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Combination Therapy of Experimental Candidiasis, Cryptococcosis and Aspergillosis in Mice

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Abstract. Combination pairs of the major systematic antimycotic drugs, amphotericin B (AmphB), 5-fluorocytosine (5-FC) and ketoconazole (Ktz) were administered to mice with experimental candidiasis, cryptococcosis and aspergillosis at a variety of combination ratios. The 3 mycoses were produced with 3 strains each of *Candida albicans, Cryptococcus neoformans,* and *Aspergillus fumigatus,* respectively, which were preselected to represent 3 different degrees of 5-FC sensitivity ('normally sensitive', 'moderately resistant', and 'definitely resistant'). The life-prolonging effect of the combinations was compared with the effect of each partner administered alone at the same and at the double dosage. Using the U test of Mann and Whitney and setting limits which on the whole were more rigorous than those of the isobole methods commonly applied to the study of drug interactions, the effects of the concentrations were classified as 'synergistic', 'additive', 'indifferent' or 'antagonistic'.

The combination *AmphB plus 5-FC* was definitely synergistic or definitely additive in all 3 candidiasis models, the most pronounced synergism occurring in the infection with the 'definitely 5-FC-resistant' *C. albicans* strain; in cryptococcosis produced by any of the 3 *C. neoformans* strains the effect was definitely additive, but only slightly additive or indifferent in the 3 aspergillosis models. The combination *AmphB plus Ktz* was slightly synergistic in candidiasis produced by one *C. albicans* strain, but definitely antagonistic in this mycosis produced by the remaining 2 strains of the same species; the combination was definitely additive or, even, slightly synergistic in the 3 cryptococcus models, but, again, antagonistic in aspergillosis produced by all 3 strains of *A. fumigatus. 5-FC plus Ktz* was additive or indifferent in the 3 candidiasis models, but throughout indifferent in cryptococcosis and aspergillosis.

Introduction

In spite of considerable progress made during the last decades, systemic antimycotic chemotherapy still presents a number of problems. This is explained on one hand, by the extreme gravity of many of the mycoses in question and on the other, by the paucity of the available drugs of which each one has its limitation (e.g. side effects, risk of resistance, or poor penetration into certain compartments of the body). Not surprisingly, there has been an increasing interest in drug combinations, with the hope that they may offer advantages such as smaller dosage of the partners, reduced incidence of resistance, and complementary pharmacokinetics of the 2 drugs, particularly with respect to penetration. Several clinical studies exist indicating that the one or the other of these expectations was fulfilled by combining amphotericin B and 5-fluorocytosine, especially in cryptococcal meningitis [4, 21, 38], Candida endocarditis [39] and invasive aspergillosis [23, 35]. Clinical experience with combinations of the most recent drug, ketoconazole, with any of the older antimycotics is still scarce. However, the results from experiments using combinations of various imidazolyl compounds (miconazole, econazole or ketoconazole) with amphotericin B were conflicting in showing synergism in some models [18, 28] but antagonism in most of the others [10–12, 33].

In this paper a comparative study is presented on the chemotherapeutic usefulness of the combinations amphotericin B plus 5-fluorocytosine, amphotericin B plus ketokonazole, and 5-fluorocytosine plus ketoconazole in murine models of candidiasis, cryptococcosis and aspergillosis.

Materials and Methods

Organisms

Candida albicans: strains H12, 13 and 140/1, *Cryptococcus neoformans:* strains 1549, 1820 and 1841, *Aspergillus fumigatus* strains 437, 636 and 638. These strains were preselected according to their sensitivity to 5-fluorocytosine in vitro: one strain of each species should represent good (normal) sensitivity, the second moderate ('partial') resistance and the third definite resistance to this drug. All these fungi are being kept in our laboratory either deep-frozen or lyophilized in order to preserve the virulence. For the inocula, fresh subcultures were always taken from the stock.

Compounds

Amphotericin B (AmphB, Squibb, UK), 5-fluorocytosine (5-FC, Roche), ketoconazole (Ktz, Janssen, Beerse, Belgium).

Fungistatic Activity of the Single Compounds in vitro

For 5-FC a chemically defined, liquid culture medium, yeast nitrogen base (Difco) was used to which 0.5% of dextrose and 2% of agar were added. Casitone agar (containing casitone 9 g, dextrose 20 g, yeast extract 10 g, KH_2PO_4 1 g, Na_2HPO_4 1 g, sodium citrate 10 g, agar 20 g/l) was used for testing Ktz and AmphB. Yeast cells (*C. albicans* and *C. neoformans*) or conidia (*A. fumigatus*) were suspended in the melted media to give a density of 1,000 cells/ml. Using 1:3 dilution steps of each drug the minimum inhibitory concentration (MIC) and the concentration producing definite relative (incomplete) inhibition were determined after 3 days of incubation at 37 °C (*C. albicans* and *A. fumigatus*) or 30 °C (*C. neoformans*).

Septic Candidiasis, Aspergillosis and Cryptococcosis in the Mouse

The mice used for these experiments were male albinos Füllinsdorf, weighing approximately 20 g. All fungi (yeasts or spores) were injected into a tail vein. The inocula per mouse were as follows. *C. albicans* strain H12: 3×10^5, strain 140/1: 4×10^5, and 13: 8×10^5 yeast cells from a 24-hour culture on Sabouraud's dextrose agar at 37 °C; *C. neoformans* strain 1549: 8×10^4,

Table I. Fungistatic activity in vitro of the single drugs, AmphB, 5-FC and Ktz on the strains of *C. albicans*, *C. neoformans* and *A. fumigatus* used to produce murine candidiasis, cryptococcosis and aspergillosis

Drug	*C. albicans* strains[1]			*C. neoformans* strains[1]			*A. fumigatus* strains[1]		
	H12	140/1	13	1841	1820	1549	437	636	638
AmphB	0.3[2]	0.3	0.6	0.15	0.15	0.3	0.3	0.6	0.6
5-FC	0.03	12.5 (1)[3]	>100	3.1	12 (0.3)	>100	3.1	100 (25)	>100 (1)
Ktz	12.5	6.3 (0.4)	12.5 (0.4)	0.5 (0.12)	0.12	0.5 (0.25)	12.5 (1.6)	25 (3.1)	12.5 (6.3)

[1] The 3 strains of each species were preselected according to their sensitivity to 5-FC: the first (on the left) should represent good (normal) sensitivity to this drug, the second (in the middle) moderate sensitivity = 'partial resistance' and the third (on the right) definite resistance.

[2] MIC in μg/ml read after 3 days of incubation at 37 °C (*C. albicans* and *A. fumigatus*) or 30 °C (*C. neoformans*).

[3] The figures in parentheses indicate the concentrations in μg/ml producing definite though only partial (relative) inhibition of growth.

strain 1820: 3×10^6, and strain 1841: 1×10^6 yeast cells from a 2- to 3-day culture on Sabouraud's dextrose agar at 30 °C; *A. fumigatus* strains 437 and 638: 4×10^6, and strain 636: 8×10^6 conidia from a 4- to 5-day culture on Sabouraud's dextrose agar at 37 °C. With these inocula septic mycoses are regularly produced in untreated control animals killing 80–90% within the observation period of 21 days. The median survival time of the controls is in the order of 6–8 days in candidiasis, 4–5 days in cryptococcosis and 6 days in aspergillosis.

Dosage Schedules

The single and combined drugs were administered 3 times in candidiasis, 5 times in cryptococcosis, and 6 times in aspergillosis. The first of these individual doses was given immediately after the infection, the second and third approximately 6 and 24 h, respectively, thereafter, and the fourth and sixth once daily on the subsequent days. Of each drug and drug combination the dosage was varied using 1:2 steps. In each experiment the number of dosage steps usually was 3–4. 10 animals were used per dosage group, and 10 animals served as untreated controls. The route of administration was per os for Ktz, and subcutaneous (s.c.) for 5-FC and AmphB.

Chemotherapeutic Activity of the Single Compounds

The effective dose 50% (ED50), i.e., the dose keeping alive 50% of the animals until the end of the observation period, was estimated graphically on probability paper. When there were survivors in the control group, the percentages of survivors in the treated groups were corrected by the formula:

$$\text{Corrected percentage} = \frac{\text{obtained percentage} - \%\text{ surviving controls}}{100 - \%\text{ surviving controls}}$$

As an additional parameter of effectiveness, the dose producing a 50% prolongation of the median survival time of the treated animals compared with the median survival time of the controls, was calculated. (In the experimental mycoses in question, a 50% prolongation of the median survival time is usually consistent with a $p < 0.05$ prolongation of the survival times of

Table II. Chemotherapeutic activity of the single drugs, AmphB, 5-FC and Ktz on murine mycoses (candidiasis, cryptococcosis and aspergillosis)

Drug	Parameter of activity[1]	Candidiasis *C. albicans* strains[2]			Cryptococcosis *C. neoformans* strains[2]			Aspergillosis *A. fumigatus* strains[2]		
		H12	140/1	13	1841	1820	1549	437	636	638
AmphB (s.c.)	ED50	0.13[3]	0.13	0.5	1.5	0.4	0.6	0.06	0.25	0.3
	dose prolonging survival by 50%	<0.060[4]	0.044	<0.060	0.051	0.12	0.11	0.038	0.060	0.063
5-FC (s.c.)	ED50	25	>200[5]	>200	>400	>400	>400	>200	>200	>200
	dose prolonging survival by 50%	8.9	>12.5	38	<25	<25	48	41	195	35
Ktz (per os)	ED50	>200	>200	200	>200	115	>200	150	>200	>100
	dose prolonging survival by 50%	58	36	25	<25	<25	<25	93	>200	40

[1] ED50: dose keeping alive 50% of the animals until the end of the observation period, and dose prolonging the median survival time of the treated animals by 50%, compared with the median survival time of the untreated controls (cf. Materials and Methods).

[2] The 3 strains of each species were preselected according to their sensitivity to 5-FC in vitro: the first (on the left) should represent good (normal) sensitivity to this drug, the second (in the middle) moderate sensitivity = 'partial resistance' and the third (on the right) definite resistance.

[3] All figures indicate doses in mg/kg.

[4] Doses with the prefix < indicate the lowest dosage used which, however, produced a still stronger effect than is required by the parameter in question.

[5] Doses with the prefix > indicate the highest dosage used which was, however, still too low to produce the effect required by the parameter in question.

the individual mice as determined in a rank test.) As dose-effect curves, cubic splines of the percentages were used according to program ICSSCU of the IMSL Library (Houston, Tex.). For the splines the following conditions were stipulated: (i) no change of the sign in the first derivation, and (ii) not more than one change of the sign in the second derivation of the spline function. Within these conditions the splines with the lowest sum of squared deviations were chosen.

Combined Chemotherapy

The combination experiments were performed with any pairs among the 3 drugs (i.e., with AmphB + 5-FC, AmphB + Ktz, and 5-FC + Ktz), whereby the 2 partners were administered immediately after each other. A variety of combination ratios was used for each pair in the aim to cover the range between subliminal and pronounced activity of the single partners. Accordingly, the dose of the AmphB component was always much smaller than the dose of the 5-FC or Ktz components, whereas the doses of the 5-FC and Ktz components were in a similar range. For example, in the experiments with *C. albicans* strain H12, each of the 3 AmphB dosages: 0.063, 0.125 and 0.25 mg/kg, was combined with each of the 3 5-FC dosages: 6.3, 12.5 and 25 mg/kg, and with each of the 4 Ktz dosages: 6.3, 12.5 25 and 50 mg/kg; and each of the 3 5-FC and 4 Ktz dosages just indicated was combined with each other. The dosages of 5-FC were higher in the

experiments with the *C. albicans* strains 140/1 and 13 and with *C. neoformans* and *A. fumigatus* strains, according to the minor susceptibility of these strains to 5-FC alone. For analogous reasons, the dosages of the Ktz component were higher in the experiments with the *C. neoformans* and, particularly, the *A. fumigatus* strains (for the detailed dosage groups see tables III–V). In each experiment the drugs were tested singly at the same dosages as those used in the combinations. In addition, the single drugs were tested at twice the highest dosage used in combination to permit comparison with the activity of the double dosage of each of the single partners. Groups of 10 mice were used for each of the combination ratios and each of the single partners.

The effect of combined chemotherapy was classified in one of the 4 categories: 'antagonism', 'indifference', 'addition' or 'synergism'. This classification was made as follows:

(1) Using the U test of Mann and Whitney, the survival times of the individual mice obtained with a given combination D_{a+b} of the 2 drugs, a plus b, were compared with the survival times obtained in the same experiment with the respective doses, D_a and D_b, of each of the single partners. With the sample sizes in question (2×10 each) the U values can vary from 0 to 100; $U = 50$ means equal distribution of the survival times; with $U < 50$ the survival times D_{a+b} are shorter, and with $U > 50$, longer than those of either D_a or D_b. Only the higher of the 2 U values, i.e. the one resulting from the comparison with the more active of the 2 single partners, was used for the following decisions: $U < 28$: antagonism; $28 \leqslant U \leqslant 72$: indifference.

(2) In the cases of $U > 72$, the survival times obtained with D_{a+b} were compared with those obtained with the double dosages of the single partner, $2D_a$ and $2D_b$. Applying the test of Mann and Whitney, $U = 50$ means equal, $U < 50$ shorter, and $U > 50$ longer survival times of D_{a+b} compared with either $2D_a$ or $2D_b$. Again, only the higher of the 2 U values was used for the decision: $U < 28$: indifference; $28 \leqslant U \leqslant 72$: addition; $U > 72$: synergism.

With the sample sizes in question (2×10), $U < 28$ and $U > 72$ reflect a significance level of $p = 0.10$ (one-tailed). This relatively high p was considered justified, since – due to the relatively small sample sizes – the risk of errors of the second kind (failure to detect actual differences) would have been too great with a lower p. Furthermore, the higher p seemed justified by the fact that the decisions for addition and synergism were based on comparisons of the combinations with the more active of the single partners.

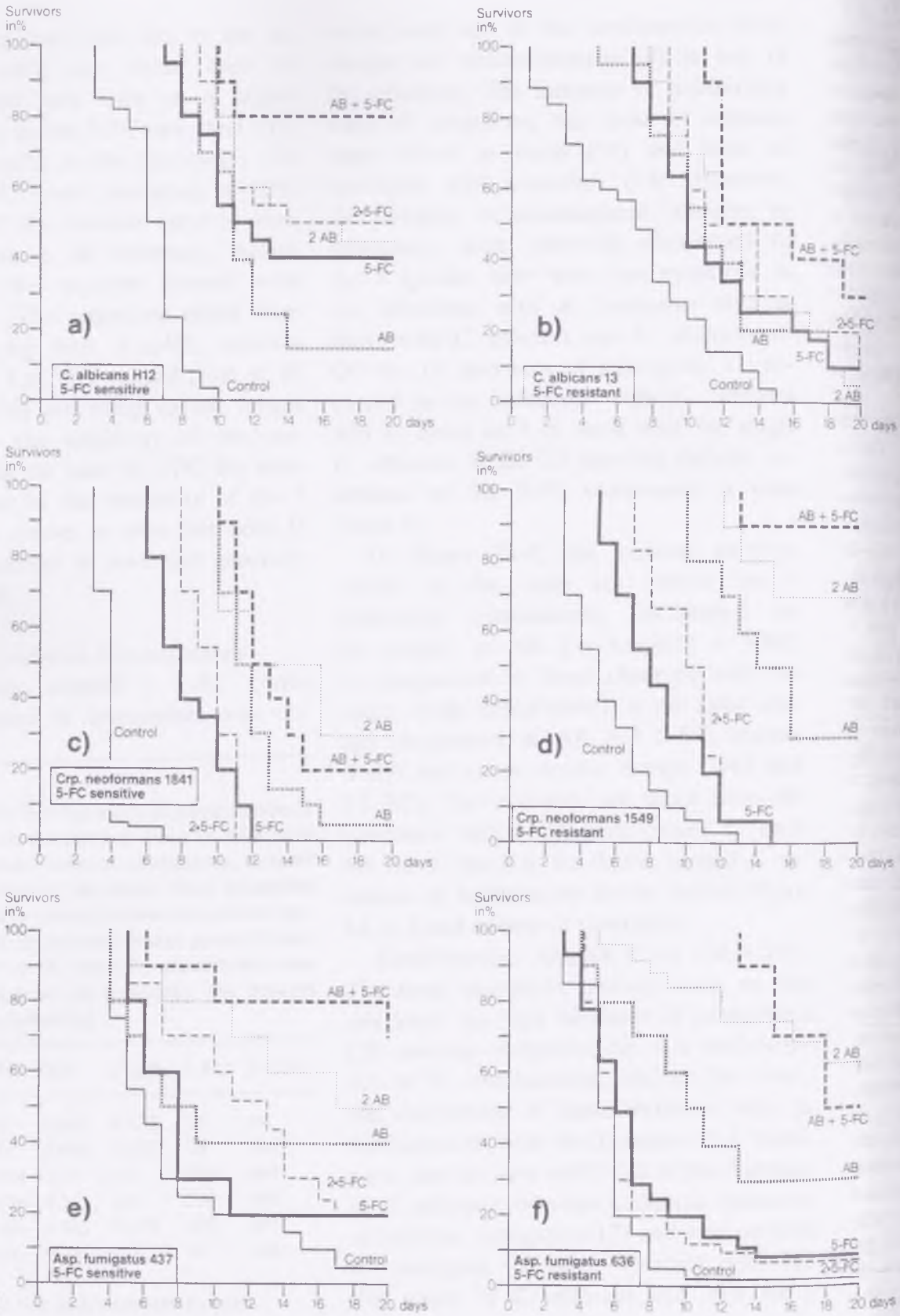
Results

Activity of the Single Compounds in vitro (table I)

The desideratum that of the 3 strains of each species one each should represent (i) high (normal) sensitivity, (ii) moderate sensitivity = 'partial resistance' and (iii) definite resistance to 5-FC, was met quite well in *C. albicans* and *C. neoformans*. In *A. fumigatus* one strain (437) was normally sensitive but the remaining 2 (636 and 638) were almost indistinguishable with the criteria used (MIC and definite relative inhibition) and both appeared to lie between 'partial' and definite 5-FC resistance. As expected, sensitivity to AmphB and Ktz was unrelated to the sensitivity to 5-FC. AmphB proved to be most potent in showing about equally low MICs against all strains of any of the 3 species. Ktz was highly active on the strains of *C. neoformans* but moderately active on those of *C. albicans* and *A. fumigatus*.

Chemotherapeutic Activity of the Single Compounds (table II)

AmphB was the most potent drug by far in that ED50ies – which usually were below 1 mg/kg – were obtained in the infections with any strains of the 3 species. With 5-FC and Ktz ED50ies were obtained only exceptionally (with 5-FC in the infection with the normally sensitive strain of


Survivors in%
a)
C. albicans H12
5-FC sensitive
AB + 5-FC
2-5-FC
2 AB
5-FC
AB
Control
b)
C. albicans 13
5-FC resistant
c)
Crp. neoformans 1841
5-FC sensitive
d)
Crp. neoformans 1549
5-FC resistant
e)
Asp. fumigatus 437
5-FC sensitive
f)
Asp. fumigatus 636
5-FC resistant
0 2 4 6 8 10 12 14 16 18 20 days

1

C. albicans only, and with Ktz in the infections with only one strain each of the three species and were of a higher order (25 mg/kg in the 5-FC case, and 115, 150 and 200 mg/kg in the Ktz cases). On the other hand, doses producing a 50% prolongation of the median survival time were measurable in all instances, including those of the mycoses treated with 5-FC and Ktz. The respective doses were below 0.1 mg/kg with AmphB, whereas with 5-FC and Ktz, they ranged from < 20 up to 200 mg/kg according to the fungus species and to the sensitivity of the particular strain. In the case of 5-FC the arbitrary differences in the sensitivity of the 3 strains of each species in vitro (see table I) were still noticeable in vivo but generally less pronounced.

Effects of Combined Chemotherapy

Combinations AmphB + 5-FC (table III). No instance of antagonism was observed with any of the combinations (total number of combinations = 89) in any of the infections. The instances of indifference were 41 altogether, but those of addition were almost as many (35) and those of synergism still numerous (13). However, the instances of enhancement (addition or synergism) were unevenly distributed to the 3 species: they were less numerous in the infections with *A. fumigatus* than in those with *C. albicans* and *C. neoformans*. Of the 13 instances of synergism, 11 occurred in the infections with *C. albicans* and as many as 7 in those with the single *C. albicans* strain 13 showing definite resistance to the 5-FC component in vitro (table I).

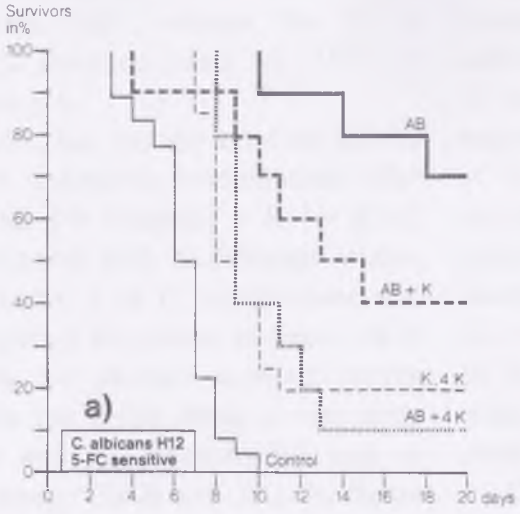
In figure 1a–f, the percent survivor curves of the mice are shown for 6 individual combinations (designated on the graphs as AB [= AmphB] + 5-FC) in comparison to those observed with the single drugs administered at the same dosage (designated as AB and 5-FC, respectively) and at the double dosage (2AB and 2·5-FC). The examples are taken from the infections with 6 different strains (2 each for the 3 species). In figure 1c and f, instances of addition are shown, and in figure 1a, b, d and e those of synergism.

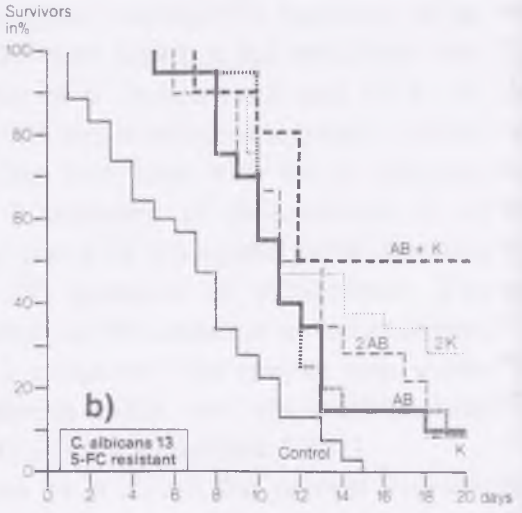
Combinations AmphB ± Ktz (table IV). The most impressive findings were, on the one hand, the high incidence of antagonism (28 instances altogether out of a total number of 72 combinations), and on the other, the distribution of these instances only to the infections with the 3 strains of *A. fumigatus* and to those with 2 out of the 3 strains of *C. albicans*, whereas numerous instances of addition (altogether 17) and even several of synergism (6) were observed with the 3rd strain of *C. albicans* and with all 3

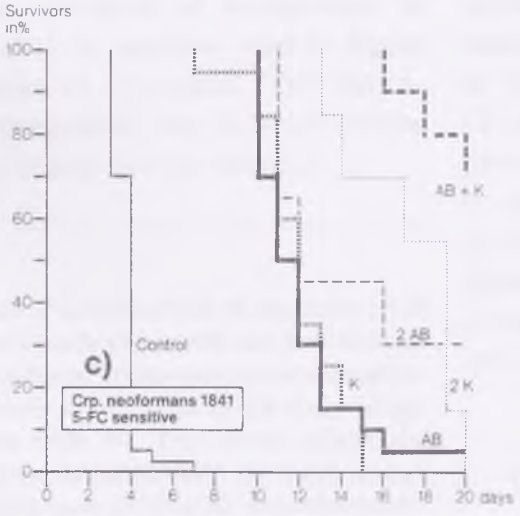
Fig. 1. Effect of combinations of amphotericin B (AB) and 5-FC on the survival times of mice with candidiasis, cryptococcosis or aspergillosis, in comparison to the effect of the single drugs (examples from table III). The curves indicate the percent survivors recorded daily for each dosage group (10 animals each, or 20 or 30, when the experiments were performed in duplicate or triplicate). The dosages (mg/kg) were the following:

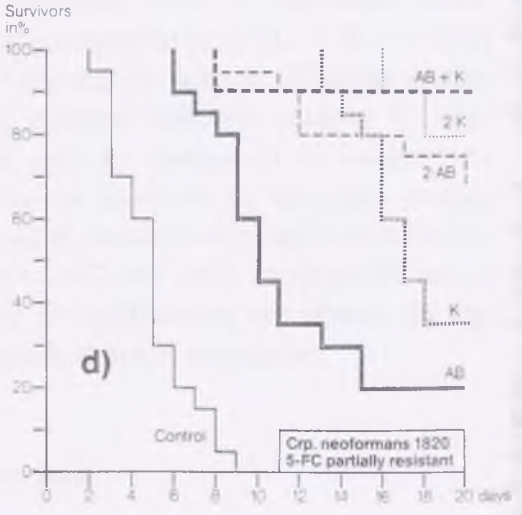
Figure	AB + 5-FC	AB	2·AB	5-FC	2·5-FC
1a	0.063 + 25	0.063	0.125	25	50
1b	0.063 + 50	0.063	0.125	50	100
1c	0.25 + 200	0.25	0.5	200	400
1d	0.5 + 200	0.5	1.0	200	400
1e	0.063 + 100	0.063	0.125	100	200
1f	0.25 + 50	0.25	0.5	50	100

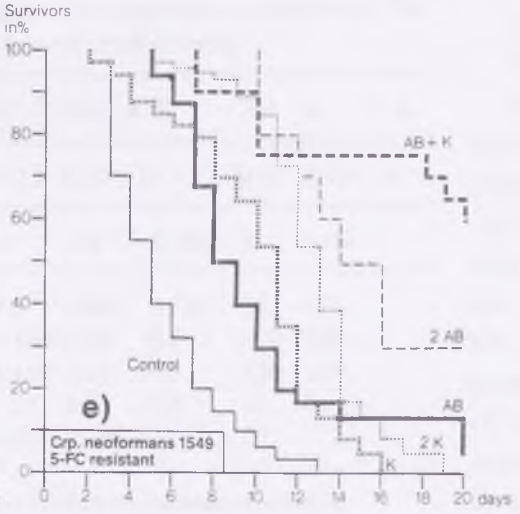
Control: group of infected, untreated animals.

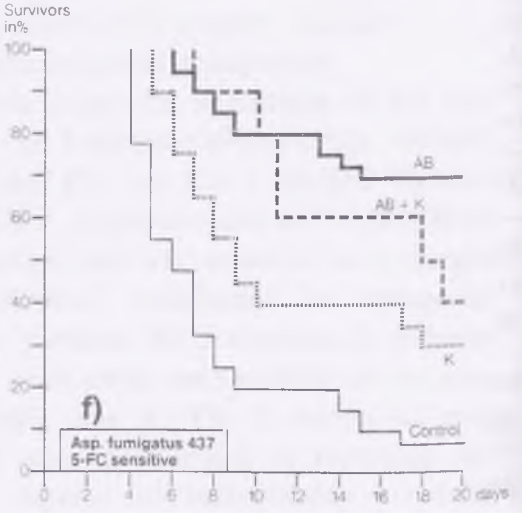

Survivors in%
AB
AB + K
K, 4 K
AB + 4 K
Control
a)
C. albicans H12
5-FC sensitive
days


Survivors in%
AB + K
2 AB
2 K
AB
K
Control
b)
C. albicans 13
5-FC resistant
days


Survivors in%
AB + K
Control
2 AB
2 K
K
AB
c)
Crp. neoformans 1841
5-FC sensitive
days


Survivors in%
AB + K
2 K
2 AB
K
AB
Control
d)
Crp. neoformans 1820
5-FC partially resistant
days


Survivors in%
AB + K
2 AB
Control
AB
2 K
K
e)
Crp. neoformans 1549
5-FC resistant
days


Survivors in%
AB
AB + K
K
Control
f)
Asp. fumigatus 437
5-FC sensitive
days

strains of *C. neoformans*. 5 out of the 6 instances of synergism were observed in the experiments with 2 strains: No. 13 of *C. albicans* (2 instances) and No. 1549 of *C. neoformans* (3).

As examples, the percent survivor curves found with 6 individual combinations (designated as AB [= AmphB] + K [= Ktz]) in the experiments with 6 different strains (2 of *C. albicans*, 3 of *C. neoformans* and 1 of *A. fumigatus*) are shown in figure 2a–f, together with the percent survivor curves observed with the single drugs at the same dosage (AB and K, respectively) and at the double dosage (2AB and 2K). In figure 2a and f are instances of antagonism, in figure 2b und d of addition, and in figure 2a and d those of synergism. (For the instances of antagonism, the survivor curves at the double dosage are not shown.)

Fig. 2. Effect of combinations of amphotericin B (AB) and ketoconazole (K) on the survival times of mice with candidiasis, cryptococcosis or aspergillosis, in comparison to the effect of the single drugs (examples from table IV). The curves indicate the percent survivors recorded daily for each dosage group (10 animals each, or 20 or 30, when the experiments were performed in duplicate or triplicate). The dosages (mg/kg) were the following:

Figure	AB + K	AB + 4·K	AB	K	4·K
2a	0.25 + 6.25	0.25 + 25	0.25	6.25	25

	AB + K	AB	2·AB	K	2·K
2b	0.063 + 25	0.063	0.125	25	50
2c	0.25 + 100	0.25	0.5	100	200
2d	0.25 + 100	0.25	0.5	100	200
2e	0.25 + 25	0.25	0.5	25	50
2f	0.125 + 50	0.125		50	

Control: group of infected, untreated animals.

Combinations 5-FC + Ktz (table V). No antagonism occurred with any of the combinations (total number 97). Instances of indifference were noted in the infections with all strains of *C. neoformans* and of *A. fumigatus* (61 combinations altogether), whereas in the infections with the *C. albicans* strains, 9 instances of enhancement (3 of addition and 6 of synergism) were observed besides 27 instances of indifference. The distribution of the instances of enhancement to the 3 strains of this species was somewhat uneven (only two observations with strain H12, but 4 with strain 140/1).

Figure 3a–d shows the percent survivor curves resulting from 4 individual combinations (designated as 5-FC + K [= Ktz]) in the experiments with 4 different strains (2 of *C. albicans* and one each of *C. neoformans* and *A. fumigatus*) in comparison to the curves observed at the same dosage (5-FC and K, respectively) and at the double dosage (2·5-FC and 2·K). In figure 3c and d instances of indifference are shown, in figure 3a and b those of synergism.

Discussion

Distinction of Synergism, Addition, Indifference and Antagonism

In this paper the interaction of any pair among the 3 systemic antimycotics, AmphB, 5-FC and Ktz, on the 3 murine mycoses candidiasis, cryptococcosis and aspergillosis was studied and was classified as 'synergistic', 'additive', 'indifferent' or 'antagonistic'. To produce the 3 mycoses, 3 different strains each were used so that the number of models was 9. The 3 strains of each species were preselected to represent different degrees of susceptibility to 5-FC

Table III. Chemotherapeutic activity of combinations of AmphB and 5-FC on murine mycoses (candidiasis, cryptococcosis and aspergillosis) in comparison to the activity of the single drugs[1])

Doses, mg/kg		Candidiasis *C. albicans* strains[2]			Cryptococcosis *C. neoformans* strains[2]			Aspergillosis *A. fumigatus* strains[2]		
AmphB + (s.c.)	5-FC (s.c.)	H12	140/1	13	1841	1820	1549	437	636	638
0.031	25	–	–	–	–	indiff	add	add	–	–
	100	–	–	–	–	indiff	indiff	indiff	–	–
0.063	6.3	add	–	–	–	–	–	–	–	–
	12.5	syn	indiff	syn	–	–	–	–	–	–
	25	syn	add	syn	–	add	add	indiff	indiff	indiff
	50	–	add	syn	add	indiff	add	add	indiff	add
	100	–	–	–	add	indiff	indiff	syn	indiff	indiff
0.125	6.3	add	–	–	–	–	–	–	–	–
	12.5	indiff	add	syn	–	–	–	–	–	–
	25	indiff	add	syn	–	add	add	indiff	indiff	indiff
	50	–	syn	add	indiff	indiff	add	indiff	indiff	indiff
	100	–	syn	add	add	indiff	indiff	indiff	indiff	indiff
	200	–	–	–	add	–	–	–	–	–
0.25	6.3	add	–	–	–	–	–	–	–	–
	12.5	indiff	indiff	syn	–	–	–	–	–	–
	25	indiff	add	syn	–	–	–	–	add	indiff
	50	–	indiff	add	indiff	add	–	–	add	indiff
	100	–	–	–	indiff	indiff	add	–	indiff	indiff
	200	–	–	–	add	indiff	add	–	–	–
0.5	50	–	–	–	–	add	–	–	–	–
	100	–	–	–	–	add	add	–	–	–
	200	–	–	–	–	add	syn	–	–	–

[1] Each effect was evaluated by comparing the survival times of 10 animals receiving the particular combination with 10 animals each receiving the single drugs at the same and at the double dosage (see Materials and Methods).

[2] The 3 strains of each species were preselected according to their sensitivity to 5-FC: the first (on the left) should represent good (normal) sensitivity to this drug, the second (in the middle) moderate sensitivity = 'partial resistance' and the third (on the right) definite resistance (cf. table I, II).

[3] indiff = indifference; add = additive effect; syn = synergism.

('sensitive', 'partially resistant' and 'definitely resistant'), since the possible existence and the prevention of resistance to 5-FC have been major arguments for the use of this drug in combination with another.

For an interaction to appear, and to reach its optimum, a certain proportion of the doses of 2 given antimicrobial drugs is usually required. Not infrequently the optimum, additive or synergistic proportion is

Table IV. Chemotherapeutic activity of combinations of AmphB and Ktz on murine mycoses (candidiasis, cryptococcosis and aspergillosis) in comparison to the activity of the single drugs[1])

Doses, mg/kg		Candidiasis *C. albicans* strains[2]			Cryptococcosis *C. neoformans* strains[2]			Aspergillosis *A. fumigatus* strains[2]		
AmphB + Ktz (s.c.)	(p.o.)	H12	140/1	13	1841	1820	1549	437	636	638
0.063	6.3	ant	–	–	–	–	–	–	–	–
	12.5	ant	indiff	indiff	–	–	–	–	–	–
	25	ant	indiff	add	indiff	indiff	indiff	–	–	–
	50	–	indiff	syn	indiff	indiff	indiff	ant	–	–
	100	–	–	–	indiff	indiff	indiff	ant	–	–
0.125	6.3	ant	–	–	–	–	–	–	–	–
	12.5	ant	ant	indiff	–	–	–	–	–	–
	25	ant	ant	indiff	add	add	add	–	–	–
	50	–	indiff	syn	add	add	add	ant	ant	ant
	100	–	–	–	add	add	add	ant	ant	ant
0.25	6.3	ant	–	–	–	–	–	–	–	–
	12.5	ant	ant	indiff	–	–	–	–	–	–
	25	ant	ant	indiff	add	add	syn	–	–	–
	50	–	ant	–	add	add	syn	–	ant	ant
	100	–	–	–	syn	add	syn	–	ant	ant
0.5	25	–	–	–	–	add	–	–	–	–
	50	–	–	–	–	indiff	–	–	ant	indiff
	100	–	–	–	–	add	–	–	ant	indiff

[1] Each effect was evaluated by comparing the survival times of 10 animals receiving the particular combination with 10 animals each receiving the single drugs at the same and at the double dosage (see Materials and Methods).

[2] The 3 strains of each species were preselected according to their sensitivity to 5-FC: the first (on the left) should represent good (normal) sensitivity to this drug, the second (in the middle) moderate sensitivity = 'partial resistance' and the third (on the right) definite resistance (cf. table I, II).

[3] indiff = indifference; add = additive effect; syn = synergism; ant = antagonism.

similar to the proportion of the active doses of the 2 drugs used alone. It was difficult to apply this rule to the present study, since depending on the parameter of activity, the proportions of the active doses of the single drugs often varied considerably. For example, for AmphB and 5-FC in the infection with *C. albicans* strain 140/1, it was 1:< 260 when comparing the ED50ies, but 1: >1,500 when comparing the doses producing a 50% prolongation of the survival time. In order to overcome this problem, a great variety of dose levels and dose proportions was used for each of the combinations in each of the models (tables III–V). Indeed, no attempt was made to determine the optimum proportions in detail.

Most commonly, the additive and syner-

Table V. Chemotherapeutic activity of combinations of 5-FC and Ktz on murine mycoses (candidiasis, cryptococcosis and aspergillosis) in comparison to the activity of the single drugs[1])

Doses, mg/kg		Candidiasis *C. albicans* strains[2]			Cryptococcosis *C. neoformans* strains[2]			Aspergillosis *A. fumigatus* strains[2]		
5-FC + (s.c.)	Ktz (p.o.)	H12	140/1	13	1841	1820	1549	437	636	638
6.25	6.25	indiff	–	–	–	–	–	–	–	–
	12.5	indiff	–	–	–	–	–	–	–	–
	25	indiff	–	–	–	–	–	–	–	–
	50	indiff	–	–	–	–	–	–	–	–
12.5	6.25	indiff	indiff	indiff	–	–	–	–	–	–
	12.5	indiff	indiff	indiff	–	–	–	–	–	–
	25	indiff	indiff	add	–	–	–	–	–	–
	50	syn	syn	indiff	–	–	–	–	–	–
25	6.25	indiff	indiff	indiff	–	–	–	–	–	–
	12.5	syn	add	indiff	–	–	–	–	–	–
	25	indiff	add	indiff	–	indiff	indiff	indiff	indiff	indiff
	50	indiff	syn	indiff	–	indiff	indiff	indiff	indiff	indiff
	100	–	–	–	–	indiff	indiff	indiff	indiff	indiff
50	6.25	–	indiff	indiff	–	–	–	–	–	–
	12.5	–	indiff	indiff	–	–	–	–	–	–
	25	–	indiff	syn	indiff	indiff	indiff	indiff	indiff	indiff
	50	–	indiff	syn	indiff	indiff	indiff	indiff	indiff	indiff
	100	–	–	–	indiff	indiff	indiff	indiff	indiff	indiff
100	12.5	–	–	–	indiff	indiff	indiff	indiff	indiff	indiff
	25	–	–	–	indiff	indiff	indiff	indiff	indiff	indiff
	50	–	–	–	indiff	–	–	–	–	–
	100	–	–	–	indiff	indiff	indiff	indiff	indiff	indiff
200	25	–	–	–	indiff	indiff	indiff	–	–	–
	50	–	–	–	indiff	indiff	indiff	–	–	–
	100	–	–	–	indiff	indiff	indiff	–	–	–

[1] Each effect was evaluated by comparing the survival times of 10 animals receiving the particular combination with 10 animals each receiving the single drugs at the same and at the double dosage (see Materials and Methods).

[2] The 3 strains of each species were preselected according to their sensitivity to 5-FC: the first (on the left) should represent good (normal) sensitivity to this drug, the second (in the middle) moderate sensitivity = 'partial resistance' and the third (on the right) definite resistance (cf. table I, II).

[3] Indiff = indifference; add = additive effect; syn = synergism.

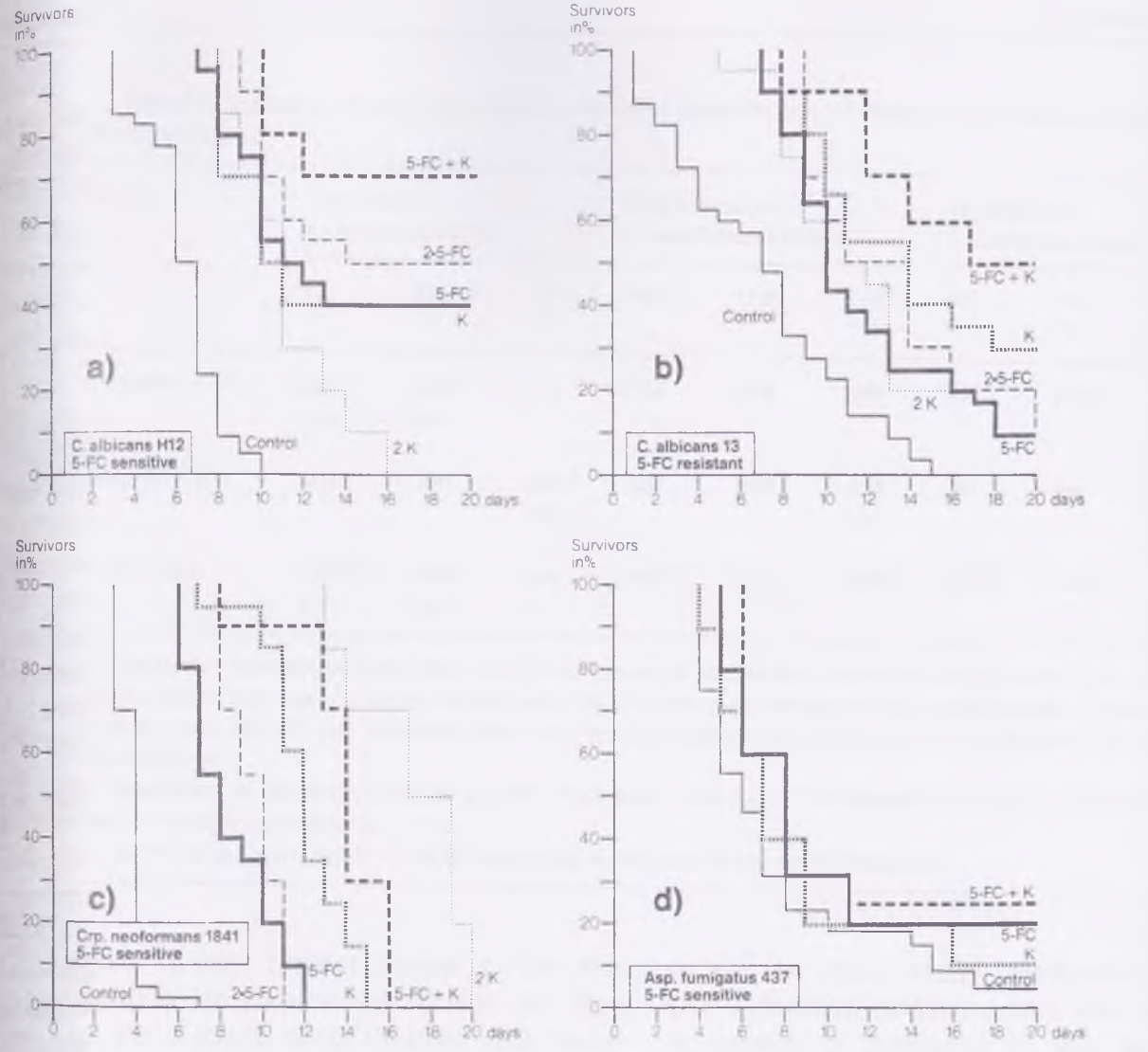


Fig. 3. Effect of combinations of 5-FC and ketoconazole (K) on the survival times of mice with candidiasis, cryptococcosis or aspergillosis in comparison to the effect of the single drugs (examples from table V). The curves indicate the percent survivors recorded daily for each dosage group (10 animals each, or 20 or 30, when the experiments were performed in duplicate or triplicate). The dosages (mg/kg) were the following:

Figure	5-FC + K	5-FC	2·5-FC	K	2·K
3a	12.5 + 50	12.5	25	50	100
3b	50 + 50	50	100	50	100
3c	200 + 100	200	400	100	200
3d	100 + 25	100	200	25	50

Control: group of infected, untreated animals.

gistic effects of 2 antimicrobials are determined, and are discriminated from each other, by means of *isobolic diagrams* [16, 24], or by similar methods analyzing the contribution made by the fraction of each partner present in the combination, to the total activity of the combination [15]. For antifungal combinations, such methods were, e.g., used by *Block and Bennett* [6], *Hamilton and Elliot* [19].

With the isobole procedures a certain endpoint of activity, e.g., an ED50, is first determined for each one of the drugs alone and for their combination at certain proportions. At the endpoint level, the fractional

Table VI. Summary of drug interaction in combined chemotherapy of murine candidiasis, cryptococcosis, and aspergillosis[1]

Drugs	Candidiasis *C. albicans* strains			Cryptococcosis *C. neoformans* strains			Aspergillosis *A. fumigatus* strains		
	12 *[2]	140/1 **	13 ***	1841 *	1820 **	1549 ***	437 *	636 **	638 ***
AmphB + 5-FC	add/[3] syn	*add/* syn	*syn*	*add*	*add*	*add*	add	indiff	indiff
AmphB + Ktz	*ant*	*ant*	add/ syn	*add*	*add*	add/ syn	*ant*	*ant*	*ant*
5-FC + Ktz	indiff/ syn	add/ syn	syn	indiff	indiff	indiff	indiff	indiff	indiff

[1] General classification from table III–V: the degrees of interaction (synergism, addition etc.) are taken for granted when they were observed with at least 2 of the individual dosages of the combinations. The abbreviated terms (syn, add etc.) are italicized when the degree in question was observed with 5 dosages of the combinations or more.

[2] Sensitivity of the fungus strains to 5-FC: * normally sensitive; ** moderately sensitive (='partially resistant'); *** definitely resistant.

[3] ant = antagonism; indiff = indifference; add = additive effect; syn = synergism.

doses of each partner present in the combination are then compared with the doses of the 2 single drugs required with monotherapy, putting the latter doses at 1.0 each. An additive effect is taken for granted if the sum of the fractional doses is 1.0, and synergism, if the sum is less than 1.0. In the usual diagram, the limit is consistent with the 'addition line' combining the 1.0 dose points of the 2 single drugs put on the abscissa and ordinate, respectively. In accordance with this, addition is not only proven for combinations containing half the dose of each component producing the defined endpoint activity, but also for those containing more than half of the 'endpoint dose' of the one of the components, as long as the fractional dose of the other component is small enough to keep the sum at 1.0. In other words, combinations are still considered additive, when the activity in question is produced by less than the double dose of the one of the single partners (which is the more active in this case), provided that a correspondingly higher dose is required of the other (less active) partner. A sum of more than 1.0 indicates indifference, as long as the fractional dose of any of the 2 partners is not greater than 1.0, but antagonism is indicated if any one is greater. In the diagram, the 'line of indifference' has 2 perpendicular parts consistent with the parallels put through the 1.0 dose points of the single drugs forming a square with the abscissa and ordinate [16]. Commonly, the effects of combinations corresponding to points between the 'addition line' and the 'line of indifference' are al-

ready considered to indicate indifference, but strictly speaking they indicate undefined 'mixtures' of indifference and addition. By some authors all points beyond the addition line (and all sums greater than 1.0) were considered evidence of antagonism [5], but these were obviously errors [26].

The isobole method can only be applied to pairs of drugs whose dose activity curves have a similar slope [17]. The fact that it does not offer statistical analysis of what is a 'same' and a 'different' effect (does, e.g., a sum of 0.9 of the fractional doses already indicate synergism?) and the somewhat unclear distinction between addition and indifference are further disadvantages of this method.

With the drugs and disease models studied in this paper, the requirement of similar dose activity curves was not fulfilled. This is reflected by the, often, very different quotients between the ED50ies and the doses producing a 50% prolongation of the survival (table II). For example, in the infection with *C. albicans* No. 140/1, this quotient was 2.4 for AmphB but > 16 for 5-FC. On the other hand, the limits we set for additive and synergistic effects were more rigorous than those used in an isobolic diagram, since we only relied on the differences with the more active of the 2 single partners, without taking into account the contribution made by the other component present in the combination. Furthermore, the limits for 'same' and 'different' effects were determined statistically. (As outlined in Materials and Methods, the U limits generally correspond to p values of less than 0.05 and a clear definition of indifference was used.) With our method, the activities were not compared at a certain endpoint (e.g., the ED50) level, but a direct comparison was made of the survival pattern of the mice observed with the combinations and single components at the dosages in question. This procedure may have favoured the appearance of incidental differences at some dosages as well as the nonappearance of true differences at some others. However, the various classes of interaction (synergism, addition etc.) were far from being distributed at random, but on the contrary were concentrated to certain pairs of drugs and certain models (table III–V). Thus, we believe that the general classification resulting from this study is reliable.

This general classification is presented in table VI for each of the 3 pairs of drugs with respect to each of the 9 disease models (3 each of candidiasis, cryptococcosis and aspergillosis). The degrees of interaction are indicated with the terms 'synergism', 'addition' etc. when they were observed with at least 2 of the individual dosages of the combinations (table III–V). The terms are italicized when the degree in question was observed at 5 instances or more.

Combination AmphB plus 5-FC

This combination was definitely synergistic or, at least, definitely additive in candidiasis. It had a definitely additive effect in cryptococcosis, whereas in aspergillosis only weak addition (one model) or indifference (2 models) was found. In candidiasis and cryptococcosis, the beneficial effect of the combination also extended to the infections with the moderately and definitely 5-FC-restistant strains of *C. albicans* and *C. neoformans*.

This general picture agrees well with the common experience with these groups of fungi. Since the first report by *Medoff* et al. [25] in 1971, beneficial (additive or syner-

gistic) antifungal effects of AmphB plus 5-FC on *C. albicans* and *C. neoformans* were widely confirmed in vitro and in animal models of candidiasis and cryptococcosis [for the first observations in vivo, see 6. 13, 36, 37]. They also extended to *C. albicans* strains 'partially resistant' to 5-FC and even were more pronounced in these [27, 30]. Still beneficial though at best additive effects were later observed with aspergilli in vitro [22] and in vivo [1]. Occasionally, lack of any improvement compared with the effects of the single components was also reported, e.g., in candida endocarditis in rabbits [7], cryptococcosis in mice [19] and aspergillus endocarditis in rabbits [27], and even a certain antagonism was claimed to have occurred in mice infected with a 5-FC-resistant strain of *C. neoformans* [19]. The latter claim seems doubtful, since as far as we understand, any lack of addition in the isobolic diagram was taken as antagonism by the authors, without considering indifference.

Combined chemotherapy with AmphB and 5-FC showed advantages over the chemotherapy with AmphB alone, or with either AmphB or 5-FC alone, in human CNS cryptococcosis [4, 21, 38], and this combination was used successfully also for severe cases of human candidiasis and invasive aspergillosis [see e.g. 8, 9, 14, 35, 39, 40], although superiority over AmphB alone was not assessed.

AmphB is fully active on 5-FC-resistant mutants, and in *C. albicans*, the 5-FC resistance frequency was found to be reduced at subinhibitory concentrations of AmphB [31]. A substantial number of the failures of 5-FC monotherapy in human mycoses (particularly CNS cryptococcosis and aspergillosis) was explained by the emergence of 5-FC resistance [see e.g. 34], whereas secondary 5-FC resistance was not or only rarely observed with the combination AmphB plus 5-FC [4, 38]. In most of the animal models used for antifungal chemotherapy including ours, the treatment period was too short for a selection of resistant mutants. (In unpublished experiments using the same treatment schedule is in the present study, we did not find any increase of resistant mutants in the organ cultures from animals dying in spite of 5-FC monotherapy.) Hence, in human chemotherapy, the prevention of secondary 5-FC resistance is a probably major, additional advantage of the AmphB plus 5-FC combination which is not revealed by the usual in vitro and in vivo models.

Combination AmphB plus Ktz

The marked differences in the interaction we observed with this combination from one model to another, were striking: Whereas the effect was definitely beneficial (mostly additive) in the infections with the 3 strains of *C. neoformans*, antagonism throughout occurred in the infections with the 3 *A. fumigatus* strains. Most surprisingly, definite antagonism was found in 2 of the candidiasis models (infections with the *C. albicans* strain H12 and 140/1), but synergism in the third (infection with *C. albicans* strain 13).

The published experience with the AmphB plus Ktz combination known to us is limited to *C. neoformans* and *H. capsulatum*. An additive effect on these fungi was found in vitro, but in murine infections the combination was not better than AmphB alone (cryptococcosis) or Ktz alone (histoplasmosis); on the other hand, in the cryptococcosis model, the combination AmphB

plus Ktz resulted in longer survival of the mice than the combination AmphB plus 5-FC [18]. Superior activity of the combination AmphB plus Ktz over each of the single partners was found in a rabbit model of cryptococcal meningitis [28].

Antagonistic effects on yeasts of the candida group (mostly, *C. albicans*) in vitro were repeatedly observed when AmphB was combined with the imidazolyl antifungals miconazole [10, 12, 33], econazole [11] and clotrimazole [10], whereas a beneficial (additive) effect on such fungi was, to our knowledge, recorded only once for the combination AmphB plus clotrimazole [2]. On *C. neoformans* (22 strains) the combination of AmphB with miconazole or econazole had an antagonistic effect in vitro [12]. The antagonism in the antifungal action of the imidazolyls on the one hand, and AmphB on the other was explained by the different role they play on the ergosterol in the membrane of the fungi: Whereas the imidazolyls inhibit ergosterol biosynthesis, AmphB must bind to ergosterol in order to act; by the action of the imidazolyls AmphB would lose its substrate [29]. No published experience is obviously available on the effect of combinations of AmphB with the various imidazolyl antifungals on in vivo models of candidiasis or cryptococcosis (except for the before-mentioned, favourable results with AmphB + Ktz in experimental cryptococcosis), and no report was found on the effects on aspergilli either in vitro or in vivo.

Combination 5-FC plus Ktz

With our murine models, an additive to moderately synergistic effect was consistently found in candidiasis (the, relatively, most pronounced synergism occurred in the infection with the definitely 5-FC-resistant strain of *C. albicans*), whereas on cryptococcosis and aspergillosis, there was neither a positive nor a negative interaction of the 2 drugs.

There are a few published reports on experiments with combinations of 5-FC and Ktz or other imidazolyl antifungals. In vitro, the combinations of 5-FC plus Ktz, 5-FC plus miconazole and 5-FC plus econazole were indifferent (neither additive nor synergistic) when tested on *C. albicans* strains fully sensitive to 5-FC, but on strains which were 5-FC-resistant at any degree, the action of all these combinations was synergistic [32]. Beneficial (mostly additive) effects on yeast-like fungi were observed with the combinations 5-FC plus Ktz (*C. albicans* including 5-FC-resistant mutants, *C. tropicalis, C. parapsilosis, T. glabrata* [3]), 5-FC plus clotrimazole (candida group [2]; *C. neoformans* [20], and 5-FC plus miconazole or econazole), *C. albicans C. neoformans* [12]). The beneficial in vivo effect of the 5-FC plus Ktz combination on murine candidiasis described in the present paper was mentioned by one of us (*A.P.*) in an earlier study in which the combinations 5-FC plus miconazole and 5-FC plus econazole were found to be indifferent (neither additive nor antagonistic) in the same disease models [32]. In murine cryptococcosis, the combination 5-FC plus Ktz was found indifferent [18], whereas a beneficial effect was observed with 5-FC plus clotrimazole [20].

Very recently, the successful treatment of 2 human cases of pulmonary aspergillosis with a combination of 5-FC plus Ktz was reported. In both cases, there was some evidence that this combination was more effective than other drugs alone, including

Ktz [23]. As AmphB, Ktz is fully active against 5-FC-resistant mutants. As mentioned before, combinations with 5-FC wich are 'indifferent' in our animal models may well be 'synergistic' in certain human mycoses thanks to the prevention of secondary 5-FC resistance.

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