2	2	2	…	…	…
Itr + Rif	…	2	2	1	1	…	…
L-AmB + Itr	…	17	17	10	7	…	…
ABLC + Itr	…	7	7	2	3	1	1
ABCD + Itr	…	1	1	…	1	…	…
L-AmB + Rif	…	1	1	1	…	…	…
ABLC + Rif	…	1	1	1	…	…	…
Ter + Itr	…	1	1	…	1	…	…
ABCD + 5-FC	…	1	1	1	…	…	…
AmB + Flu	…	1	1	…	1	…	…
L-AmB + Vor	…	1	1	…	1	…	…
L-AmB + 5-FC	…	3	3	2	1	…	…
AmB + Itr, followed by AmB + 5-FC	…	1	1	1	…	…	…
AmB + 5-FC, followed by AmB + Itr	…	2	2	1	…	1	…
L-AmB + 5-FC + Itr	…	1	1	1	…	…	…
AmB + 5-FC, followed by AmB + Itr, followed by ABLC + Itr	…	1	1	1	…	…	…
AmB + 5-FC, followed by AmB + Rif	…	1	1	1	…	…	…
AmB + Rif, followed by AmB + 5-FC	…	1	1	1	…	…	…
5-FC + Ket, followed by AmB + 5-FC	…	1	1	1	…	…	…
AmB + Rif + 5-FC	…	6	6	5	1	…	…
AmB + Ket +5-FC	…	1	1	1	…	…	…
AmB + Itr + 5-FC	…	3	3	3	…	…	…
AmB + Rif + Ket	…	1	1	…	…	1	…
ABCD + Itr + 5-FC	…	1	1	…	1	…	…
Total, no. (%)[a]	78	171	249	158 (63)	84 (34)	6 (2)	1 (<1)

NOTE. ABCD, amphotericin B colloidal dispersion; ABLC, amphotericin B lipid complex; AmB, amphotericin B; Flu, fluconazole; Itr, itraconazole; Ket, ketoconazole; L-AmB, liposomal amphotericin B; Rif, rifampin; Ter, terbinafine; Vor, voriconazole; 5-FC, 5-fluorocytosine.

[a] No. of cases treated with combination therapy or no. of cases treated (% of total cases identified through MEDLINE).

tericin B. The clinical outcomes of the 18 different sequential therapies used are also stratified according to the case report outcomes (table 7). The sequence with the greatest patient improvement was amphotericin B followed by voriconazole, with itraconazole followed by amphotericin B the least effective. Only 4 separate laboratory reports focused on sequential therapy during 1990–2001 (table 8), and they generally found antagonistic interactions.

DISCUSSION

Rationale for combination therapy. Combination antibacterial therapy was first used 3 decades ago to treat febrile neutropenic patients, with great success [18]. As the incidence of IA increases and mortality remains high, clinicians need newer approaches to therapy. There are several foreseeable advantages to combination antifungal therapy: a widened spectrum and potency of drug activity, more-rapid antifungal effect, synergy, lowered dosing of toxic drugs, and a reduced risk of antifungal resistance [19]. Although each individual antifungal agent has limitations, combinations might prove more effective, as seen with the now standard highly active antiretroviral therapy used with HIV-infected patients.

The available antifungals for IA target 4 different cell functions: cell membrane integrity (polyenes), ergosterol biosynthesis (azoles and allylamines), DNA synthesis (pyrimidine an-

Table 5. Drug interactions observed in 27 in vitro studies of antifungal combinations against *Aspergillus*.

Antifungal combination	Type of interaction, no. of studies			
	Synergy	Additivity	Indifference	Antagonism
AmB + 5-FC	2	3	3	1
AmB + Rif	3	0	1	0
AmB + Itr	1	2	2	2
Itr + Caf	1	…	…	…
AmB + Caf	3	1	…	…
AmB + Ket	…	…	1	2
AmB + Ter	…	1	2	1
Itr + Ter	3	1	…	…
Flu + Ter	1	1	1	…
Vor + Ter	1	…	…	…
5-FC + Ter	…	…	1	1
AmB + Flu	…	…	1	…
AmB + L-nystatin	…	1	…	…
AmB + Mif	2	1	1	…
L-AmB + Mif	…	…	1	…
Itr + Mif	1	1	1	…
5-FC + Mif	1	1	…	…
AmB + Mic	2	…	…	…
Ket + Mic	…	…	…	1
AmB + DU-6859a[a]	1	…	…	…
Flu + DU-6859a	…	…	1	…
AmB + Rib	1	1	…	…
NikkZ + Itr	1	…	…	…
NikkZ + Flu	…	…	1	…
NikkZ + Mif	1	…	1	…
NikkZ + anidulafungin	1	…	…	…
AmB + histatin 5	…	…	1	…
Itr + NC1175	…	1	…	…
Caf + NC1175	…	1	…	…
AmB + NC1175	…	…	1	…
Vor + Mif	…	1	…	…
Vor + Caf	…	1	…	…
Vor + AmB	…	…	1	…
AmB + Azm	1	…	…	…
Total, no. (%)[b]	27 (36)	18 (24)	21 (28)	8 (11)

NOTE. AmB, amphotericin B; Azm, azithromycin; Caf, caspofungin; Flu, fluconazole; Itr, itraconazole; Ket, ketoconazole; L, liposomal; Mic, miconazole; Mif, micafungin; NikkZ, nikkomycin Z; Rib, rifabutin; Ter, terbinafine; Vor, voriconazole; 5-FC, 5-fluorocytosine.

[a] Experimental fluoroquinolone.

[b] No. of combinations tested that produced the indicated interaction (% of all combinations tested that produced any interaction).

alogues, and rifampin), and cell wall integrity (echinocandins and chitin synthase inhibitors). Although antifungals are targeted against specific cell functions, many drugs also have pleiotropic mechanisms of activity that may inhibit other elements of fungal homeostasis [19]. For instance, azoles also inhibit many cytochrome P-450–dependent enzymes of fungal respiration and amphotericin B generates oxidative species that damage fungal mitochondrial function and enhance macrophage fungal killing [20]. These subtle effects theoretically could be enhanced with a second antifungal or could act antagonistically as a class of drugs affects the targets for another class of drugs.

Table 6. Drug interactions observed in 18 animal-model studies of antifungal combinations against invasive aspergillosis.

Antifungal combination	Type of interaction, no. of reports			
	Synergy	Additivity	Indifference	Antagonism
AmB + 5-FC	1	2	5	…
AmB + Rif	1	…	1	…
AmB + Itr	…	…	4	1
Itr + 5-FC	1	1	1	…
Flu + 5-FC	…	…	1	…
AmB + Caf	…	1	…	…
AmB + cilofungin	…	…	…	1
AmB + Ket	…	…	1	3
5-FC + Ket	…	…	1	…
AmB + Flu	…	…	1	…
AmB + Mif	1	1	2	…
L-AmB + Mif	…	…	1	…
AmB + L-AmB	…	1	…	…
AmB + DU-6859a[a]	1	…	…	…
NikkZ + Mif	…	1	…	…
Total, no. (%)[b]	5 (14)	7 (20)	18 (51)	5 (14)

NOTE. AmB, amphotericin B; Caf, caspofungin; Flu, fluconazole; Itr, itraconazole; Ket, ketoconazole; L-AmB, liposomal amphotericin B; Mif, micafungin; NikkZ, nikkomycin Z; Rif, rifampin; 5-FC, 5-fluorocytosine.

[a] Experimental fluoroquinolone.

[b] No. of combinations tested that produced the indicated interaction (% of all combinations tested that produced any interaction).

The possible combination regimens with so many new antifungals with activity against *Aspergillus* [4] is astounding. At present 8 systemic antifungals are approved or in advanced clinical trials for treatment of IA, and this list excludes other drugs without primary antifungal activity (e.g., rifampin and 5-fluorocytosine) that have been used historically in combination therapy. The number of antifungals increases to 11 by including the 3 different amphotericin B lipid preparations that have shown therapeutic equivalency [21–26]. Three other currently-available antifungals have also been studied in combination but are not yet ready for clinical study (nikkomycin Z and NC-1175) or the pharmaceutical company has halted investigation (liposomal nystatin).

Synergy is defined as improved antifungal activity with a magnitude greater than the expected sum of the activities of the individual agents. Antagonism is defined as activity less than that of the least active drug. Additivity is defined as improvement in antifungal activity but no greater than the sum of the activity of the individual drugs, whereas indifference is defined as a combination no more effective than the single most active agent alone [7, 27]. Synergy generally occurs through 3 possible mechanisms: sequential inhibition of different steps of a common biochemical pathway, simultaneous inhibition of cell wall and cell membrane targets, or use of a cell wall- or cell membrane-active drug to enhance penetration of a second antifungal [19]. Only the terbinafine-azole combination is predicted to inhibit sequential steps in ergosterol biosynthesis. The third type might explain the cases of increased activity with amphotericin B plus rifampin, 5-fluorocytosine, or quinolones.

The most-cited predictions of antagonism are azole inhibition of ergosterol-binding sites for amphotericin B or lipophilic itraconazole blocking amphotericin B interaction with sterol components of the cell membrane by interacting with the cell surface [28]. Although this is not inherently true antagonism, because the loss of polyene activity can be balanced by gain from the azole activity, it may be viewed as clinically antagonistic if it does not improve clinical response and increases cost and toxicity [19]. However, not all azoles similarly antagonize amphotericin B: the hydrophilic fluconazole often will not accumulate in the lipid-rich membrane environment [7].

In vitro antifungal testing. Preceding any discussion about combination antifungal therapy must be critical evaluation of its foundation built on unstandardized methodology from early in vitro antifungal susceptibility testing. Studies have used diverging and imprecise parameters for estimation of growth inhibition or measurement [29], as well as varying in vitro conditions. Despite recent advances in standardization, the science of antifungal susceptibility testing remains behind antibacterial testing [30, 31]. Bacterial susceptibility testing now convincingly guides the clinical choice of antibacterial therapy, and the hope is that antifungal testing for molds will reach that same level of confidence and clinical utility. Mold testing is presently done under the NCCLS in vitro susceptibility testing method M38-A [31].

Aside from the difficulties in standardizing testing of filamentous fungi, the antifungals themselves have unique in vitro properties. For instance, azoles sometimes cause partial inhibition, resulting in the absence of a clear MIC end point value [33]. However, this difficulty with azoles is not as great a problem with molds as it is with yeasts [32], and the M38-A standard uses as a reference point ~50% inhibition of the growth control for azole MICs[32]. The present in vitro susceptibility testing method (M38-A) [31] also cannot easily identify amphotericin B-resistant *Aspergillus fumigatus* isolates but can identify *Aspergillus* isolates that are resistant to even very high azole concentrations [34]. When echinocandins are tested by broth-based assays with use of conventional MIC criteria, *Aspergillus* species would be categorized as resistant because examination would show partial growth [32]. However, although echinocandin activity against *Aspergillus* does not give classic MICs in vitro by dilution techniques, it does demonstrate clear morphological inhibition in vitro [35].

Testing the in vitro activity of lipid formulations of polyenes is also controversial, because in general lipid formulations enhance in vivo activity by improving tolerability and potentially

Table 7. Outcomes of 34 cases of clinical sequential antifungal therapy.

Sequence of antifungals administered			Treatment outcome, no. of cases		
First	Second	Third	Improvement	Died	No improvement
Itr	AmB		4	6	…
Itr	AmB	Vor	1	…	…
Ket	Flu		1	…	…
Itr	ABCD		1	…	…
Rif	5-FC		1	…	…
AmB + 5-FC	Itr		…	…	1
Itr	AmB	Scz	1	…	…
Flu	AmB + 5-FC + Flu		1	…	…
Mic	5-FC		1	…	…
AmB	Vor		3	…	…
Flu	Itr		…	…	1
AmB	Itr	Vor	1	…	…
Itr	Vor		2	…	…
L-AmB	Vor		…	1	…
Flu	L-AmB		1	…	…
ABLC + Itr	Vor		1	…	…
AmB	L-nystatin		3	2	…
5-FC	AmB + 5-FC + Rif		1	…	…
Total, no. (%)			23 (68)	9 (26)	2 (6)

NOTE. Cases treated with amphotericin B followed by itraconazole were excluded. ABCD, amphotericin B colloidal dispersion; ABLC, amphotericin B lipid complex; AmB, amphotericin B; Flu, fluconazole; Itr, itraconazole; Ket, ketoconazole; L, liposomal; Mic, miconazole; Rif, rifampin; Scz, saperconazole; Vor, voriconazole; 5-FC, 5-fluorocytosine.

changing drug targeting. Moreover, the active moiety in the lipid-complexed drugs is amphotericin B, and the lipid component is but an altered delivery vehicle that delivers amphotericin B in a toxicologically superior way. Some authorities therefore believe that in vitro testing should be done only on the parent compound [36].

Three principal methods have been used to determine *Aspergillus* MICs: broth macrodilution, broth microdilution, and agar dilution testing. Variations exist that use several different basic media, pH, buffers, temperature, incubation periods, and conidia inocula. End-point determination also varies; microdilution tests are usually read microscopically or photometrically (each of which has limitations), whereas macrodilution results are usually determined with a subjective visual end point [37].

In vitro combination testing. Drug interactions are complex events that are difficult to assess and detect objectively unless they are very pronounced [38]. Using the same combinations, different authors have observed a spectrum from antagonism to synergy, depending on the methodology and analysis used [6]. Many of the published claims of synergy between antifungals are also potentially founded on criteria too lenient to determine interactions, and the clinical relevance of synergy or antagonism is undefined. The mathematical definitions themselves are borrowed from antibacterial research. However, even an additive interaction may be clinically significant, because it might allow decreased doses of a drug and therefore potentially lowered toxicity.

Unfortunately, past in vitro studies of combination antifungal therapy include these historical inconsistencies and difficulty in interpretation of in vitro testing. Additionally, there is no agreed standard method for testing combination therapy, and in vitro synergy does not necessarily correlate with clinical responsiveness. For example, there might also be synergistic toxicity not seen until animal models or human trials [39]. Interpretation of in vitro combination interactions with amphotericin B can also be difficult, because amphotericin B is not stable in vitro [40]. As an example of the problems with in vitro testing of antifungal combinations, the well-accepted combination of amphotericin B plus 5-fluorocytosine has been reported as antagonistic in vitro against *Candida albicans* [41, 42].

Two laboratory techniques are frequently used to determine the effects of combination therapy: checkerboard dilutions and time-kill studies. Time-kill studies, although more laborious, provide a more detailed description of the rate and extent of antifungal activity over time [7]. However, checkerboard dilutions, which report only a single growth end point at a single

Table 8. Summary of reported in vitro and in vivo sequential antifungal therapy for invasive aspergillosis.

Type of study and infection; reference	Year	Evaluation method	Antifungal combination	Observed effect
In vitro				
Schaffner and Frick [13]	1985	MFC	Ket followed by AmB	Antagonism
Schaffner and Bohler [15]	1993	MFC	Itr followed by AmB	Antagonism
Maesaki et al. [16]	1994	Checkerboard	Mic followed by AmB	Synergism
			Flu followed by AmB	Antagonism
			Ket followed by AmB	Antagonism
			Itr followed by AmB	Antagonism
In vivo, murine				
Disseminated; Schaffner and Frick [13]	1985	Survival	Ket followed by AmB + Ket	Antagonism
Disseminated; Schaffner and Bohler [15]	1993	Survival	Itr followed by AmB	Antagonism
Pulmonary; Lewis et al. [17]	2001	Survival, lung burden	Itr followed by AmB	Antagonism

NOTE. AmB, amphotericin B; Flu, fluconazole; Itr, itraconazole; Ket, ketoconazole; MFC, minimum fungicidal concentration; Mic, miconazole.

time point, are easier to perform and standardize between laboratories and thus are more commonly reported. Concerns with checkerboard testing include the rapid fungicidal action of amphotericin B compared with the slower-acting azoles, with simultaneous exposure likely obscured by the rapid activity of amphotericin B. However, neither method of in vitro testing simulates the decline in drug concentrations in vivo after a dose is administered, thereby creating unrealistic drug concentrations in the testing system [7].

To calculate in vitro combination antifungal results, a drug dilution series is created. When sufficient growth is found in the no-drug control assay tubes, the MIC is read as the concentration of drug in the first assay tube in a dilution series that shows no growth. Next, a volume of suspension from each tube showing inhibition is plated on agar, and the concentration of drug yielding no growth or only 1 colony is designated as the minimum fungicidal concentration (MFC) [37]. Different studies have used varying MFC definitions, largely reflecting different methodologies regarding the volume subcultured from the assay tube. These small changes will alter the defined end point, from ⩾96% killing to 100% killing [37].

In a checkerboard titration, 2 drugs are tested in serial dilutions and in combinations of these dilutions together to find concentrations of each drug, both alone and in combination, that produce the desired effect [43]. The nature of these interactions can be expressed numerically or via a plot or geometric curve. With the latter method, a graph is constructed with axes being drug concentrations geometrically expressed as a fraction of the MIC, with the intercept representing the no-drug control. An isobole is a line joining the points that represent all combinations with the same effect, including equally effective concentrations of monotherapy. When the line is straight, the combination is additive, whereas a concave isobole (deviation to the left of this line) denotes synergism and a convex isobole (deviation to the right) denotes an interaction less than additive or indifference [44]. By drawing intersecting straight lines through the experimental points, one can identify a point at which the combined fractional inhibitory concentrations (FICs) reach a minimum (and therefore a point of maximal effectiveness), and thus a single number can quantify the degree of interaction of a pair of compounds [45]. The advantage of plotting FICs rather than actual concentrations of the drugs is that it produces symmetrical axes and normalizes differences between 2 drugs whose concentrations required for inhibition may vary greatly, in absolute terms. Additionally, it will be reproducible in the face of small differences in the range of combinations tested.

Drug interaction assessed with the checkerboard titration method specifically involves tubes containing 2 drugs, with the volume of each drug concentration halved and the concentration doubled. Thus, the final concentration of each is the same as that in the tubes containing only a single drug [37]. To calculate the FIC for a drug, the tube with the lowest concentration of a drug inhibiting growth in the presence of the other is selected. That concentration is divided by the MIC of that drug. Therefore, the FIC is the concentration of the inhibitor in the combination divided by the amount of inhibitor that would be required to give the same degree of inhibition by itself [45]. The FIC index (FICI) [43, 45] is the lowest sum of the FICs for each drug (A and B) in any 1 assay tube, calculated by the following formula: FICI = [(MIC A in combination) /MIC A] + [(MIC B in combination)/MIC B].

Interpretation of the FICI can vary, but a logical interpretation is as follows. An additive effect is represented by an FICI of 1; therefore, the drugs behave as if they are merely complementary, and inhibition requires combining, for example, one-half the MIC of one drug to one-half of the MIC of the other drug, or the same effect that would be produced by doubling

the half-MIC of either drug alone (the straight line in the example given above). Any combination producing an FICI of <1 is a combination superior to this, or synergy. An important synergistic interaction is arbitrarily defined as an FICI of <0.5. A FICI of 2 would represent indifference. This is a situation in which there was no contribution of either drug's inhibitory activity to that of the other; one drug inhibits at its MIC, and no amount of the other drug, up to the second drug's MIC, will produce inhibition when added to any concentration of the first drug less than the first drug's MIC. A combination producing an FICI of >2 (i.e., more than both drugs' MICs in combination is required to produce inhibition) is therefore antagonism.

An FICI of >1 and <2 is a more complex area to conceptualize, and we term it "subadditive." An isobologram passing through this region passes through tubes in which the 2 drugs in combination produce an effect greater than either alone but less than if the 2 drugs' effects were additive. This is analogous to the equation $5 + 8 = 11$: the combined effect is greater than the effect of either drug alone but less than it would be if the 2 drugs were fully complementary. If the 2 drugs are studied in only 2-fold dilutions in the checkerboard and in no smaller increments, as is commonly the case, it becomes difficult to capture some of these interactions, particularly subadditive interactions.

By convention, the FICI is expressed as the highest combination of FICs (i.e., the highest point in the isobologram) in indifferent, subadditive, and antagonistic interactions, as opposed to the lowest combination of FICs in additive or synergistic interactions. Sometimes unusual (inconsistent) patterns of interaction are seen when the isobologram is not symmetrical. An example would be synergistic interactions (concavity) in only 1 part of the curve, such as when low concentrations of drug A are combined with high concentrations of drug B, and antagonistic interactions (convexity) in another part, such as when high concentrations of drug A are combined with low concentrations of drug B. This situation is best disposed of by the neutral term "indifference." Although FICI remains the most common expression of interaction, some authors have obtained more consistent results with 3-dimensional response surface modeling [46].

The largest in vitro combination review reported on 54 *Aspergillus* isolates [37] and noted that 92% of 39 tests of amphotericin B plus rifampin revealed synergy and 8% of tests revealed indifference, despite uniform resistance to rifampin monotherapy. Of 26 tests of amphotericin B plus 5-fluorocytosine, 23% revealed synergy, 4% revealed additivity, 50% revealed indifference, and 23% revealed antagonism. Of 5 tests of amphotericin B plus itraconazole, 2 showed synergy, 1 showed an additive effect, and 2 showed indifference. Marked superiority of amphotericin B plus rifampin over amphotericin B plus 5-fluorocytosine was seen in repeat testing.

Animal model antifungal combination testing. Animal model testing is preferred over in vitro analysis because of greater predictive value for human pharmacokinetic effects, including tissue penetration and toxicities. In vivo testing can also compare various disease location models, analyze different host immune states, and take into account differing pharmacokinetics of the drugs. In addition, histological examination can examine fungal sterilization. Although in vitro testing might suggest an effective antifungal, the dose needed to achieve the desired effect might be unachievable safely in vivo or the drug may not penetrate infected tissue adequately. Unfortunately, many animal model studies lack the statistical power to discern subtle differences in outcome, and it can be difficult to define synergy in an animal model system.

As is the case with in vitro studies, comparing animal model studies is difficult because of variability in methodology, including different animal species and varying immune states. Animal models may not mimic acquisition and extent of IA, and there is no accepted standard of interpreting synergy in survival terms in the various species of animal models. Most models involve 1 of 4 different species: mice, rabbits, guinea pigs, and rats. Many *Aspergillus* animal models involve iv injection of conidia into healthy animals or those immunosuppressed with cytotoxic agents, but ip injection has been studied. Whereas such models mimic systemic aspergillosis, there is an important role for respiratory tract infection models, with inoculations of conidial suspensions given intranasally, intratracheally, or aerosolized to better mimic the most common form of human infection [47]. Intracerebral infection, to mimic cerebral aspergillosis, has also been studied [48].

The isobologram method used in vitro to determine the contribution to the total activity made by each fraction of the partner in the combination may be applied in vivo to pairs of drugs whose dose-effect curves are similar. With an isobole procedure, an end point of activity, for example, an ED_{50}, is determined for each drug alone and then for the combination at a certain proportion. The fractional doses of each partner are then compared with the 2 single drugs, which are assigned a value of 1 each. Additive effect is assumed if the sum of the fractional doses is 1, and synergy is assumed if the sum is <1 [49].

Clinical antifungal combination testing. The in vitro and in vivo interactions must continue to be questioned and improved because of so many confounding patient variables, leaving clinical experience still the most accurate tool. A number of factors contribute to clinical efficacy, including the complex interaction among fungal virulence, pharmacokinetics and availability of antifungal at the site of infection, intrinsic or acquired fungal resistance, and the host immune condition and its interaction with the therapeutic agents. However, clinical

relevance might best be related to patient factors (e.g., recovery of neutropenia, cessation of glucocorticoid therapy) and not intrinsically related to the susceptibility of the fungus itself. No clinical study to date has answered convincingly whether combination therapy is more beneficial than therapy with amphotericin B alone [50]. In fact, only 1 prospective trial of combination therapy to treat patients has been reported [10], whereas only 1 other has been done (unpublished data).

Clinical research of combination therapy for IA is not without its pitfalls and inconsistencies. The clinical spectrum of aspergillosis is vast, and the previous lack of uniform definitions for diagnosis and response have limited effective study [51]. However, a recent consensus document of definitions of invasive fungal infections has been developed as a joint effort between the NIAID Bacteriology and Mycoses Study Group and the European Organization for Research and Treatment of Cancer, taking into account mycological data, clinical manifestations, and host factors [52]. One of the largest difficulties in clinical comparative antifungal trials is the various underlying conditions. For instance, remission status of an underlying malignancy is crucial, as demonstrated in survival of patients with disseminated candidiasis [53]. Baseline mortality is also related to location of the underlying disease and subsequent antifungal tissue penetrance. Further complicating any future clinical trial is the reality that many patients receive antifungal prophylaxis or empirical therapy [54], which in the case of amphotericin B leads to detectable levels of the drug for weeks, even after the drug is discontinued [55].

The previous largest review in 1990 assessed 2121 published cases of IA treatment and evaluated 446 cases in 379 patients. Of patients treated for >14 days, 63 patients were treated with amphotericin B plus 5-fluorocytosine and 68% of those patients responded to treatment, whereas 26 patients were treated with amphotericin B plus rifampin and 65% responded [9]. However, after patients treated for <14 days were included, results were considerably poorer [56]. Another review of 142 patients included 34 patients treated with amphotericin B plus 5-fluorocytosine, who demonstrated a 60% cure rate, and 17 patients with amphotericin B plus rifampin, who demonstrated a 53% cure rate, higher than that for amphotericin B monotherapy (46% cure rate) [27].

Amphotericin B plus 5-fluorocytosine. 5-fluorocytosine is a fluorinated analogue of cytosine first synthesized in 1957 as a potential anti-tumor agent [57], first used to treat human disease in 1968 [58], and initially approved for use in 1972 [22]. 5-fluorocytosine has little inherent anti-*Aspergillus* activity [59], and most reports detail clinical failure of monotherapy [60]. Its antimycotic activity likely results from the rapid conversion of 5-fluorocytosine into 5-fluorouracil within susceptible fungal cells [61, 62]. The compound 5-fluorouracil has 2 mechanisms of action via its phosphorylated metabolites: incorporation into fungal RNA in place of uridylic acid to inhibit fungal protein synthesis and inhibition of thymidylate synthetase to inhibit fungal DNA synthesis [62]. The latter appears to be the dominant mechanism.

The toxicity of 5-fluorocytosine is hypothesized to be due to its conversion to 5-fluorouracil; there are reports of patients receiving 5-fluorocytosine for antifungal treatment who have serum 5-fluorouracil levels in the range found after chemotherapeutic doses [63]. It is thought the conversion occurs as a result of host intestinal microbes. Additionally, 5-fluorocytosine may exacerbate myelosuppression in patients with neutropenia, and toxic levels may develop when administered in combination with amphotericin B because of the nephrotoxicity of amphotericin B and the decreased renal clearance of 5-fluorocytosine [11]. In a report of 10 patients treated with amphotericin B plus 5-fluorocytosine, there was no marrow suppression (5-fluorocytosine levels, 30–60 μg/mL) and a shorter overall duration of marrow aplasia, raising the speculation that enhanced antifungal therapy results in more effective control and thus less potential suppression related to IA [64].

The commonly fungistatic 5-fluorocytosine is thought to enhance the antifungal activity of amphotericin B, especially in anatomic sites where amphotericin B penetration is often suboptimal, such as CSF, heart valves, and the vitreous [9]. The 5-fluorocytosine molecule penetrates well into most body sites because it is small, highly water-soluble, and not bound by serum proteins to a great extent [62]. One explanation for the synergism detected with amphotericin B plus 5-fluorocytosine is that the membrane-permeabilizing effects of low concentrations of amphotericin B facilitate penetration of 5-fluorocytosine to the cell interior [65]. By use of a *C. albicans* model, Beggs and Sarosi [66] suggested that synergism actually results from sequential and not combined action, with amphotericin B acting alone until its gradual oxidation results in its depletion, at which point 5-fluorocytosine acts on surviving fungal cells.

In vitro combination studies for *Aspergillus* were first documented in 1974 [67] with use of 7 clinical isolates and demonstrated an additive effect with amphotericin B plus 5-fluorocytosine. A 1982 in vitro study assayed viable fungal biomass by bioluminescence spectrophotometry and showed an additive effect with amphotericin B plus 5-fluorocytosine against *A. fumigatus,* but synergy was seen in only 1 5-fluorocytosine–resistant isolate. Generally results were additive when combinations of amphotericin B with 5-fluorocytosine, ketoconazole, or miconazole were used. This included triple and quadruple therapy with amphotericin B plus 5-fluorocytosine plus ketoconazole plus miconazole. However, results with the same combination against 3 different isolates varied from synergy to overt antagonism [38].

The first animal study evaluated an *A. fumigatus* rabbit endocarditis model in 1975 [68]. The 3-day survival was not

significantly different between the treatment regimens of amphotericin B or amphotericin B plus 5-fluorocytosine. The extent of infection in vegetations was also not significantly lower in the combination group. A rat model showed that 100% of animals (10 of 10) treated with amphotericin B (4 mg/kg/day) survived at day 7, and combinations of amphotericin B (4 mg/kg/day) plus 5-fluorocytosine did not worsen survival, compared with amphotericin B monotherapy [69]. Another rabbit model reported no antagonism with combination amphotericin B plus 5-fluorocytosine. However, in their model, amphotericin B monotherapy was superior to combination therapy [70]. Often, enhancement is seen only with 5-fluorocytosine–susceptible strains [71, 72], such as with itraconazole plus 5-fluorocytosine.

Based on the first in vitro report in 1972 of synergistic combination antifungal therapy for yeasts [73], the first clinical case reports of amphotericin B plus 5-fluorocytosine combination therapy against pulmonary aspergillosis were published in 1973 [74] and then later in 1974 [75], reporting treatment of a patient with endocarditis who was originally described in 1971 [76]. Each publication reported clinical improvement with the combination therapy; then in 1975, the first triple-antifungal therapy was used with amphotericin B plus 5-fluorocytosine plus rifampin to treat a patient with renal aspergillosis, who also showed improvement [77].

Case series of amphotericin B plus 5-fluorocytosine treatment show clinical improvement [64, 78], but some no better than with amphotericin B monotherapy. There are also published reports of clinical improvement only after addition of 5-fluorocytosine [79]. In a case of cerebral IA that extended during treatment with amphotericin B and showed no improvement with the addition of rifampin, clinical resolution appeared after addition of 5-fluorocytosine [80]. Another patient had sinoorbital disease that deteriorated during amphotericin B monotherapy but showed dramatic improvement after addition of rifampin and 5-fluorocytosine [81]. However, not all studies report success and lack of toxicity. In a series of 15 patients after renal transplantation, 6 were treated with amphotericin B and 9 with amphotericin B plus 5-fluorocytosine. Only 1 of the 6 patients treated with amphotericin B but 7 of the 9 treated with amphotericin B plus 5-fluorocytosine survived. However, 4 of those 7 survivors treated with amphotericin B plus 5-fluorocytosine rejected their renal allografts [82].

The only published prospective clinical study of combination therapy for pulmonary IA included 18 patients with documented systemic infection [10]. Only 1 of 9 patients receiving amphotericin B monotherapy survived, and 2 of 9 treated with amphotericin B plus 5-fluorocytosine survived. The study was terminated early because of poor outcomes in both arms. However, outcome might have been poor because entry criteria demanded confirmed fungal infection, which caused delay of standard empirical antifungal therapy. An additional possible confounder was the low dose of amphotericin B used (0.5 mg/kg/day).

Amphotericin B plus rifampin. Rifampin, approved by the US Food and Drug Administration (FDA) in 1982, has broad-spectrum activity against both gram-negative and gram-positive bacteria, as well as some species of mycobacteria, that is based on inhibition of DNA-dependent RNA polymerase [83]. Although rifampin and its analogues alone have no inherent antifungal activity, it is postulated that amphotericin B's action on the fungal cell membrane allows rifampin's entry and activity. Early experiments with protoplasts of yeast cells showed strong inhibition of RNA synthesis with rifampin, suggesting that if cells were made more permeable, they would be susceptible to rifampin [84].

In vitro work with *Saccharomyces cerevisiae* demonstrated clear dose-dependent synergy, whereas rifampin alone had no lethal effect and low-dose amphotericin B only slowed growth. However, inhibition of RNA synthesis was not complete, even in the presence of high levels of rifampin. Acrylamide gel analysis indicated that the RNA formed in the presence of rifampin was unmethylated and unstable, with much of the RNA found in polyribosomes rather than in ribosomal precursors; this suggested that rifampin preferentially inhibits the synthesis of ribosomal RNA [83].

Similar to 5-fluorocytosine, rifampin is not without side effects. Rifampin is one of the most potent inducers of the cytochrome P-450 3A enzyme system [85]. Induction leads to greatly enhanced metabolism of cyclosporine, resulting in decreased blood levels, which can lead to graft-versus-host disease or graft rejection [86]. Even a single dose of rifampin can have a profound effect [87], and short-term rifampin therapy can lead to lowered cyclosporine levels for 48 h [88]. Rifampin not only induces the metabolism of cyclosporine but also decreases its bioavailability to a greater extent than would be predicted from the increased metabolism, likely through an induction of the intestinal cytochrome P-450 enzymes [89]. Rifampin also induces clearance and reduces bioavailability of tacrolimus, although not as extensively as it does cyclosporine [90], leading to lowered blood levels [91] and graft dysfunction [92]. For instance, there has been a report that heart transplant rejection occurred after rifampin was added to an amphotericin B regimen [93].

Coadministration of rifampin with azoles, although nearly consistently demonstrating enhanced activity in vitro, should be discouraged in humans because of the potent P-450 enzyme–inducing properties of rifampin, which can result in clinically ineffective azole concentrations [7]. Even subsequent use of itraconazole is precluded by the use of rifampin for >3 days [11, 94], and a course of rifampin can produce accelerated

metabolism of itraconazole for 3 weeks after the itraconazole is stopped [95].

Rifabutin is a semisynthetic derivative of rifamycin S [96] closely related to rifampin, albeit more difficult to use in clinical practice, yet has a broader spectrum of activity and accumulates at higher tissue concentrations [97]. Rifabutin was examined in vitro in combination with amphotericin B with 26 isolates of *A. fumigatus* and *Aspergillus flavus* and demonstrated synergy in 77% of isolates (20 isolates), additivity in 23% (6), and no antagonism [96]. Amphotericin B MICs were reduced 2- to 8-fold on combination with rifabutin, and in many cases amphotericin B–resistant isolates were rendered susceptible. Similarly, the amphotericin B plus rifabutin combination reduced rifabutin MICs for all isolates 8- to 256-fold. The level of labeled uridine incorporation into RNA was unaffected with either amphotericin B (0.25 μg/mL) or rifabutin compared with incorporation in a drug-free control. However, when combination therapy consisting of amphotericin B with rifabutin at 1, 2, or 4 μg/mL was tested, incorporation was decreased by 21%, 54%, and 68%, respectively. Labeled methionine–assessed protein synthesis revealed a reduction in incorporation by 22% and 25% compared with amphotericin B monotherapy after treatment with amphotericin B combined with rifabutin at 2 and 4 μg/mL, respectively. This inhibition of protein synthesis was also very rapid, with 75% of total reduction in the first 1 h of coincubation. It is important to note that these tests were done with concentrations of rifabutin within the range of those achievable in human tissue, suggesting clinical relevance to the interaction [96].

One of the early in vitro studies in 1976 [98], which evaluated by results, MICs, inhibition of RNA synthesis, and dry-weight increase, demonstrated synergy in all 6 strains tested with amphotericin B plus rifampin. Synergy was also seen with amphotericin B plus 5-fluorocytosine in 3 of 3 strains of *A. fumigatus* and 1 of 3 strains of *A. flavus,* with additive effects in the other 2 *A. flavus* strains. When MIC was used as a measure of susceptibility, the concentrations of rifampin and 5-fluorocytosine needed to show synergy with amphotericin B were well above clinically achievable concentrations, although when the effects were measured at the level of RNA inhibition and dry-weight increase, clinically achievable levels showed significant effects. A later in vitro study of 3 clinical *Aspergillus* strains confirmed fungicidal synergy with amphotericin B plus rifampin, with amphotericin B MICs decreased 2–10-fold. Amphotericin B plus 5-fluorocytosine was indifferent in effect, and amphotericin B plus ketoconazole demonstrated no antagonism. Additionally, rifampin combinations demonstrated much greater fungicidal activity than did 5-fluorocytosine combinations [99].

Studies in a disseminated IA murine model in 1977 [100] demonstrated statistically significant synergy in reduction in deaths with amphotericin B plus rifampin at clinically achievable levels. However, when the infectious inoculum was increased, the effectiveness of the combination therapy was not significant. Improvement in survival with the combination amphotericin B plus 5-fluorocytosine versus amphotericin B monotherapy was also significant. However, in no case was infection completely eradicated, even in long-term survivors. Further animal studies of combination therapy showed indifference and some antagonism with amphotericin B plus itraconazole, indifference or additive effects with amphotericin B plus 5-fluorocytosine, and synergy or additive reactions with itraconazole plus 5-fluorocytosine [71]. In a rat model, amphotericin B at 1 mg/kg/day was no better than placebo, and after the addition of rifampin to that lower dose of amphotericin B (1 mg/kg/day), survival was somewhat worse (<20% survival), although not statistically significant. Combination of rifampin with amphotericin B (2 mg/kg/day) did not statistically alter survival compared with amphotericin B monotherapy at 2 mg/kg/day (50% vs. 60% survival) [69].

Another clinical report in 1976 [101] of success with amphotericin B plus rifampin first outlined the potential mechanisms of action of these 2 agents against *Aspergillus.* The authors postulated that amphotericin B increased the permeability of the fungal membrane to allow increased penetration of rifampin, which then inhibited the fungal RNA polymerase [83, 102]. As with 5-fluorocytosine, there are clinical reports of disease improvement only after addition of rifampin. There is 1 report of necrotizing otitis externa that showed no response to amphotericin B therapy, but the patient became asymptomatic and achieved negative results of culture with the addition of rifampin [103]. Another patient with cerebral IA treated with amphotericin B plus 5-fluorocytosine had no benefit until his therapy was changed to amphotericin B plus rifampin, along with intraventricular amphotericin B, resulting in a cure [104]. A patient with sinusitis also did not show improvement until rifampin was added to amphotericin B therapy [105].

Amphotericin B plus itraconazole. The most intriguing combinations are amphotericin B with azoles. In fact, the first clinical antifungal combination that did not involve amphotericin B plus 5-fluorocytosine or rifampin was not used until 1987 [106]. Fluconazole was one of the first azoles to be used in experimental models of aspergillosis. An in vivo rabbit systemic model showed no mortality with amphotericin B at both 0.5 mg/kg/day and 1.5 mg/kg/day. Mortality increased to 40% (4 of 10 animals) in those treated with fluconazole at either 60 mg/kg/day or 120 mg/kg/day but dropped to 9% (1 of 11) among animals given fluconazole (60 mg/kg/day) plus amphotericin B (0.5 mg/kg/day) [70]. Semiquantitative organ culture results also showed that amphotericin B at 1.5 mg/kg/day was most effective at reducing tissue burden, and the combination of amphotericin B (0.5 mg/kg/day) and fluconazole (60

mg/kg/day) was as effective as lower-dose amphotericin B (0.5 mg/kg/day) monotherapy. Importantly, there was no evidence of antagonism on the basis of semiquantitative organ culture results with amphotericin B plus fluconazole or amphotericin B plus 5-fluorocytosine, whereas treatment with fluconazole plus 5-fluorocytosine was superior to no treatment and treatment with 5-fluorocytosine [70].

With the failure of fluconazole for effective *Aspergillus* activity, the focus of attention with respect to azoles turned to itraconazole. First publicly described in 1983 [107, 108] and approved for treatment of *Aspergillus* in 1992, itraconazole (Sporanox; Ortho-Biotech) inhibits the fungal cytochrome $P\text{-}450_{14DM}$ (also known as lanosterol 14α-demethylase), which catalyzes a late step in ergosterol biosynthesis. Itraconazole's fungicidal activity is not as efficient as that of amphotericin B, because inhibition of sterol synthesis takes longer than directly creating channels in the cell membrane [109]. Itraconazole also possesses important drug interactions, with a well-known ability to inhibit the metabolism and therefore increase the blood level of cyclosporine [110] and tacrolimus [111], which may result in death [112]. Interestingly, the combination of itraconazole and tacrolimus has been reported as synergistic against azole-resistant *C. albicans* strains [113].

The most debated combination scheme for treatment of IA is amphotericin B plus itraconazole, and the theoretical risks of antagonism with this combination have been reviewed [42]. The proposed mechanism of antagonism stems from the very aspect that is often the foundation of potential synergy: different methods of action. The repeated concern is that the polyene amphotericin B, which functions by binding to ergosterol in the cell membrane, will be antagonized with an azole, which inhibits a late enzyme step in ergosterol synthesis. Therefore, instead of attacking the fungal membrane at 2 different steps for a synergistic interaction, the concern is that the azole will remove the target for the polyene. If this is commonly the case, pretreatment with itraconazole would be expected to have a much more deleterious effect than concurrent treatment, and this has been demonstrated in vitro [114].

The amphotericin B–azole interaction is difficult to assess by use of present combination checkerboard testing, because the 2 antifungal classes possess different time courses of activity [115]. Amphotericin B possesses more-rapid fungicidal activity, often complete in 6 h [19, 116]. If the fungus is exposed to both antifungals simultaneously, the activity of amphotericin B is likely to obscure the effect of the slower agent and preempt the detection of azole-induced antagonism [19, 117].

Scheven and Schwegler [28] further proposed that the lipophilic azoles (itraconazole or ketoconazole), but not the hydrophilic azoles (fluconazole), antagonize the fungicidal effects of amphotericin B because of accumulation in the fungal cell membrane. They found that the azole-induced depletion of ergosterol in the membrane required at least 1 h and that complete exchange of ergosterol by its methylated precursors occurs after ~6 h of exposure.

A novel proposed mechanism is interference of amphotericin B with a cell membrane–associated permease that is likely to be necessary for itraconazole's entry into the cell [118]. Another study has suggested that 2 itraconazole-resistant isolates, generated by exposure to miconazole, have a decreased permeability to itraconazole as opposed to an efflux mechanism [119]. This observation is based on a fall in intracellular [^{3}H]itraconazole concentrations after exposure to the respiratory inhibitor carbonyl cyanide *m*-chlorophenyl hydrazone. Few other observations of reduced permeability have been made, and more studies are therefore required to resolve the mechanisms of decreased drug accumulation in *A. fumigatus.* If amphotericin B is damaging the cell membrane, it may be having effects on the proteins there that maintain homeostasis, hence the leakage of potassium. Interference with itraconazole influx is a possibility. This effect would not be relevant for azoles with no intrinsic anti-*Aspergillus* activity, so multiple different effects could be occurring.

The difference in effects of the azoles is hypothesized to be due to an affinity of fluconazole for intracellular ligands that is comparably weaker that the affinities of the lipophilic azoles, possibly by means of a nonspecific Van der Waals-type bond [28]. Therefore, lipophilic azoles block the interaction of amphotericin B at the cell membrane by adsorbing to the cell surface, whereas water-soluble azoles do not accumulate in the cell membrane and thereby allow amphotericin B to bind to cell membrane ergosterol [120]. Thus, the interaction is more complicated than merely inhibiting ergosterol synthesis and reducing amphotericin B targets and could also include inhibition of sterol synthesis by both 14α-demethylase–dependent and 14α-demethylase–independent mechanisms [121]. Other studies showed that the antagonistic effect with amphotericin B plus itraconazole was significantly less pronounced than with amphotericin B plus ketoconazole, with either concurrent treatment [72] or sequential therapy beginning with the azole [13]. This is hypothesized to be due to the better chemotherapeutic efficacy of itraconazole against *Aspergillus* infection, compared with ketoconazole

Although there is more experience with *Candida* and combination antifungal experiments, the results are just as conflicting, likely because of varying methodologies. There are reports of both synergy [122, 123] and antagonism, [124] including positive interactions with amphotericin B and fluconazole [122] and negative interactions with itraconazole [120]. Azole–amphotericin B coincubation resulted in antagonism for 4 azoles: fluconazole, itraconazole, ketoconazole, and miconazole. However, to achieve a similar degree of antagonism, the concentration of fluconazole required was at least 2 orders of

magnitude higher than the concentration of ketoconazole or miconazole [125]. Fluconazole also increased the amphotericin B concentration needed to show activity 2.3-fold by the quantitative agar double-diffusion method [126].

An in vitro study of 15 clinical *A. fumigatus* isolates examined the activity of amphotericin B, miconazole, fluconazole, ketoconazole, and itraconazole. Monotherapy with itraconazole was the most effective and fluconazole the least active. Combination therapy with amphotericin B plus miconazole had the greatest synergistic effect, whereas amphotericin B combined with fluconazole, ketoconazole, or itraconazole demonstrated generally a subadditive effect or antagonism. Of the dual azole combinations, miconazole plus itraconazole or ketoconazole plus itraconazole demonstrated significant antagonism (in 67% of strains). Fluconazole plus itraconazole, ketoconazole, or miconazole demonstrated a subadditive effect or antagonism [16].

Combination therapy with amphotericin B plus itraconazole against murine cerebral aspergillosis did show a trend toward better survival that was not statistically significant. In that experiment, mice were treated with either amphotericin B, itraconazole formulated in cyclodextrin, or a combination of the 2 drugs. Fifteen days after infection, the amphotericin B treatment group had a 40% survival rate, and mice treated with itraconazole either once or twice a day had only a 10% survival rate; all brains of surviving mice revealed *Aspergillus*. The combination of amphotericin B plus itraconazole showed a 70% survival rate, but not statistically better than either monotherapy arm [127].

Clinical therapy with amphotericin B and azoles has been extensively reviewed [42]. Despite continuously voiced concerns regarding amphotericin B with azoles, amphotericin B plus itraconazole or fluconazole is a common treatment for some fungal infections around the world [42]. A recent epidemiological survey of treatment practices for IA revealed that 19 (3%) of 595 patients were concurrently treated with amphotericin B plus itraconazole for IA [2]; however, specific outcomes for this subpopulation of patients were not reported. Another practice survey describing the contemporary position in Europe examined 39 patients treated with amphotericin B plus itraconazole and found that 56% of patients were alive without IA, 28% were alive with IA, and only 15% had died. The mortality figure is important, because the analogous values for treatment with monotherapy were higher: with amphotericin B, 46% mortality; with liposomal amphotericin B, 46%; and with itraconazole, 50%. However, these 39 patients mostly received sequential therapy; initially concurrent therapy was only used in some instances [3].

A retrospective clinical case series of 21 patients examining concurrent therapy with amphotericin B (1 mg/kg/day) plus itraconazole (400 mg/kg/day, capsules or suspension) demonstrated no clinical antagonism, with a cure or improvement rate of 82% (9 of 11 patients) in the combination arm and an improvement rate of only 50% (5 of 10) with amphotericin B monotherapy. Two patients (18%) either had therapy failure or had no clinical or radiographic change with combination therapy, compared with 5 patients (50%) in the amphotericin B group. Mortality in the combination group was 27% (3 patients) versus 50% (5 patients) in the amphotericin B group [8]. Therapeutic differences were not statistically significant but did show improved outcome in the combination group. Of note, 10 of 11 patients completed their combination therapy with itraconazole alone. However, in practice, they were receiving combination treatment for several days to weeks on the basis of the pharmacodynamics of amphotericin B, with levels (potentially subtherapeutic) detected 3–6 weeks after the last dose [55].

As with the other combinations, there are reports of previous clinical failure followed by success only after addition of itraconazole. A patient with pulmonary and cerebral IA did not show improvement with treatment with amphotericin B plus 5-fluorocytosine but was clinically and radiographically cured with itraconazole treatment [128]. Another patient's sinusitis progressed with amphotericin B and surgical debridement, but disease was cured with liposomal amphotericin B and itraconazole treatment [129]. A patient with chronic granulomatous disease (CGD) who had pulmonary IA and rib osteomyelitis was treated with amphotericin B plus 5-fluorocytosine, but after developing vertebral osteomyelitis, a regimen of amphotericin B plus itraconazole therapy was begun, and the patient showed clinical deterioration. After the itraconazole dose was increased and amphotericin B lipid complex treatment was begun, he was cured [130]. A patient with common variable immunodeficiency with a hepatic abscess due to *Aspergillus terreus* predictably did not show improvement under treatment with liposomal amphotericin B, even with granulocyte-macrophage colony-stimulating factor, but his fever resolved and he showed clinical improvement 2 days after itraconazole was added, and he remained well 2 years after therapy [131].

These successful reports have many confounders, including recovery from neutropenia, drug dose, cytokine use, lowered immunosuppression, and adjunctive surgery. Another patient being treated with amphotericin B plus 5-fluorocytosine had worsening osteomyelitis on MRI, and after his treatment regimen was changed to liposomal amphotericin B plus itraconazole plus intralesional amphotericin B plus granulocyte transfusions, he was still no better. However, after granulocyte-macrophage colony-stimulating factor on alternate days was added to his therapeutic regimen, there was a remarkable decrease in his osteomyelitis over the next 2 months, as shown by MRI [132]. Conventional doses of liposomal amphotericin B or caspofungin had no effect on a patient's cerebral IA, but the patient did show a response to high-dose liposomal am-

photericin B (15 mg/kg/day) plus itraconazole and an accompanying marked decrease in immunosuppressive medications [133].

There are also case reports showing clinical failure of combination amphotericin B plus itraconazole. A report involving 2 liver transplant patients noted good in vitro activity of each drug when administered independently but a significant decrease in activity when administered in combination. In addition, the patients' serum, in which the drug was present, was poorly inhibitory against the isolates, and both patients experienced clinical deterioration during double antifungal therapy [134].

NEWER COMBINATION THERAPY

The persistent dismal mortality of IA and emergence of resistant strains [135] underscores the need for new classes of antifungals with newer targets [136]. In the last few years, we have seen an explosion of newer antifungals in development and testing and reignited the idea of combination therapy for IA. Now, with several entire new classes of antifungals with novel mechanisms of action, the possible combination permutations increase. However, we are still in the infancy of testing these agents as monotherapy for IA and consequently have very few reports on combination use.

Echinocandins. An entirely new class of antifungals, the echinocandins and the amino-containing pneumocandin analogues, are cyclic hexapeptide agents that interfere with cell wall biosynthesis by noncompetitive inhibition of 1,3 β-D-glucan synthase, an enzyme absent in mammalian cells but present in fungi [137, 138]. The first studies of this new drug class were with cilofungin, a now-discontinued echinocandin derivative, and showed considerable in vivo activity against *Aspergillus.* However, in combination with amphotericin B, antagonism resulted, which was associated not only with increased mortality but also with statistically earlier deaths (20% earlier), compared with amphotericin B monotherapy [139]. However, this combination was synergistic in a candidiasis model [140].

Caspofungin. Caspofungin (Cancidas; Merck), an echinocandin, is currently approved by the FDA as only the third parent drug for treatment of IA, presently indicated because of refractory aspergillosis or intolerance to other therapies. In vitro susceptibility of *A. fumigatus* by spectrophotometric and radiometric assays revealed that caspofungin plus amphotericin B and caspofungin plus itraconazole were synergistic against *A. fumigatus,* whereas amphotericin B plus itraconazole, NC1175 (an experimental conjugated styryl ketone) plus itraconazole, and NC1175 plus caspofungin had additive effects [141].

In another in vitro analysis of 14 clinical *Aspergillus* isolates, each species *(A. fumigatus, A. flavus, Aspergillus niger,* and *A. terreus)* gave similar results. Caspofungin plus amphotericin B produced synergistic or additive results for more than one-half of *Aspergillus* isolates, with no antagonism seen [142]. Further unspecified in vitro data reveal that amphotericin B combined with caspofungin is not antagonistic and in fact is possibly additive or synergistic [143]. In 1 study, several 2-drug combinations were evaluated in vitro against *A. fumigatus,* and amphotericin B plus caspofungin provided synergistic susceptibility indices, with the caspofungin combination slightly more active [144].

In a chronically immunosuppressed murine model of disseminated aspergillosis, real-time PCR was used to evaluate combination therapy with caspofungin plus amphotericin B. The mean kidney fungal burden was significantly reduced with monotherapy with both amphotericin B and caspofungin, and combination therapy reduced the *A. fumigatus* kidney burden to levels less than (in 10 of 16 animals) or equal to (in 6 of 16) the results seen with the antifungals given alone. This suggested an additive interaction, with no evidence of antagonism [145].

A phase I pharmacokinetic study evaluated drug interactions with caspofungin and itraconazole in 8 healthy volunteers and showed that the 2 drugs were unaltered with coadministration. The study also reaffirms that caspofungin is not subject to drug interactions based on CYP3A4 inhibition [146], and no adjustment is needed for concurrent use of amphotericin B with caspofungin [147].

Micafungin. In vitro analysis of 10 clinical *Aspergillus* isolates tested with another new echinocandin, micafungin (Fugisawa Healthcare), plus liposomal amphotericin B revealed no antagonism in all 10 isolates. All showed indifference and no potentiation for killing, but in 7 there was a greater partial inhibition when both drugs were present versus the partial inhibition with each drug alone, however, not achieving the definition of synergy [148]. Recent in vitro susceptibility data obtained by a checkerboard method indicate synergistic activity with micafungin plus amphotericin B or caspofungin plus amphotericin B. This study suggests that combination therapy with an echinocandin plus a polyene or azole may be more effective than monotherapy with the newer agents [144]. Another in vitro checkerboard study revealed synergistic or additive effects for micafungin plus amphotericin B in 65% of *A. fumigatus* strains, whereas micafungin plus itraconazole showed synergy or additivity in 45% and micafungin plus 5-fluorocytosine in 55%.

A murine model showed significantly higher survival with micafungin plus amphotericin B compared with monotherapy with each drug, including significant reduction in lung fungal burden and clearance of galactomannan antigen [14]. In a murine pulmonary IA model, the survival rate at 6 days was 62% with micafungin (1 mg/kg), 54% with amphotericin B (0.25 mg/kg), and 100% with a combination of micafungin plus amphotericin

B. Histological examination of the lungs of the monotherapy groups showed hyphae at 6 days, whereas the group treated with the combination showed no hyphal growth. Here the pathological findings correlated with the in vivo findings [149].

A systemic murine aspergillosis model compared micafungin monotherapy at 1, 3, or 10 mg/kg versus a suboptimal micafungin dose (3 mg/kg) alone or combined with amphotericin B, itraconazole, or nikkomycin Z (an experimental chitin synthase inhibitor). Combination therapy with the suboptimal dose of micafungin plus amphotericin B or micafungin plus itraconazole as well as monotherapy with amphotericin B, micafungin, or itraconazole prolonged survival. The lower doses of micafungin or nikkomycin Z were not efficacious alone, but the suboptimal dose of micafungin plus nikkomycin Z protected all mice, significantly superior to either alone. Fungal burden in the brain was 70% cleared with itraconazole, whereas with micafungin plus itraconazole it was 80% cleared. Although no animal was free of kidney infection, the micafungin plus nikkomycin Z as well as amphotericin B and itraconazole monotherapy regimens significantly reduced burden. Micafungin plus nikkomycin Z showed significant additive efficacy, whereas neither micafungin plus itraconazole nor micafungin plus amphotericin B resulted in significant improvement over monotherapy with itraconazole or amphotericin B. No antagonism was seen with any combination [150].

In a neutropenic rabbit model of IA, therapy with micafungin (1 mg/kg) plus amphotericin B (0.1 mg/kg) resulted in a 55% survival rate (5 of 9 animals), which decreased to 44% (4 of 9) with micafungin plus liposomal amphotericin B (0.5 mg/kg), compared with 33% (3 of 9) with monotherapy with amphotericin B and 22% (2 of 9) with liposomal amphotericin B. However, there were no statistically significant differences in survival, mean infarct score, or lung weight when micafungin plus amphotericin B or liposomal amphotericin B was compared with monotherapy with any of the 3 drugs. Further checkerboard, time kill, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays all demonstrated that amphotericin B plus micafungin interacted in vitro neither synergistically nor antagonistically. This 2-part study concluded that there was no synergy or antagonism in vitro or in vivo [151].

Voriconazole. Voriconazole (VFend; Pfizer) is a new second-generation triazole synthetic derivative of fluconazole which was first described in 1995. Voriconazole recently demonstrated superior clinical efficacy over amphotericin B in a pivotal clinical trial involving 392 patients at 92 centers in 19 countries over 3 years, comparing initial randomized therapy with voriconazole versus amphotericin B. Patients who received voriconazole initially had statistically significantly better complete or partial response (53% of patients) versus those receiving amphotericin B (32%) after 12 weeks of total therapy. Survival was also improved: 71% of patients given voriconazole survived, compared with 58% of those given amphotericin B [152]. Analysis in an open, noncomparative multicenter study of 116 patients treated with voriconazole as primary therapy (60 patients) or salvage therapy (56 patients) yielded encouraging results: 14% of patients had a complete response, 34% a partial response, and 21% a stable response to voriconazole, whereas 31% failed to respond to therapy [153]. These crucial studies led to FDA approval and likely will modify the landscape of the treatment for IA, with voriconazole surpassing amphotericin B as the antifungal agent of choice for IA.

In 1 study, several 2-drug combinations were evaluated in vitro against *A. fumigatus.* Voriconazole plus micafungin and voriconazole plus caspofungin were additive, whereas voriconazole plus amphotericin B was indifferent. Evaluation by FIC index or radiometric assay yielded similar interaction results [144].

An open-label, randomized trial of healthy adult males showed that voriconazole levels are affected by combination use with rifampin or rifabutin. Higher doses of voriconazole led to recovery of the voriconazole level with the rifabutin combination, but only partial recovery was obtained with the rifampin combination [154].

Liposomal nystatin. Nystatin, a tetraene diene macrolide, was the first polyene antifungal and was licensed for use in 1951 against superficial *Candida* infections [155]. Previous problems with solubility and toxicity with parenteral use limited nystatin to topical use [36], but recent liposomal reformulation (Nyotran; Antigenics) has reduced toxicity and preserved antifungal activity in vitro [156, 157]. Two large trials for treatment of IA have been closed because of lack of enrollment, and it is likely that the pharmaceutical company will not pursue further research with liposomal nystatin at present (E. Hawkins, Antigenics, personal communication, 2002).

One in vitro combination evaluation with 3 isolates of *A. fumigatus* showed that liposomal nystatin plus amphotericin B had additive effects against 2 of the 3 isolates [158]. There have been limited case reports of use of liposomal nystatin monotherapy and no clinical reports of combination therapy.

Nikkomycin Z. The nikkomycins are nucleoside peptide antibiotics that act as competitive analogues of the substrate uridine dinucleotide phosphate–*N*-acetylglucosamine for the enzyme chitin synthase [138, 159]. Loss of cell wall chitin, a polysaccharide found in medically important fungi but not present in mammalian cells, leads to osmotic lysis. Nikkomycin Z is not clinically available, so research is limited to the laboratory. An in vitro study revealed fungicidal synergy between nikkomycin Z and itraconazole, resulting in a $\geqslant$4-fold decrease in MICs of both drugs for *A. fumigatus* and *A. flavus* but not other *Aspergillus* species [160]. The postulated mechanism for this synergy is the loss of membrane integrity caused by the

azole facilitating uptake of the nikkomycin, or the azoles themselves interrupting chitin synthesis or precursor transport to the cell wall.

Glucan and chitin are structurally linked in the fungal cell wall, and this leads to the theoretical hypothesis that a dual-target approach could enhance effect. A glucan synthase inhibitor (anidulafungin) and a chitin synthase inhibitor (nikkomycin Z) showed marked in vitro synergy for both inhibition and killing against 5 isolates of *A. fumigatus* [161]. In another study, *A. fumigatus* isolates previously resistant in vitro to monotherapy with cilofungin, the discontinued echinocandin, showed a high degree of synergism when cilofungin and nikkomycin Z were combined [162].

In another in vitro study, a checkerboard assay demonstrated synergy between nikkomycin Z plus micafungin against *A. fumigatus* and indifference against *A. flavus, A. terreus,* and *A. niger.* Significant synergistic hyphal damage against *A. fumigatus* was demonstrated over a wide range of drug concentrations, and the synergistic effect was most pronounced after 12 h of incubation and sustained through 24 h of incubation [163].

Terbinafine. Since its introduction into clinical practice in 1991, clinicians have used oral terbinafine (Lamisil; Novartis Research Institute) mainly for dermatophyte infections of the skin and nails [164]. Terbinafine may hold promise for use in combination IA therapy. In vitro interactions with terbinafine plus itraconazole revealed synergistic or additive results against 9 clinical *Aspergillus* isolates. Terbinafine plus fluconazole was also synergistic against *A. fumigatus, A. terreus,* and *A. flavus* and indifferent with *A. niger* isolates. Amphotericin B plus terbinafine as well as 5-fluorocytosine plus terbinafine were generally indifferent or antagonistic. The MFCs of combinations were generally in accord with MICs, although the killing of *A. fumigatus* by amphotericin B plus terbinafine in combination was enhanced. This study highlighted the promise of terbinafine combinations with azoles, but not amphotericin B or 5-fluorocytosine, for combination therapy for IA [165].

An in vitro study of 4 clinical isolates of *A. fumigatus* and 1 of *A. niger* showed an additive interaction between amphotericin B plus terbinafine against 1 strain and synergy against the other 4 strains. For the strains against which synergy was demonstrated, the presence of a low concentration of amphotericin B resulted in a 2-step reduction in the terbinafine MIC, but the reverse effect did not occur [166]. This raises the issue of possibly classifying this interaction as additive and not synergistic. The combinations of terbinafine plus itraconazole and terbinafine plus voriconazole showed exceptional fungicidal synergy against all 5 isolates. Although fluconazole had no inherent activity, it did lower the terbinafine MIC, resulting in an additive to synergistic interaction [166].

An in vitro checkerboard study evaluated terbinafine, amphotericin B, and itraconazole against 4 itraconazole-susceptible and 3 itraconazole-resistant *A. fumigatus* isolates. Growth measurements were concordant between FIC indices and an interaction coefficient alpha determined by a computer program by means of the universal response surface approach of Greco 166a. The amphotericin B plus itraconazole combination demonstrated slight antagonism, and amphotericin B plus terbinafine demonstrated indifference. Use of itraconazole plus terbinafine against itraconazole-susceptible strains indicated synergism; however, no coefficient could be reliably obtained for the itraconazole-resistant strains. This study revealed that the most potent combination was itraconazole plus terbinafine, which was also active against itraconazole-resistant strains [167].

The only other clinical combination antifungal study for IA involved a small, randomized study comparing amphotericin B plus placebo with amphotericin B plus terbinafine (750 mg/day) (unpublished data). This study showed that mortality was significantly higher in the combination group.

Combinations with antibacterials. DU-6859a, a fluoroquinolone with bactericidal activity based on inhibition of bacterial DNA gyrase (topoisomerase II) but no antifungal activity, had clear in vitro synergistic activity with amphotericin B against 3 strains of *A. fumigatus* at lower concentrations but displayed antagonism at higher concentrations in different media. A mouse model study showed that DU-6859a potentiated the effect of amphotericin B in a dose-dependent fashion, but clinical studies remain to be done [168].

In vitro testing of *Aspergillus* isolates with azithromycin, a protein synthesis inhibitor also with no inherent antifungal activity, showed a synergistic interaction with amphotericin B, leading to a 2- to 10-fold reduction in amphotericin B MICs. Assessment of fungal protein synthesis also revealed 68% reduction with combination therapy but no reduction with each agent used alone [169]. Finally, use of imipenem may also influence amphotericin B treatment, because 1 in vitro study showed a decrease in susceptibility of *A. fumigatus* strains to amphotericin B when tested in combination with imipenem; however, there was no direct chemical interaction between the 2 agents [170].

TRIPLE THERAPY

It is inherently unlikely that the maximal degree of synergy attainable with antifungals is present with combinations of only 2 drugs [43]. However, reports for animal models and clinical studies of even double-combination antifungal therapy for IA are scarce. Because nearly all of these models use 2-drug therapy, very little is known about triple- or quadruple-drug therapy. Moreover, the full panoply of interactions of a range of concentrations for the interacting drugs, as is possible in checkerboard assays, becomes almost inoperably difficult in matrices exceeding 2 dimensions.

A 1982 in vitro study included triple therapy with amphotericin B plus 5-fluorocytosine plus miconazole or ketoconazole as well as amphotericin B plus ketoconazole plus miconazole. Interactions were generally synergistic with amphotericin B plus 5-fluorocytosine plus ketoconazole and additive with the miconazole combinations. Quadruple therapy with amphotericin B plus 5-fluorocytosine plus ketoconazole plus miconazole was also tested and yielded a generally synergistic result, with no isolates showing indifference or antagonism [38].

In vivo triple therapy with 5-fluorocytosine plus amphotericin B plus itraconazole is reported as not curative in aspergillosis and more deleterious than the combination 5-fluorocytosine plus itraconazole [171]. This triple therapy was tested in a murine model with 5-fluorocytosine–susceptible and –resistant strains [72], and 10 of 16 susceptible strains showed indifference and the remainder antagonism. Five of the 8 resistant strains showed indifference, 1 an additive effect, and 2 an antagonistic effect.

There are few case reports of triple antifungal therapy for IA, and as expected, they are skewed toward success. A patient's cerebral disease worsened during treatment with amphotericin B, showed no improvement with the addition of rifampin, but had clinical resolution after addition of 5-fluorocytosine [80]. The condition of a patient with sinoorbital disease deteriorated during amphotericin B treatment but dramatically improved after addition of rifampin and 5-fluorocytosine [81]. The potential benefit would be added clinical activity through synergistic or additive interactions, but toxicity might also be increased. In addition, there is an increase in the chance of drugs interacting antagonistically.

SEQUENTIAL THERAPY

In the quest for optimal IA therapy, some clinicians have experimented with concurrent combination antifungal therapy with different drug classes. However, reports of various patterns of sequential antifungal therapy raise another issue of antifungal interactions—the appropriate and safe sequence of agents. The long half-life of amphotericin B confounds matters, so even sequential use has an element of concurrent therapy [172]. Given the conflicting reports on combination therapy, the issue of sequential therapy generates further confusion. Additionally, the literature on polyenes and azole interactions is limited, and information regarding newer classes is understandably scarce.

The most practical experience is with the sequence of amphotericin B followed by itraconazole. There seem to be many instances of initial therapy with amphotericin B followed by itraconazole with generally no harm seen [2, 12, 172, 173]. A widely accepted regimen uses amphotericin B to treat a patient's acute disease until neutropenia resolves and then itraconazole maintenance therapy is used for antifungal coverage [2, 174]. Because this sequence appears safe and is, in fact, recommended in recent guidelines [11], we instead explored the outcomes with other less commonly used sequences of therapy. As with concurrent therapy, the most debated sequence is an azole followed by amphotericin B. Antagonism is postulated to be due to azole inhibition of fungal ergosterol synthesis and subsequent exhaustion of the target for amphotericin B, with loss of antifungal effect of amphotericin B [175, 176].

In a survey of the treatment regimens of 595 patients with IA during 1994–1995, sequential therapy with amphotericin B followed by itraconazole was used to treat 93 patients (16%), whereas itraconazole followed by amphotericin B was used for only 10 patients (2%) [2]. That survey found that sequential therapy (amphotericin B followed by itraconazole) showed higher response rates compared with amphotericin B monotherapy. However, this possibly reflected a selection bias, because the healthier patients were the ones that survived long enough to receive oral itraconazole therapy. Seventy percent of the patients (130 of 187) treated with amphotericin B monotherapy were considered severely immunosuppressed, compared with 52% (48 of 93) receiving sequential amphotericin B followed by itraconazole and only 17% (10 of 58) receiving itraconazole alone. They found that clinical complete or partial responses to antifungal therapy were significantly lower in the amphotericin B monotherapy group (32% of patients) than in the group treated with sequential amphotericin B followed by itraconazole (54%) or itraconazole alone (57%). This is possibly not surprising, because the amphotericin B alone group had the highest number of severely immunosuppressed patients. However, the number of patients in that survey treated with concurrent amphotericin B plus itraconazole or itraconazole followed by amphotericin B was too small for meaningful conclusions [2].

Studies with *Candida* may help to shed some light on the mechanistic issues of antagonism between polyenes and azoles. Preincubation of *C. albicans* with fluconazole before exposure to amphotericin B resulted in dramatic decreases in amphotericin B fungicidal activity; no amphotericin B antagonism was seen after incubation for 1–2 h in fluconazole, but amphotericin B was antagonized after incubation for 3–4 h. After preexposure to fluconazole, *C. albicans* also remained resistant to amphotericin B for up to 3 days [177]. In another study, simultaneous in vitro administration of fluconazole did not affect amphotericin B activity against *C. albicans,* but marked antagonism was seen when isolates were exposed to fluconazole for $\geqslant$8 h before addition of amphotericin B. Additionally, after removal of fluconazole from culture, amphotericin B activity was partially delayed but fungicidal activity was restored [178].

If fluconazole acts only by depleting membranes of ergosterol and causes tolerance to amphotericin B by removing its target, then adding ergosterol to the media during the fluconazole

incubation may allow replacement and thus restore the target and susceptibility for amphotericin B. However, *C. albicans* cells exposed to fluconazole in a medium containing the highest level of ergosterol (35 μg/mL) were as resistant to subsequent amphotericin B exposure as were control cells in an ergosterol-free medium. This suggests that either exogenous ergosterol uptake did not occur or ergosterol depletion is not the only mechanism involved in this interaction [177].

Removal of the *C. albicans* cell membrane with methanol solutions revealed again that fluconazole was not bound at detectable amounts. Preincubation with lipophilic azoles and then amphotericin B increased viable yeast cell counts up to 10,000 times compared with amphotericin B alone, whereas fluconazole did not remarkably antagonize amphotericin B. Preincubation with other lipophilic substances did not produce any comparable antagonism. Because of a lack of antagonizing effects of lipophilic agents other than azoles on the fungicidal activity of the subsequently applied amphotericin B, the antagonism hypothesis based strictly on the cell membrane is incorrect and the dependence of antagonism on the azole moiety is clear [28].

After penetration of the cellular envelope, large quantities of lipophilic azoles bind to intracellular domains and are released from the reservoir only after removal of the azole from the incubation medium. This period of intracellular azole attachment could be sufficient for complete blockade of cytochrome P-450. In contrast, a smaller intracellular binding capacity would be expected for fluconazole because of its greater hydrophilicity. In another study, there was a postantifungal effect of the lipophilic azoles ketoconazole and miconazole, which caused impaired growth for >24 h, but a similar effect could not be consistently shown for itraconazole. In contrast, no postantifungal effect of growth inhibition was found for fluconazole [125]. The absence of a postantifungal effect of fluconazole also supports the hypothesis of differential binding. Insertion of lipophilic side chains of certain azoles into the cell membrane may influence affinity of the membrane for amphotericin B, and such impairment could involve conjugated sterol bonds [125].

Sequential therapy with an azole followed by amphotericin B for *Aspergillus* infection has been the focus of several experiments. An important in vitro study of 6 clinical isolates of *A. fumigatus* showed that pretreatment with ketoconazole at clinically relevant levels uniformly and dose dependently suppressed the fungicidal, but not fungistatic, activity of amphotericin B (2 mg/kg). Antagonism was also increased with prolonged ketoconazole incubation. However, when ketoconazole was added at the same time as amphotericin B, antagonism was minimal but reproducible. The authors also showed that after preexposure, when ketoconazole was added again with amphotericin B, the MFC of amphotericin B was further increased [13].

Another in vitro study of 15 clinical *A. fumigatus* isolates showed that pretreatment with amphotericin B followed by miconazole or fluconazole resulted in a greater synergistic effect than when the drugs were given simultaneously. However, pretreatment with either ketoconazole, fluconazole, or itraconazole and then amphotericin B generally showed antagonism, whereas pretreatment with miconazole produced a somewhat synergistic effect [16]. This work confirmed earlier findings [13] in which pretreatment with ketoconazole followed by amphotericin B antagonized amphotericin B's effects. Another study also showed that pretreatment with fluconazole antagonized amphotericin B's action but that the isolate regained susceptibility 1 h after the fluconazole was washed off [179].

One in vitro study examined 12 clinical *A. fumigatus* isolates by use of the Etest (AB Biodisk) to avoid some of the nonstandardization of broth microdilution techniques. The investigators observed an overall increase in the amphotericin B MIC (a net change of 2–32 μg/mL) for all isolates when the isolates were preexposed to noninhibitory concentrations of itraconazole, but not fluconazole; the increase was more pronounced with higher concentrations of itraconazole. This antagonism was specific for itraconazole, because preexposure of 3 isolates to subinhibitory concentrations of H_2O_2, a known growth inhibitor, or fluconazole either decreased (H_2O_2) or did not change (fluconazole) the amphotericin B MIC after sequential administration of amphotericin B. Also, preexposure to itraconazole appeared to result in a greater increase in the amphotericin B MIC than did concomitant administration of itraconazole. However, preexposure to subinhibitory concentrations of amphotericin B decreased the itraconazole MIC [114]. This sequential antagonism was also first documented to be fully reversible with culture incubation in a drug-free environment for 24 h, presumably because of recovery of the ergosterol content of the fungal membrane for amphotericin B action. This is important, because some patients receive itraconazole prophylaxis before amphotericin B therapy.

In a murine model of pulmonary IA, oral itraconazole (50 mg/kg b.i.d.) was given for 3 days, with serum drug concentrations monitored, before treatment with amphotericin B at 4 different dosages. At all time points, fungal lung burden was statistically significantly higher in those animals pretreated with itraconazole, as measured by both colony-forming unit counts and chitin assay. Also, fewer itraconazole-pretreated mice than those not pretreated were alive at 96 h (0–20% vs. 60%–80% survival) when treated with amphotericin B doses of 1 and 3 mg/kg/day, showing that even higher-dose amphotericin B did not reverse the antagonism [17].

In another murine model, ketoconazole pretreatment was done 24 h before amphotericin B treatment. Seven of 13 mice

pretreated with ketoconazole died, and this antagonistic effect was increased with 48 h of pretreatment, confirming the in vitro findings. Even when ketoconazole therapy was stopped when amphotericin B began, the effect was still there. These survival differences with pretreatment were also verified by organ culture [13]. A possible explanation of the effect of ketoconazole on amphotericin B is that ketoconazole has been shown to have a direct membrane-damaging effect independent from interference with ergosterol synthesis [180].

One study describes a renal transplant patient with a diagnosis of IA whose treatment was initially itraconazole and who showed some clinical improvement. Therapy was then changed to amphotericin B because of hepatotoxicity; over the next 10 days, the patient's condition markedly deteriorated, and the patient died, despite removal of the transplanted kidney and reduction of immunosuppression. The same authors report that amphotericin B lost its in vitro activity against 6 *A. fumigatus* isolates after the isolates were exposed to subfungicidal concentrations of itraconazole, even if itraconazole treatment was stopped before amphotericin B treatment was begun. They confirmed their clinical and in vitro findings in a systemic neutropenic mouse model and found that amphotericin B and itraconazole monotherapy were both superior to combination or sequential therapy. However, there was no significant difference in treatment with itraconazole (100 mg/kg/day) for days 0–2 followed by amphotericin B (2 mg/kg/day) for days 2–6 versus itraconazole for days 0–2 followed by amphotericin B for days 2–6 [15].

Individual clinical sequential antifungal case reports show a spectrum of outcomes with little standardization. One study analyzed 7 heart transplant recipients with pulmonary IA, who were divided into 2 groups. Four patients were treated with itraconazole for 28 days, and their conditions worsened, but the conditions of 3 showed improvement when treatment was changed to amphotericin B. The second group of 3 patients all did well with amphotericin B monotherapy [181]. Another report showed that itraconazole was ineffective in a patient with pulmonary IA, but that the patient's condition improved when treatment was changed to amphotericin B [182]. Amphotericin B plus 5-fluorocytosine showed no effect against a case of cerebral IA, but after low-dose ketoconazole was added to the regimen for 1 month and then high-dose ketoconazole was administered, there was clinical improvement [183]. A patient with CGD, rib osteomyelitis, and a pulmonary lesion was unsuccessfully treated with amphotericin B plus 5-fluorocytosine, but after the regimen was changed to itraconazole, the lesion decreased in size [184]. Another patient with CGD with pulmonary IA was also unsuccessfully treated with amphotericin B plus 5-fluorocytosine therapy but showed improvement with itraconazole therapy [185]. A child with CGD and pulmonary IA deteriorated during 6 weeks of amphotericin B treatment and granulocyte transfusions but showed improvement after 4 weeks of voriconazole monotherapy. The patient continued iv voriconazole treatment and then oral therapy for 8.5 months, followed by itraconazole prophylaxis, and remained disease-free 2 years later [186].

GENERALIZATIONS

With the continued poor efficacy of conventional therapies for IA, clinicians are looking for unique strategies that use both newer antifungals as well as potentially immunomodulatory therapy [4]. Now with increased therapeutic options, combination antifungal practices will play a major role in this new generation of treatment. This is the most comprehensive review undertaken that evaluates the practice patterns and outcomes of combination and sequential antifungal therapy for IA.

The clinician should be aware of the bias of the reported literature, possibly historically angled toward reports of success. This is further offset with the countless reports of successful or failed combination therapies used to treat patients, including by the present authors, that were never published. The interpretation of cause and effect is also crucial, as exemplified in a case of cerebral IA treated with low-dose ketoconazole followed by amphotericin B plus 5-fluorocytosine that did not improve but did improve after addition of high-dose ketoconazole [183]. Because ketoconazole has no appreciable activity against *Aspergillus,* and the report states that amphotericin B plus 5-fluorocytosine was used for 1 month before the addition of ketoconazole, it is possible the clinical course and the addition of ketoconazole were coincidental. This emphasizes that it is somewhat difficult to evaluate all reports of sequential failure with one agent followed by success with another, because the contribution of the initial therapy to the eventual good outcome can sometimes be questioned.

As outlined herein, because of varying laboratory conditions and definitions, there is at present no universal standard for conducting studies or interpreting results of combination in vitro antifungal treatment. For example, several older studies report only MIC data and do not use checkerboard techniques to analyze drug interactions. Additionally, the laboratory and clinical relevance are potentially suspect. The fact that a combination is synergistic in vitro does not, in itself, guarantee its usefulness in vivo, because the combination may be too toxic for the host or the required concentrations may not be achievable [43]. As with any opportunistic infection, host immune status is paramount. Another important point in interpreting combination studies is the inherently low activity of some antifungals, such as terbinafine or fluconazole, against *Aspergillus.* Therefore, a slight increase in activity when these 2 agents are combined would quantitatively be viewed as synergistic; yet, in practice, even this synergistic combination might be far inferior

to monotherapy with amphotericin B, which has well-established efficacy.

Our review of all available in vitro combination reports (Appendix A, table A2) revealed generally more positive interactions than did the in vivo studies. For instance, rates of in vitro synergy (36% of interactions) and additivity (24%) were higher than in vivo synergy (14%) and additivity (20%). There is more in vivo indifference (51% of interactions) and antagonism (14%), as well, compared with in vitro indifference (28%) and antagonism (11%). This echoes concerns that in vitro data do not accurately represent the in vivo interactions, but it also points to the difficulty in defining synergy in an animal model.

The limited number of in vitro and in vivo studies makes it difficult to draw firm conclusions and make firm recommendations, yet some trends are apparent. For instance, it appears, from analyzing in vitro studies, that the historically used combination of amphotericin B plus rifampin generally displayed positive interactions. However, these in vitro results do not take into account a clinical situation in which toxicity and drug interaction could be substantial. Amphotericin B plus itraconazole appears generally indifferent, whereas amphotericin B plus ketoconazole shows largely antagonism. The newer echinocandins are promising, with both amphotericin B plus caspofungin and amphotericin B plus micafungin showing generally synergistic or additive interactions. Other trends included the observation that terbinafine plus amphotericin B was not effective in combination, whereas the azoles appear to have more favorable results with terbinafine [165].

In vivo studies (Appendix A, table A3) support more of an indifferent effect with amphotericin B plus 5-fluorocytosine and amphotericin B plus rifampin. Important is the observation that amphotericin B plus itraconazole appears largely indifferent and even antagonistic in vivo, nearly exactly following in vitro results. In vivo work with amphotericin B plus micafungin also confirms in vitro positive or indifferent interactions, whereas there are limited in vivo data on amphotericin B plus caspofungin. These animal models may more accurately represent the actions inside a human host, taking into account antifungal pharmacokinetics and tissue penetrance.

The clinical outcomes (table 4) for IA from 1966 to 2001 show general improvement in 63% of patients. A review of all individual combinations reports is very weighted toward the inclusion of more therapies from years earlier and generally showed patient improvement with amphotericin B plus 5-fluorocytosine or amphotericin B plus rifampin. Interestingly, amphotericin B plus itraconazole was generally indifferent in clinical outcome, which matches both in vitro and in vivo indifference. Clinical reports of combinations with the newer antifungals are too scarce to make generalizations about, although both the second-generation triazoles and the echinocandins seem promising. Here it remains unclear if an agent such as voriconazole, shown to be more effective than amphotericin B as initial monotherapy [152], will be further potentiated with agents from a difficult drug class for use in those patients whose infections are recalcitrant to therapy.

Outcomes with sequential antifungal therapy (table 7) were, again, divided nearly equally for patients receiving itraconazole followed by amphotericin B. Most other reports regarding sequential therapy noted that improvement was demonstrated, yet most reports included only a single patient. Of note, the sequences concluding with voriconazole were effective, but again it is unclear if this is simply a factor of the effectiveness of voriconazole monotherapy.

CONCLUSION

Since 1958, the most common treatment for IA has been a regimen of the relatively toxic amphotericin B, often administered to patients in the late stages of their disease. Unfortunately, amphotericin B is only moderately effective against IA [56], and therapy may be ineffective in the absence of bone marrow recovery. Literature and research on combination antifungal therapy and interactions is still in its infancy, and to date no clinical study has convincingly answered the question of combination therapy or sequential therapy. Studies with amphotericin B and itraconazole, for instance, have demonstrated a range of effects from synergy to antagonism. Now with entire new classes of antifungals with novel actions, the option of combination therapy needs to be investigated.

Concerns about interpreting any laboratory study persist, although, now that there are in vitro standards, investigators can tackle questions with results viewed as comparable [42]. However, laboratory results still need to be correlated with clinical outcomes. It is premature to recommend combination antifungal therapy for general use; rather, each patient needs to be addressed individually. The clinician faces a difficult situation when a critically ill patient's condition worsens during monotherapy. What is needed is careful and detailed prospective observation of the effects of combination and sequential therapy to establish a database of experience, eventually leading to clinical combination antifungal trials for IA.

APPENDIX A

Table A1. Summary of 249 reports of clinical combination antifungal therapy for invasive aspergillosis (IA).

Reference	Year	No. of patients	Underlying condition(s)	Type(s) of IA	Treatment	Evaluation method(s)	Outcome(s)
Atkinson and Israel [74]	1973	2	Immunocompetent; sarcoidosis	Pulmonary	AmB + 5-FC	CXR	Improvement
Carrizosa et al. [75]	1974	1	Aortic valve prosthesis	Endocarditis	AmB + 5-FC	Clinical	Improvement
Warshawsky et al. [77]	1975	1	NIDDM	Renal	AmB + 5-FC + Rif	Culture	Improvement
Ribner et al. [101]	1976	1	AML	Pulmonary	AmB + Rif	CXR	Improvement
Gordon and Holzman [187]	1976	1	IVDA	Meningitis	AmB + 5-FC	CT	Improvement
Kyriakides et al. [188]	1976	2	Renal transplantation	Pulmonary	AmB + 5-FC + Rif	CXR	Improvement
Sinclair et al. [189]	1978	5	AML	Pulmonary	AmB + 5-FC	CXR	Improvement (3); died (2)
Beyt et al. [190]	1978	1	AML	Pulmonary	AmB + Rif	CXR	Improvement
Codish et al. [191]	1979	1	Immunocompetent	Pulmonary	AmB + 5-FC	CXR	Improvement
Mikulski et al. [192]	1979	1	ALL	Pulmonary, endocarditis, myocardial abscess	AmB + Rif + 5-FC	CXR	Died
Luce et al. [193]	1979	1	AML	Pericarditis	AmB + Rif	CXR	Died
Perlmutter et al. [194]	1980	1	NIDDM	Cerebral	AmB + Rif	CT	Died
Borkin et al. [195]	1980	1	ALL	Pulmonary	AmB + Rif	CXR	Died
Yu et al. [81]	1980	1	Immunocompetent	Sinusitis, orbital	AmB + Rif + 5-FC	CT	Improvement
Sekhar et al. [80]	1980	1	Immunocompetent	Cavernous sinus thrombosis	AmB + Rif + 5-FC	CT	Improvement
Doft et al. [196]	1980	1	IVDA	Endophthalmitis	AmB + 5-FC	Electroretinography	Improvement
Corrado et al. [197]	1980	1	CGD	Rib osteomyelitis	AmB + Rif	CXR	Improvement
Drexler et al. [198]	1980	1	Aortic valve prosthesis	Endocarditis	AmB + 5-FC	Clinical	Died
Ramos-Gabatin and Jordan [199]	1981	1	Immunocompetent	Pituitary	AmB + 5-FC	CT	Improvement
Ahmad et al. [200]	1981	1	Immunocompetent	Mediastinitis	AmB + 5-FC	CXR	Died
Walsh and Bulkley [201]	1982	2	CLL; CML	Pericarditis	AmB + 5-FC	Clinical	Died
Henze et al. [202]	1982	1	ALL	Pulmonary, cerebral	AmB + 5-FC	CXR, CT	Improvement
Lazzarin and Capsoni [203]	1982	1	CGD	Rib osteomyelitis	AmB + Rif	CXR	Improvement
Tack et al. [204]	1982	1	Hemilaminectomy	Vertebral osteomyelitis	AmB + 5-FC	Myelography	Improvement
Mawk et al. [205]	1983	2	Immunocompetent	Vertebral osteomyelitis	AmB + 5-FC	Myelography	Improvement (1); no improvement (1)
Daly et al. [206]	1983	1	AML	Pulmonary	AmB + Rif	CXR	Improvement
Weiland et al. [82]	1983	9	Renal transplantation	Pulmonary	AmB + 5-FC	CXR	Improvement (7); died (2)
Berkow et al. [207]	1983	1	ALL	Sinusitis	AmB + 5-FC	Clinical	Died
McKee et al. [208]	1984	1	G6PD	Vertebral osteomyelitis	AmB + Rif	CT	Improvement
Swerdlow et al. [209]	1984	1	AML	Sinusitis	AmB + Rif	CT	Improvement
Rodenhuis et al. [210]	1984	1	Healthy	Pulmonary	AmB + 5-FC	CXR	Improvement
Vieira et al. [211]	1984	1	Near drowning	Pulmonary	AmB + 5-FC	CXR	Improvement
Wagner et al. [212]	1985	1	Mitral valve prosthesis	Endocarditis	AmB + 5-FC	Clinical	Improvement
Landoy et al. [213]	1985	1	Aplastic anemia	Sinusitis	AmB + Rif	CT	Improvement
Fuchs et al. [105]	1985	1	Immunocompetent	Sinusitis	AmB + Rif	CT	Improvement
Modry et al. [93]	1985	2	Heart transplantation	Disseminated, pulmonary	AmB + Rif	Clinical	Died (1); improvement (1)
Van de Wyngaert et al. [214]	1986	1	Immunocompetent	Meningitis	AmB + Rif, followed by AmB + 5-FC	Myelography	Improvement
Mullen et al. [215]	1986	1	IVDA	Cerebral, endocarditis	AmB + 5-FC	CT, Echo	Died

(continued)

Table A1. ***(Continued.)***

Reference	Year	No. of patients	Underlying condition(s)	Type(s) of IA	Treatment	Evaluation method(s)	Outcome(s)
Daenen et al. [216]	1986	1	AML	Pulmonary	AmB + 5-FC, followed by Ket	Clinical	Improvement
Rhine et al. [217]	1986	1	Neonatal	Cerebral	AmB + Rif + intraventricular AmB	CT	Improvement
Spiteri et al. [218]	1986	1	Immunocompetent	Pulmonary	AmB + 5-FC	CXR	Improvement
Davies et al. [106]	1987	1	NIDDM	Renal	5-FC + Ket, followed by AmB + 5-FC	IV pyelography	Improvement
Burch et al. [64]	1987	10	AML	Pulmonary	AmB + 5-FC	CT	Improvement (9); died (1)
Ruutu et al. [219]	1987	3	AML (1); BMT (2)	Pulmonary	AmB + 5-FC	CXR	Died
Denning and Williams [79]	1987	1	Lymphoma	Pulmonary	AmB + 5-FC	CXR	Improvement
Kwong et al. [183]	1987	1	SLE	Cerebral	AmB + Ket + 5-FC	CT	Improvement
Allo et al. [220]	1987	5	AML (3); ALL (1); aplastic anemia (1)	Invasive cutaneous	AmB + 5-FC	CT	AML: improvement (2), died (1); ALL: died (1); aplastic anemia: died (1)
Bradley et al. [221]	1987	1	Immunocompetent	Sinusitis, orbital	AmB + 5-FC	CT	Improvement
Karp et al. [222]	1988	10	AML	Pulmonary	AmB + 5-FC	CT	Improvement
Cunningham et al. [103]	1988	1	Immunocompetent	Necrotizing otitis externa	AmB + Rif	CT, culture	Improvement
Stanley et al. [223]	1988	1	CLL	Mastoiditis	AmB + 5-FC	Culture	Died
Neijens et al. [184]	1989	1	CGD	Pulmonary, osteomyelitis	AmB + 5-FC	Scintigraphy	No improvement
Goodman and Coffey [224]	1989	1	AML	Cerebral	AmB + Rif	CT	Improvement
van't Wout et al. [185]	1990	1	CGD	Pulmonary	AmB + 5-FC, followed by Itr	CXR	No improvement
Katz et al. [225]	1990	1	Heart transplantation	Pulmonary	AmB + Rif, followed by L-AmB monotherapy	CXR	Improvement
Nussaume et al. [226]	1990	1	Aortic prosthesis	Aortic graft	Itr + 5-FC	Clinical	Improvement
Dupont [227]	1990	7	AML (3); ALL (3); myeloma (1)	Sinus (3); pulmonary (4)	AmB + 5-FC (2); AmB + Itr (5)	CXR	Improvement
Blomley et al. [228]	1990	1	Emphysema	Pulmonary	Itr + Rif	CXR, sputum	Died
Talbot et al. [229]	1991	6	AML	Sinusitis	AmB + Rif (4); AmB + 5-FC (2)	CT	Improvement with Rif (2); died (4)
Kloss et al. [128]	1991	1	CGD	Pulmonary, cerebral	AmB + 5-FC + Itr	CT	Improvement
Brincker et al. [78]	1991	10	AML (9); CML (1)	Pulmonary	AmB + 5-FC	CXR	Improvement
Kumar et al. [230]	1991	1	Immunocompetent	Skin	AmB + Rif + Ket, followed by Itr	Clinical	Improvement after Itr
Green et al. [104]	1991	1	Liver transplantation	Cerebral	AmB + 5-FC, followed by AmB + Rif + intraventricular AmB (h/o 5-FC + AmB)	CT	Improvement
Groll et al. [231]	1992	1	ALL	Pulmonary	AmB + 5-FC	CT	Died
Marterre et al. [232]	1992	1	ALL	Bowel invasion	AmB + 5-FC	Biopsy	Improvement
Lortholary et al. [233]	1993	3	HIV	Pulmonary (2); sinusitis (1)	AmB + Itr	BAL	Died
Hummel et al. [234]	1993	1	Heart transplantation	Osteomyelitis	AmB + 5-FC	CT	Improvement
Kline et al. [130]	1994	1	CGD	Pulmonary, rib and vertebral osteomyelitis	AmB + 5-FC, AmB + Itr, ABLC + Itr	CXR, MRI	Improvement
Verweij et al. [10]	1994	9	Acute leukemia	Pulmonary	AmB + 5-FC	CXR, sputum	Died (7); improvement (2)
Kerkmann et al. [235]	1994	1	Alcohol abuse	Cerebral	AmB + 5-FC	CT	Improvement

(continued)

Table A1. ***(Continued.)***

Reference	Year	No. of patients	Underlying condition(s)	Type(s) of IA	Treatment	Evaluation method(s)	Outcome(s)
Cortet et al. [236]	1994	6	Heart transplantation (3); steroids (1); hairy cell leukemia (1); unknown (1)	Spondylodiscitis	AmB + 5-FC	CT	Improvement
van Ede et al. [237]	1994	1	AML	Pulmonary, pericarditis	AmB + Itr	CXR	Improvement
Matsuzono et al. [238]	1995	1	CGD	Cerebral, pulmonary	AmB + 5-FC + Flu	CXR, MRI	Improvement
Teh et al. [239]	1995	3	HIV	Sinusitis	AmB + Itr	CT	Died
Coleman et al. [240]	1995	1	ALL	Cerebral	L-AmB + Itr	CT	Improvement
Desselle et al. [241]	1995	1	AML	Pulmonary	AmB + Rif	CT	Died
Merino et al. [242]	1995	1	ALL	Pulmonary	AmB + Itr	CXR	Improvement
Michailov et al. [243]	1996	5	BMT	Pulmonary	AmB + Itr	CT	Improvement (3); died, AML (2)
Nampoory et al. [244]	1996	2	Renal transplantation	Pulmonary	AmB + Rif	CT	Improvement (1); died (1)
Naim-Ur-Rahman et al. [245]	1996	7	Not reported	Sinus, cerebral	AmB + 5-FC	CT	Died (2); improvement (5)
Darras-Joly et al. [246]	1996	3	Ependymoma (1); bladder carcinoma (1); mastoidectomy (1)	Cerebral	ABCD + Itr (1); AmB + 5-FC (1); L-AmB + 5-FC + Itr (1)	Clinical	Died (1); died, malignant hypercalcemia (1); improvement (1)
Janssen et al. [247]	1996	7	Non-Hodgkin lymphoma (2); ALL (1); CML (1); AML (2); near-drowning (1)	Pulmonary	AmB + Itr	BAL	Died (6); improvement (1)
Dal Conte et al. [248]	1996	1	HIV	Pulmonary	Itr + 5-FC	BAL	Improvement
Sessa et al. [249]	1996	1	Chronic renal failure, dialysis	Pulmonary	L-AmB + Rif	CT	Improvement
Hovi et al. [250]	1996	1	AML	Osteomyelitis	AmB + 5-FC, followed by AmB + Itr	CT	Improvement
Basler et al. [251]	1997	1	BMT	Cerebral	L-AmB + Itr	CT	Improvement
Taillandier et al. [252]	1997	1	Heart-lung transplantation	Osteomyelitis	AmB + 5-FC	MRI	Improvement
Karim et al. [253]	1997	1	Immunocompetent	Pulmonary	AmB + Itr	CXR	Died
Proctor and Jackson [254]	1997	1	BMT	Cerebral	AmB + Rif	CT	Improvement
Iemmolo et al. [255]	1998	1	Liver transplantation	Cerebral	L-AmB + Itr	MRI	Improvement
Levy et al. [256]	1998	1	ALL	Pulmonary	AmB + Itr	CT	Died
Clancy and Nguyen [257]	1998	3	Immunocompetent	Sinusitis	AmB + Itr + 5-FC (1); ABCD + Itr + 5-FC (1); ABLC + Itr (1)	CT	Improvement (1); died (2)
Lamy et al. [258]	1998	5	AML	Pulmonary	AmB + Itr	CT	Improvement
Rieske et al. [259]	1998	1	AML	Sinusitis	ABCD + 5-FC	MRI	Improvement
Kummerle and Wedler [260]	1998	1	HIV	Renal	AmB + 5-FC	CT	Improvement
Segal et al. [261]	1998	2	CGD	Pulmonary	AmB + 5-FC; ABLC + Itr	CT	Died (1); improvement (1)
Renard et al. [262]	1998	1	Immunocompetent	Cerebral	Itr + Rif	CT	Improvement
Weishaar et al. [263]	1998	3	IVDA	Endophthalmitis	AmB + 5-FC	Clinical	Improvement
Johnson et al. [264]	1999	1	HIV	Sinoorbital, cerebral	ABLC + Itr	CT, MRI	Died
Bajjoka et al. [134]	1999	2	Liver transplantation	Pulmonary, epidural abscess	L-AmB + Itr	CT	Died
Streppel et al. [129]	1999	1	Immunocompetent	Sinusitis	L-AmB + Itr	CT	Improvement
Boots et al. [265]	1999	1	Immunocompetent	Tracheobronchitis	AmB + Itr + 5-FC	BAL	Improvement

(continued)

Table A1. ***(Continued.)***

Reference	Year	No. of patients	Underlying condition(s)	Type(s) of IA	Treatment	Evaluation method(s)	Outcome(s)
Viertel et al. [266]	1999	1	Kidney transplantation	Uveitis, endocarditis	AmB + 5-FC	Echo	Died, heart failure
van Ooij et al. [267]	2000	1	AML	Spondylodiscitis	AmB + Itr, followed by AmB + 5-FC	Clinical	Improvement
Abu Jawdeh et al. [132]	2000	1	Monocyte-killing defect	Vertebral osteomyelitis	AmB + 5-FC, followed by AmB + Itr	MRI	No improvement until GM-CSF
Jaing et al. [268]	2000	1	AML	Pulmonary, sinusitis	AmB + Itr	CT	Improvement
Mylonakis et al. [269]	2000	1	HIV	Cerebral, sinusitis	Ter + Itr	CT	Died
Binder and Ruchel [270]	2000	1	AML	Pulmonary, cerebral	AmB + Itr	CT, MRI	Died
Roy et al. [271]	2000	1	Non-Hodgkin lymphoma	Skin, pulmonary, cerebral	ABLC + Itr	Not specified	Improvement
Gumbo et al. [272]	2000	3	CLL (1); bronchiolitis obliterans (1); bronchiectasis (1)	Endocarditis + chorioretinitis (1); endocarditis + anterior uveitis (1); endocarditis + cerebral + endophthalmitis (1)	AmB + Itr (1); AmB + 5-FC (1); AmB + Rif (1)	Echo, CT	Died
Grandiere-Perez et al. [273]	2000	1	AML	Pulmonary, spondylodiscitis	AmB + Itr	CT, MRI	Improvement
Cuccia et al. [274]	2000	2	Ependymoma (1); aplastic anemia (1)	Cerebral	L-AmB + 5-FC (1); AmB + 5-FC (1)	CT	Improvement (1); died (1)
Ng et al. [275]	2000	1	ALL	Cerebral	L-AmB + 5-FC	MRI	Improvement
van Landeghem et al. [276]	2000	1	Congenital heart defect	Meningitis	L-AmB + 5-FC	CT, MRI	Died
Silva et al. [277]	2000	1	AML	Pulmonary	ABLC + Rif + aerosolized AmB	CT	Improvement
Nenoff et al. [278]	2001	1	Testicular tumor, IDDM	Orbital, meningitis	AmB + 5-FC	CT	Died
Apostolidis et al. [279]	2001	1	ITP	Pulmonary, cerebral	L-AmB + Itr	CT	Improvement
Endo et al. [280]	2001	1	Pituitary adenoma	Cerebral	AmB + Flu	CT	Died, cerebral infarction
Sevilla et al. [281]	2001	9	BMT	Invasive, not specified	L-AmB + Itr (8); L-AmB + Vor (1)	Not specified	L-AmB + Itr: improvement (3), died (5); L-AmB + Vor: died (1)
Symoens et al. [282]	2001	1	Lung transplantation	Pulmonary	AmB + Itr	CT	Died
Gupta et al. [283]	2001	1	CGD	Vertebral osteomyelitis	AmB + Itr	MRI	Improvement
Trachana et al. [131]	2001	1	Common variable immunodeficiency	Hepatic lesions	L-AmB + Itr	CT	Improvement
Baddley et al. [284]	2001	1	BMT	Pulmonary, cerebral	ABLC + Itr	CT	Died
Govender and Kumar [285]	2001	2	COPD	Pulmonary	AmB + Itr	CT	Died
Bulpa et al. [286]	2001	1	CGD	Cerebral	AmB + Itr	MRI	Improvement
Saulsbury [287]	2001	3	Immunocompetent	Spondylitis	AmB + 5-FC	Radiography	Improvement
Kontoyiannis et al. [133]	2001	1	BMT	Pulmonary	L-AmB + Itr	CT	Improvement
Hwang et al. [288]	2001	1	AML	Hepatosplenic	ABLC + Itr	CT	Worsening

NOTE. ABCD, amphotericin B colloidal dispersion; ABLC, amphotericin B lipid complex; ALL, acute lymphocytic leukemia; AmB, amphotericin B; AML, acute myeloid leukemia; BAL, bronchoalveolar lavage; BMT, bone marrow transplantation; CGD, chronic granulomatous disease; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; COPD, chronic obstructive pulmonary disease; CT, computed tomography; CXR, chest radiography; Echo, echocardiography; Flu, fluconazole; GM-CSF, granulocyte-macrophage colony-stimulating factor; G6PD, glucose-6-phosphatase deficiency; h/o, history of; IDDM, insulin-dependent diabetes mellitus; ITP, idiopathic thrombocytopenic purpura; Itr, itraconazole; iv, intravenous; IVDA, iv drug abuser; Ket, ketoconazole; L-AmB, liposomal amphotericin B; MRI, magnetic resonance imaging; NIDDM, noninsulin-dependent diabetes mellitus; Rif, rifampin; SLE, systemic lupus erythematosus; Ter, terbinafine; Vor, voriconazole; 5-FC, 5-fluorocytosine.

Table A2. Summary of 27 reports of in vitro combination antifungal therapy for *Aspergillus* species.

Reference	Year	Treatment	Evaluation method(s)	Outcome(s)
Fields et al. [67]	1974	AmB + 5-FC	MIC	Add
Kitahara et al. [98]	1976	AmB + Rif; AmB + 5-FC	MIC, RNA synthesis, dry weight	Syn; Syn
Lauer et al. [289]	1978	AmB + 5-FC	Checkerboard	Ind
Odds [38]	1982	AmB + 5-FC; AmB + Mic; Ket + Mic	MIC, bioluminescence spectrophotometry	Add; Syn; Ant
Hughes et al. [99]	1984	AmB + Rif; AmB + 5-FC; AmB + Ket	MIC	Syn; Ind; Ant
Schaffner and Frick [13]	1985	AmB + Ket	MFC	Ant
Perfect et al. [162]	1992	NikkZ + Cilofungin	Broth microdilution checkerboard	Syn
Denning et al. [37]	1992	AmB + Rif; AmB + 5-FC; AmB + Itr	Checkerboard	Syn/Ind; Syn/Add/Ind/Ant; Syn/Add/Ind
Maesaki et al. [16]	1994	AmB + Mic; AmB + Itr; AmB + Flu; AmB + Ket	Checkerboard	Syn; Ind; Ind; Ind
Nakajima et al. [168]	1995	AmB + DU-6859a; Flu + DU-6859a	Modified microdilution checkerboard	Syn; Ind
Nguyen et al. [169]	1997	AmB + Azithromycin	Checkerboard, nucleotide incorporation	Syn
Clancy et al. [96]	1998	AmB + Rifabutin	Macrodilution checkerboard, RNA synthesis, protein synthesis	Syn, Add
Li and Rinaldi [160]	1999	NikkZ + Itr; NikkZ + Flu	Broth macrodilution checkerboard	Syn; Ind
Stevens [148]	1999	L-AmB + Mif	Broth macrodilution checkerboard	Ind
Petraitis et al. [151]	1999	AmB + Mif	Checkerboard, time kill, MTT assay	Ind
Jessup et al. [158]	1999	AmB + L-nystatin	Broth microdilution checkerboard	Add
Stevens [161]	2000	NikkZ + anidulafungin	Broth macrodilution checkerboard	Syn
van't Hof et al. [290]	2000	Histatin 5 + AmB	Checkerboard	Ind
Kontoyiannis et al. [114]	2000	AmB + Itr	Etest	Ant
Te Dorsthorst et al. [167]	2000	AmB + Itr; AmB + Ter; Itr + Ter	Broth microdilution checkerboard, MTT assay	Ant; Ind; Syn
Manavathu et al. [141]	2000	AmB + Caf; Itr + Caf; AmB + Itr; NC1175 + Itr; NC1175 + Caf; AmB + NC1175	Checkerboard, XTT/MTT assays	Syn; Syn; Add; Add; Add; Ind
Arikan et al. [142]	2000	AmB + Caf	Checkerboard	Syn/Add
Kohno et al. [14]	2000	AmB + Mif; Itr + Mif; 5-FC + Mif	Broth microdilution checkerboard	Syn/Add; Syn/Add/Ind; Syn/Add
Chiou et al. [163]	2001	Mif + NikkZ	Broth macrodilution checkerboard, MTT assay	Syn/Ind
Manavathu et al. [144]	2001	AmB + Mif; AmB + Caf; Vor + Mif; Vor + Caf; AmB + Vor	Checkerboard	Syn; Syn; Add; Add; Ind
Mosquera et al. [165]	2001	Itr + Ter; Flu + Ter; AmB + Ter; 5-FC + Ter	Broth microdilution checkerboard	Syn/Add; Syn/Ind; Ind/Ant; Ind/Ant
Ryder and Leitner [166]	2001	AmB + Ter; Itr + Ter; Vor + Ter; Flu + Ter	Checkerboard	Add; Syn; Syn; Add

NOTE. This table includes only devoted in vitro studies and therefore excludes individual case reports in which MICs or combination results are also presented. Add, additivity; AmB, AmB, amphotericin B; Ant, antagonism; Caf, caspofungin; Flu, fluconazole; Ind, indifference; Itr, itraconazole; Ket, ketoconazole; L, liposomal; MFC, minimum fungicidal concentration; MIC, minimum inhibatory concentration; Mic, miconazole; Mif, micafungin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NikkZ, nikkomycin Z; Rif, rifampin; Syn, synergy; Ter, terbafine; Vor, voriconazole; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)2H-tetrazolium-5-carboxanilide sodium salt; 5-FC, 5-fluorocytosine.

Table A3. Summary of 18 reports of in vivo concurrent combination antifungal therapy for invasive aspergillosis (IA).

Reference	Year	Model	Type of IA	Treatment	Evaluation method(s)	Outcome(s)
Carrizosa et al. [68]	1975	Rabbit	Endocarditis	AmB + 5-FC	Survival, vegetations	Ind
Arroyo et al. [100]	1977	Murine	Disseminated	AmB + Rif; AmB + 5-FC	Survival, organ culture	Syn; Syn
Polak et al. [49]	1982	Murine	Disseminated	AmB + 5-FC; AmB + Ket; 5-FC + Ket	Survival	Ind/Add; Ant; Ind
Schaffner and Frick [13]	1985	Murine	Disseminated	AmB + Ket	Survival, organ culture	Ant
Polak [71]	1987	Murine	Disseminated	AmB + Itr; AmB + 5-FC; Itr + 5-FC	Survival	Ind/Ant; Ind; Ind/Add/Syn
Longman and Martin [291]	1987	Rabbit	Endocarditis	AmB + 5-FC	Survival, vegetations	Add
Van Cutsem [292]	1990	Guinea pig	Disseminated	AmB + Itr; AmB + Ket	Survival, organ culture	Ind; Ind
Schmitt et al. [69]	1991	Rat	Disseminated	AmB + Rif; AmB + 5-FC; AmB + Ket	Survival	Ind; Ind; Ant
Denning and Stevens [139]	1991	Murine	Disseminated	AmB + cilofungin	Survival, organ cultures	Ant
George et al. [70]	1993	Rabbit	Disseminated	AmB + Flu; AmB + 5-FC; 5-FC + Flu	Survival, target organs	Ind; Ind; Ind
Nakajima et al. [168]	1995	Murine	Disseminated	AmB + DU-6859a	Survival, organ burden	Syn
Petraitis et al. [151]	1999	Rabbit	Pulmonary	AmB + Mif; L-AmB + Mif	Survival, lung burden	Ind; Ind
Kohno et al. [14]	2000	Murine	Pulmonary	AmB + Mif	Survival, lung burden	Add
Nakajima et al. [149]	2000	Murine	Pulmonary	AmB + Mif	Survival, lung burden	Syn
Chiller et al. [127]	2001	Murine	CNS	AmB + Itr	Survival, brain burden	Ind
Becker et al. [293]	2000	Rat	Pulmonary	L-AmB + AmB	Survival, galactomannan	Add
Douglas et al. [145]	2001	Murine	Disseminated	AmB + Caf	Survival, kidney burden	Add
Capilla Luque et al. [150]	2001	Murine	Disseminated	AmB + Mif; AmB + Itr; NikkZ + Mif	Survival, brain and kidney burden	Ind; Ind; Add

NOTE. Add, additivity; AmB, AmB, amphotericin B; Ant, antagonism; Caf, caspofungin; CNS, central nervous system; Flu, fluconazole; Ind, indifference; Itr, itraconazole; Ket, ketoconazole; L-AmB, liposomal AmB; Mif, micafungin; NikkZ, nikkomycin Z; Rif, rifampin; Syn, synergy; 5-FC, 5-fluorocytosine.

Table A4. Summary of 34 reports of sequential antifungal therapy for invasive aspergillosis (IA), 1990–2001.

Reference	Year	No. of patients	Underlying condition(s)	Type(s) of IA	Treatment	Evaluation method(s)	Outcome(s)
Warshawsky et al. [77]	1975	1	NIDDM	Renal	5-FC, followed by AmB + 5-FC + Rif	Urine culture	Improvement
Verweij et al. [294]	1999	1	Competent	Meningitis	Itr, followed by AmB, followed by Vor	CT	Improvement
Weishaar et al. [263]	1998	1	IVDA	Endophthalmitis	Ket, followed by Flu	Clinical	Improvement
Marks et al. [295]	1996	1	Renal transplantation	Pulmonary	Itr, followed by ABCD	CT	Improvement
Nussaume et al. [226]	1990	1	Aortic graft	Aortic graft	Rif, followed by 5-FC	Clinical	Improvement
Tumbarello et al. [296]	1993	1	HIV	Pulmonary	Itr, followed by AmB	CXR	Died
van't Wout et al. [185]	1990	1	CGD	Pulmonary	AmB + 5-FC, followed by Itr	CXR	No improvement until Itr
Girmenia et al. [297]	1995	1	HIV, ALL	Pulmonary	Itr, followed by AmB	CT	Died
Khoo et al. [298]	1995	1	Competent	Sinusitis	Itr, followed by AmB, followed by saperconazole	CT	Improvement
Decker and Parenti [299]	1991	1	HIV	Pulmonary	Itr, followed by AmB	CXR	Died
Libanore et al. [300]	1994	1	HIV	Sinusitis	Itr, followed by AmB	CT	Died
Matsuzono et al. [238]	1995	1	CGD	Cerebral, pulmonary	Flu, followed by AmB + 5-FC + Flu	CXR, MRI	Improvement
Morioka et al. [301]	1990	1	Chronic subdural hematoma	Cerebral	Mic, followed by 5-FC	CT	Improvement
van't Hek et al. [186]	1998	1	CGD	Pulmonary	AmB, followed by Vor	CT	Improvement
Nanas et al. [181]	1998	4	Heart transplantation	Pulmonary	Itr, followed by AmB	CT, CXR	Improvement (3); died (1)
Galimberti et al. [182]	1998	1	Nephrotic syndrome	Pulmonary	Itr, followed by AmB	CXR	Improvement
Karim et al. [253]	1997	1	Competent	Cerebral	Flu, followed by Itr	CT	No improvement
Schwartz et al. [302]	1997	1	ALL	Cerebral	AmB, followed by Itr, followed by Vor	MRI	Improvement
Verweij et al. [303]	2000	1	CGD	Pulmonary	AmB, followed by Vor	CT	Improvement
Lortholary et al. [233]	1993	1	HIV	Pulmonary	Itr, followed by AmB	Clinical, BAL	Died
Kessler et al. [304]	1997	1	Lung transplantation	Pulmonary	Itr, followed by Vor	BAL, biopsy	Improvement
Wilson et al. [305]	2000	1	Malaria	Pulmonary, cerebral	L-AmB, followed by Vor	CT, BAL	Died
Garcia et al. [306]	1998	1	Renal transplantation	Cerebral	Flu, followed by L-AmB	CT	Improvement
Machetti et al. [307]	2000	1	BMT	Cerebral	AmB, followed by Vor	CT, MRI	Improvement
Hwang et al. [288]	2001	1	AML	Hepatosplenic	ABLC + Itr, followed by Vor	CT	Improvement
Krupova et al. [308]	2001	5	ALL (1); AML (2); BMT (1); choriocarcinoma (1)	Pulmonary	AmB, followed by L-nystatin	Clinical	Improvement (4); died (1)
Swift and Denning [309]	1998	1	Competent	Skull osteitis	Itr, followed by Vor	CT	Improvement

NOTE. ABCD, amphotericin B colloidal dispersion; ABLC, amphotericin B lipid complex; ALL, acute lymphocytic leukemia; AmB, amphotericin B; AML, acute myeloid leukemia; BAL, bronchoalveolar lavage; BMT, bone marrow transplantation; CGD, chronic granulomatous disease; Competent, immunocompetent host; CT, computed tomography; CXR, chest radiography; Flu, fluconazole; Itr, itraconazole; IVDA, intravenous drug user; Ket, ketoconazole; L, liposomal; Mic, miconazole; MRI, magnetic resonance imaging; NIDDM, non–insulin-dependent diabetes mellitus; Rif, rifampin; Vor, voriconazole; 5-FC, 5-fluorocytosine.

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