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# Efficacy and Pharmacodynamics of Flucytosine Monotherapy in a Nonneutropenic Murine Model of Invasive Aspergillosis

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The therapeutic efficacy of flucytosine (5FC) monotherapy and the pharmacodynamic index predictive of efficacy were evaluated in a nonneutropenic mouse model of acute invasive aspergillosis. Mice were infected intravenously with an *Aspergillus fumigatus* isolate (the median MICs of 5FC were 128 µg/ml under the standard condition, 0.5 µg/ml at pH 6.0, and 0.031 µg/ml at pH 5.0) 2 h prior to the start of therapy and were treated for 7 days with different 5FC dosing regimens. The total doses ranged from 50 to 800 mg/kg of body weight/day and were administered at 6-, 12-, and 24-h intervals. The efficacy was assessed by means of survival. The survival rates of the treatment groups ranged from 40 to 90%, while the survival rate of the control group was 20%. The efficacy found depended primarily on the total daily dose. However, the power of our sample size may have been too low to exclude an effect of dose fractionation. The pharmacodynamic index that most strongly correlated with the efficacy was the area under the serum concentration-time curve and MIC ratio ($R^2$ = 0.86). We conclude that 5FC monotherapy is efficacious in a murine *Aspergillus fumigatus* infection model.

Flucytosine (5FC) is the fluorinated analogue of cytosine which was first used for the treatment of human invasive mycoses in 1968 (21). The drug has a narrow spectrum of activity and is seldom used as monotherapy due to the emergence of resistance. 5FC monotherapy is presently used only for the treatment of some cases of chromoblastomycosis and uncomplicated lower urinary tract candidiasis and vaginal candidiasis. In all other cases, 5FC is used in combination with other agents, usually amphotericin B, for the treatment of systemic mycoses (24). Its use for the treatment of infections caused by filamentous fungi is controversial because there is currently insufficient scientific evidence for such an indication.

Filamentous fungi are usually not susceptible to 5FC in vitro (16). However, in a recent study (22) we demonstrated that the in vitro activity of 5FC against *Aspergillus fumigatus* increased when the medium pH was lowered from 7.0, which is the pH recommended by the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) (16), to 5.0. For most *A. fumigatus* isolates, high MICs at pH 7.0 (MIC range, 2 to >256 µg/ml; median MIC, 16 µg/ml) were converted to very low MICs at pH 5.0 (MIC range, 0.031 to 0.5 µg/ml; median MIC, 0.125 µg/ml). At pH 5.0 the MICs were below the concentrations achievable in vivo. Based on these findings, we speculated that 5FC monotherapy could be effective for the treatment of infections caused by filamentous fungi.

We therefore investigated whether 5FC treatment prolonged survival in a nonneutropenic murine model of acute invasive aspergillosis. In this model, the duration of 5FC treatment was 7 days, and the therapeutic efficacy was assessed by survival rather than by CFU quantitation, because CFU counts do not accurately reflect the number of viable cells in infections caused by filamentous fungi. Due to the filamentous nature of these organisms, a large fungal mass is often indistinguishable from single-cell conidial forms when the organisms are spread on agar plates, since both will usually yield one colony (13).

Pharmacodynamic studies for antibacterials have shown that the dosing regimen may have an impact on the effect. The pharmacodynamic index, such as the area under the serum concentration-time curve (AUC) and MIC ratio (AUC/MIC) or the percentage of time that the levels of drug in serum remain above the MIC ($T > MIC$), that correlates with efficacy has been established for most antibacterials (11). However, the pharmacodynamic index predictive of efficacy of antifungals has primarily been studied in murine models of candididiasis (1–5, 7, 15). To our knowledge there is only one other published study in which the pharmacodynamics of an antifungal agent in a murine model of aspergillosis has been determined (25). Apart from studying the efficacy of 5FC monotherapy in a nonneutropenic murine model of acute invasive aspergillosis, we therefore also determined the pharmacodynamic index that correlates with efficacy by varying the dose levels and the dosing intervals.

(The results presented here were partly presented at the 44th Interscience Conference on Antimicrobial Agents and Chemotherapy, 2004, Washington, D.C.)

## MATERIALS AND METHODS

**Microorganism.** A clinical isolate of *Aspergillus fumigatus* (AZN 8196) was used in all experiments. The isolate was obtained from the private collection of the Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. The isolate was stored in glycerol broth at −80°C and was revived by subculturing it twice on Sabouraud dextrose agar supplemented with 0.02% chloramphenicol for 5 to 7 days at 35°C.

**Antifungal agent.** 5FC was obtained as a powder from Valeant Pharmaceuticals (Zoetermeer, The Netherlands). Drug dilutions were prepared on the day of

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study by dissolving the powder in distilled water. High concentrations of 5FC (approximately 10 g/liter and higher) sometimes deflocculate. This was dissolved by heating it for no longer than 30 min at a maximum of 80°C (manufacturer's instruction).

**In vitro susceptibility testing.** The MICs for the organism at pH 5.0, 6.0, and 7.0 were determined by using a broth microdilution technique described previously (23) and based on the M38-A method of the Clinical and Laboratory Standards Institute (16). Determinations were performed in triplicate.

**Animals.** Female CD-1 outbred mice (Charles River Laboratories, Sulzfeld, Germany) weighing 20 to 29 g were used for all studies. Animal studies were conducted in accordance with the recommendations of the European Community (Directive 86/609/EEC, 24 November 1986), and all animal research procedures were approved by the institutional animal care and use committee of Radboud University.

**Infection model.** Conidia were harvested by washing the agar surface of Sabouraud dextrose agar with sterile saline containing 0.05% Tween 80. The conidial suspensions were filtered three times through sterile gauze folded two times to remove the hyphae, counted in a hemacytometer, and adjusted to the required concentration in sterile saline containing 0.05% Tween 80. Preliminary studies were performed to determine the 90% lethal dose ($LD_{90}$), which was $1 \times 10^7$ CFU/mouse. The mice were infected with the $LD_{90}$ by injection of 0.1 ml of the conidial suspension into the orbital vein.

**Pharmacokinetics.** The single-dose pharmacokinetics of 5FC were determined for individual uninfected nonneutropenic CD-1 mice following intraperitoneal administration of doses of 25 and 100 mg/kg of body weight in 0.2-ml volumes. For each dose examined, groups of two mice were sampled by retro-orbital puncture, and the samples were collected in heparinized tubes (Becton Dickinson, Franklin Lakes, NJ) 5 min, 15 min, 30 min, 45 min, 60 min, 90 min, 2 h, 3 h, 4 h, 5 h, 6 h, and 8 h after administration. As soon as possible, but not later than 10 min after collection, the samples were centrifuged (model EBA 12 centrifuge; Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 1,500 × g for 5 min at a temperature of 4°C. The plasma was subsequently removed, transferred into polypropylene tubes, and stored at −70°C until the drug levels were determined by high-performance liquid chromatography at Apotheek Haagse Ziekenhuizen (The Hague, The Netherlands). The lower level of detection for this assay was 0.6 mg/liter, and the assay variance was ≤6%. Pharmacokinetic parameters, including the elimination half-life, were estimated by using a one-compartment model with first-order absorption via a nonlinear least-squares techniques (MW-Pharm, Mediware, Groningen, The Netherlands). The AUC was calculated by use of the trapezoidal rule. For doses for which no kinetics were determined, pharmacokinetic parameter values were extrapolated from the values obtained in the actual studies. Total levels in serum were used for all calculations because of the negligible amount of protein binding to 5FC (1).

**In vivo efficacy.** Nonneutropenic mice were infected with *A. fumigatus* AZN 8196 2 h prior to the start of therapy. Groups of 10 mice each were treated for 7 days with different 5FC dosing regimens by using twofold increasing total doses administered at 6-h (q6h), 12-h (q12h), and 24-h (q24h) intervals. Total doses ranged from 50 to 800 mg/kg/day. The drug was administered intraperitoneally in 0.2-ml volumes. Control mice were infected but received only distilled water. The animals were checked twice daily for mortality and for clinical signs. The mice were observed for 8 days after the end of treatment.

**Data analysis.** The data for the control groups were pooled for analysis. Mortality data were compared by the log rank test. Statistical analyses were performed by using GraphPad Prism, version 4.00, for Windows (GraphPad Software, San Diego, CA). Statistical significance was defined as a $P$ value <0.05.

The $T > MIC$ was determined by using MicLab 2.33 (Medimatics, Maastricht, The Netherlands). The percentage of survivors in the treated groups was corrected for the percentage of survivors in the control group by the following formula: (percentage of survivors in the treated group − percentage of survivors in the control group)/(100% − percentage of survivors in the control group) (19). The relationship between the in vivo efficacy (corrected survival) and the pharmacodynamic indices $T > MIC$, AUC/MIC, and the peak level/MIC was determined by nonlinear regression analysis. A sigmoid dose-response model (with variable slope) was used. The goodness of fit was checked by determination of the $R^2$ value, and analysis was carried out with the GraphPad Prism software.

## RESULTS

**In vitro susceptibility testing.** The median MICs of 5FC for *A. fumigatus* AZN 8196 were 0.031 μg/ml at pH 5.0, 0.5 μg/ml at pH 6.0 (MIC range, 0.25 to 1 μg/ml), and 128 μg/ml at pH 7.0 (MIC range, 32 to 128 μg/ml).
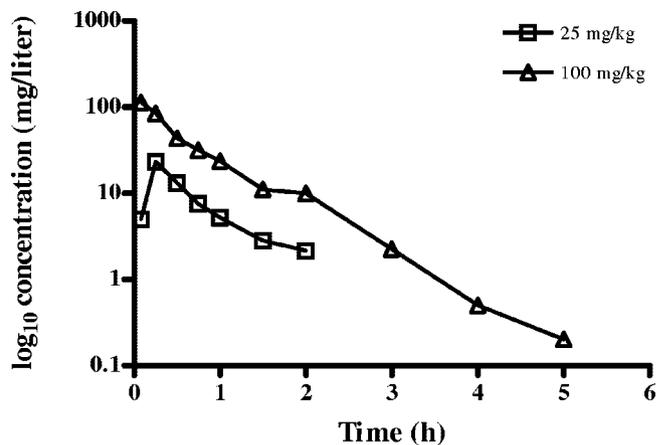


FIG. 1. Serum flucytosine concentrations after intraperitoneal administration of 25 and 100 mg/kg in uninfected nonneutropenic mice. Each symbol represents the geometric mean level in plasma for two mice.

**Pharmacokinetics.** The time course of the levels of 5FC in the sera of uninfected nonneutropenic mice following intraperitoneal administration of doses of 25 and 100 mg/kg are shown in Fig. 1. The pharmacokinetics were linear and were well described by a one-compartment model. Peak levels were achieved within 0.2 h for each dose and ranged from 16.3 to 87.8 μg/ml. The elimination half-life did not change significantly with higher doses and ranged from 0.51 to 0.57 h. The AUC, as determined by use of the trapezoidal rule, ranged from 15 to 74.3 mg · h/liter with the lowest and highest doses, respectively.

**Animal model.** The survival curve for the control mice (Fig. 2) demonstrates that *A. fumigatus* caused acute and lethal infections in nonneutropenic control mice. Eighty percent of the control mice died within 2 to 9 days. After infection the *A. fumigatus* isolate rapidly disseminated into all organs, including the kidneys and brain (Fig. 3).

**In vivo efficacy.** The survival and the median survival time of mice infected with *A. fumigatus* AZN 8196 and treated with different 5FC dosing regimens are shown in Table 1. The rates
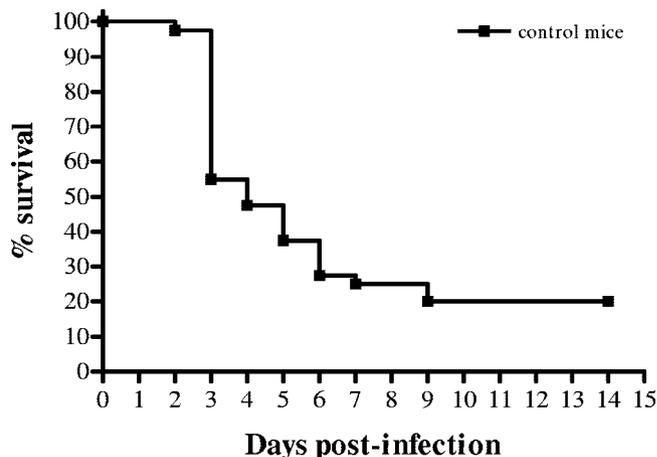


FIG. 2. Cumulative mortality for nonneutropenic untreated mice infected with *A. fumigatus* AZN 8196.
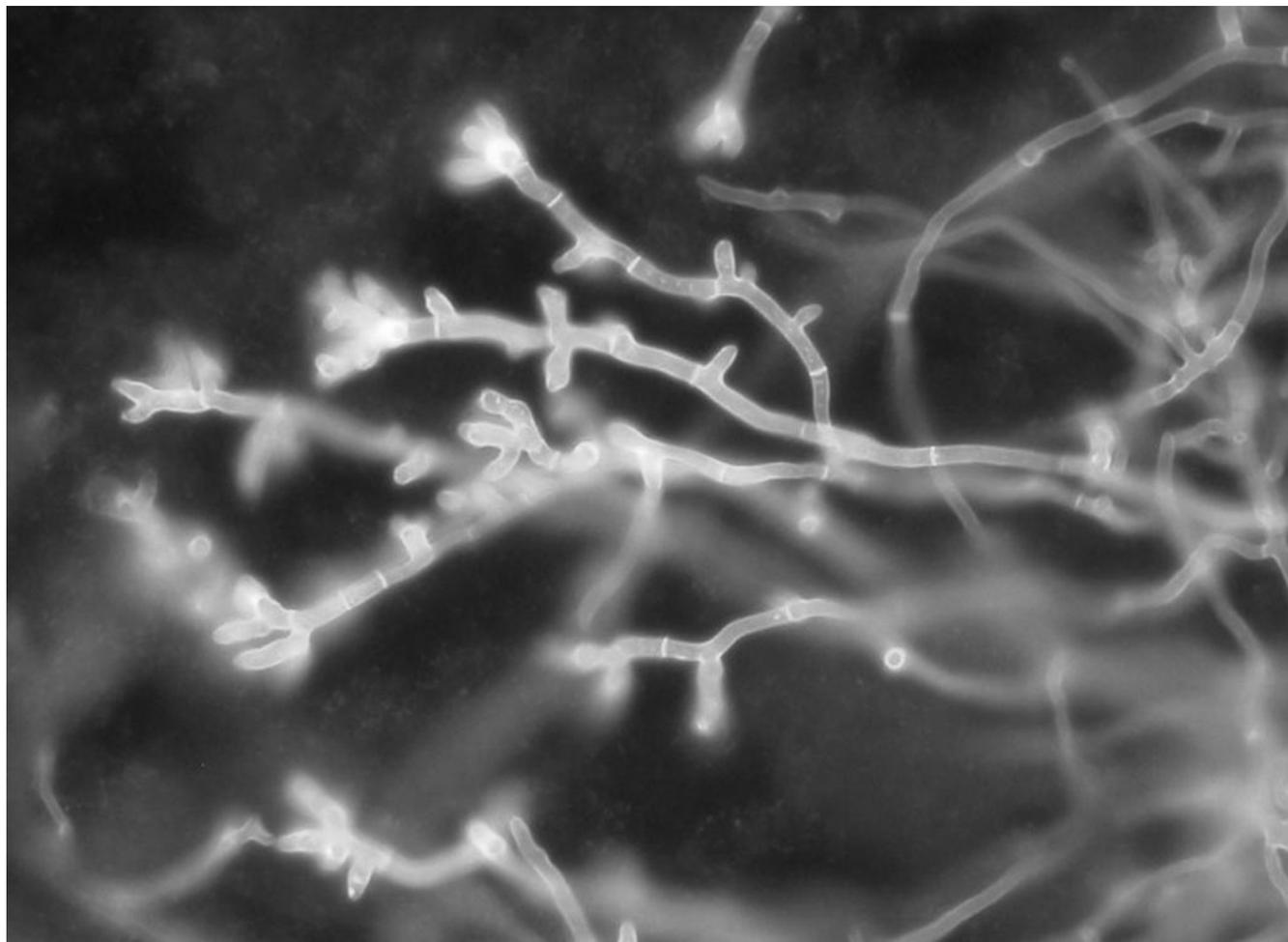
FIG. 3. Blankophor preparation of brain tissue infected with *A. fumigatus*.

of survival in the 5FC treatment groups ranged from 40 to 90%, depending on the dosing regimen used. For 7 of the 11 5FC treatment groups, the rate of survival was significantly prolonged compared to that of control mice ($P < 0.05$).

Figure 4 shows the dose-response curves for the q6h and q12h dosing intervals. The dose-response curve for the q24h dosing interval is not shown because only two dosing regimens were tested. Figure 4 shows that for both dosing intervals the corrected survival increased when the dose (mg/kg) increased. The corrected survival rate increased from 33.3 to 85.7% with escalating 5FC doses for the q6h dosing interval (dose range, 12.5 mg/kg to 200 mg/kg) and from 14.3 to 71.4% for the q12h dosing interval (dose range, 25 mg/kg to 200 mg/kg). Figure 4 also shows that for the same total daily dose (mg/kg/day), the corrected survival of the q6h dosing interval was similar to or higher than the corrected survival of the q12h dosing interval. For example, a total daily dose of 50 mg/kg resulted in 14.3% corrected survival with dosing every 12 h and 33.3% corrected survival with dosing every 6 h. A total daily dose of 100 mg/kg resulted in 42.9% corrected survival with dosing every 12 h and 44.4% corrected survival with dosing every 6 h. However, when the dose-response curves were compared by linear regression analysis, no statistically significant differences were found be-

TABLE 1. Survival times for mice infected with *A. fumigatus* AZN 8196 and treated with different 5FC dosing regimens[a]

| Treatment group | Total daily dose (mg/kg/day) | Survival (%) | Median (range) survival time (days) |
|---|---|---|---|
| 200 mg/kg/q6h | 800 | 90 | 14 (4–14)[b] |
| 100 mg/kg/q6h | 400 | 90 | 14 (5–14)[b] |
| 50 mg/kg/q6h | 200 | 70 | 14 (5–14)[b] |
| 25 mg/kg/q6h | 100 | 50 | 14 (3–14)[c] |
| 12.5 mg/kg/q6h | 50 | 40 | 6 (3–14) |
| | | | |
| 200 mg/kg/q12h | 400 | 80 | 14 (3–14)[b] |
| 100 mg/kg/q12h | 200 | 40 | 8 (3–14) |
| 50 mg/kg/q12h | 100 | 60 | 14 (6–14)[b] |
| 25 mg/kg/q12h | 50 | 40 | 9.5 (3–14) |
| | | | |
| 200 mg/kg/q24h | 200 | 50 | 14 (4–14)[d] |
| 100 mg/kg/q24h | 100 | 60 | 14 (3–14)[d] |
| | | | |
| Control group | | 20 | 4 (2–14) |

[a] Treatment was started 2 h after infection and continued for 7 days.
[b] $P < 0.01$ (compared with controls).
[c] $P < 0.1$ (compared with controls).
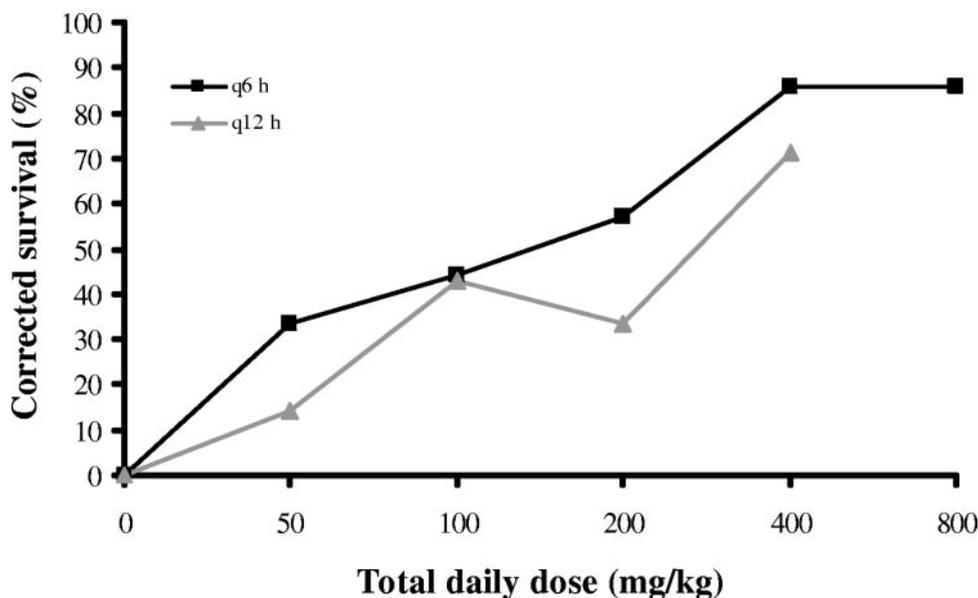[d] $P < 0.05$ (compared with controls).

FIG. 4. Relationship between the total daily dose for the q6h and the q12h dosing intervals and corrected survival (proportion of deaths in control group) in a nonneutropenic murine model of disseminated aspergillosis.

tween both the slopes and the intercepts ($P > 0.05$). The efficacy was therefore concluded to be primarily dependent on the total daily dose.

The relationship between the in vivo efficacy (corrected survival) and each of the pharmacodynamic indices, $T > $ MIC, AUC/MIC, and peak level/MIC, for the MICs found at pH 6.0 and 7.0 are shown in Fig. 5. The AUC/MIC appeared to be the most important pharmacodynamic index in predicting the efficacy. The $R^2$ value was 0.86 at both pH 6.0 and pH 7.0. For the peak/MIC the $R^2$ value was 0.70 at both pH values, and for $T > $ MIC the $R^2$ values were 0.74 at pH 6.0 and 0.51 at pH 7.0.

## DISCUSSION

In this study we showed that 5FC monotherapy is effective in a nonneutropenic murine model of acute invasive aspergillosis with a prolonged treatment period of 7 days and that the AUC/MIC was the pharmacodynamic index that most strongly correlated with the efficacy.

In order to determine the efficacy of 5FC monotherapy, we developed a nonneutropenic murine model of invasive aspergillosis. Nonneutropenic mice were injected intravenously with *A. fumigatus* conidia. This leads to an acute infection and generates mortality in 80% of the control mice within a short time period (2 to 9 days). After intravenous challenge, the *A. fumigatus* isolate rapidly disseminates into all organs, including the kidneys and brain. The mice were treated for 7 days with different 5FC dosing regimens (treatment was started 2 h after inoculation), and the efficacy of 5FC treatment was assessed by means of survival.

Experimental models of invasive aspergillosis have been described in mice, rats, guinea pigs, and rabbits. There are two models that are used worldwide, namely, a normal or immunocompetent model and an immunosuppressed model. In both models the disseminated infection is induced by an intravenous injection of *Aspergillus* conidia. The first is a model of acute disseminated aspergillosis, while the second is a more chronic model (18). Both models can be used in order to determine the efficacy of antifungal treatment. Since the normal or immunocompetent or acute model is simple and less time-consuming than the immunosuppressed or chronic model, we used the former one. We also considered it important to provide treatment for a prolonged period of time, 7 days, to more simulate the clinical situation. A neutropenic model has the disadvantage of being increasingly prone to superinfections.

The efficacy of antifungal treatment is usually assessed by means of survival or CFU quantitation in selected organs, mostly the kidneys, liver, lungs, or brain. However, CFU counts do not accurately reflect the number of viable cells for filamentous fungi such as *A. fumigatus*. Due to the filamentous nature of these organisms, a large fungal mass composed of hundreds of cells may be recorded only as a single unit by the traditional CFU methodology (13). Bowman et al. recently showed that a quantitative PCR assay could be used to measure the fungal burden in organs and thus monitor the progression of infection and assess the efficacy of antifungal therapy (9). However, quantitative PCR does not discriminate between viable and dead cells and may lead to incorrect conclusions with regard to the efficacy of antifungal therapy. Thus, although there is a practical limitation in the relatively large number of mice needed in order to determine the dose-response relationship by using survival analysis, we consider survival the most reliable method at present.

In our nonneutropenic murine model of invasive aspergillosis, the efficacy of 5FC monotherapy was well demonstrated. The survival in all treatment groups was higher (range, 40 to 90%) than the survival of the control mice (20%). Unfortunately, 100% survival in the treatment groups was not found even at the highest doses. One reason could be that we did not give the maximum effective dose. Alternatively, 100% survival can perhaps not be obtained even with the highest doses and/or
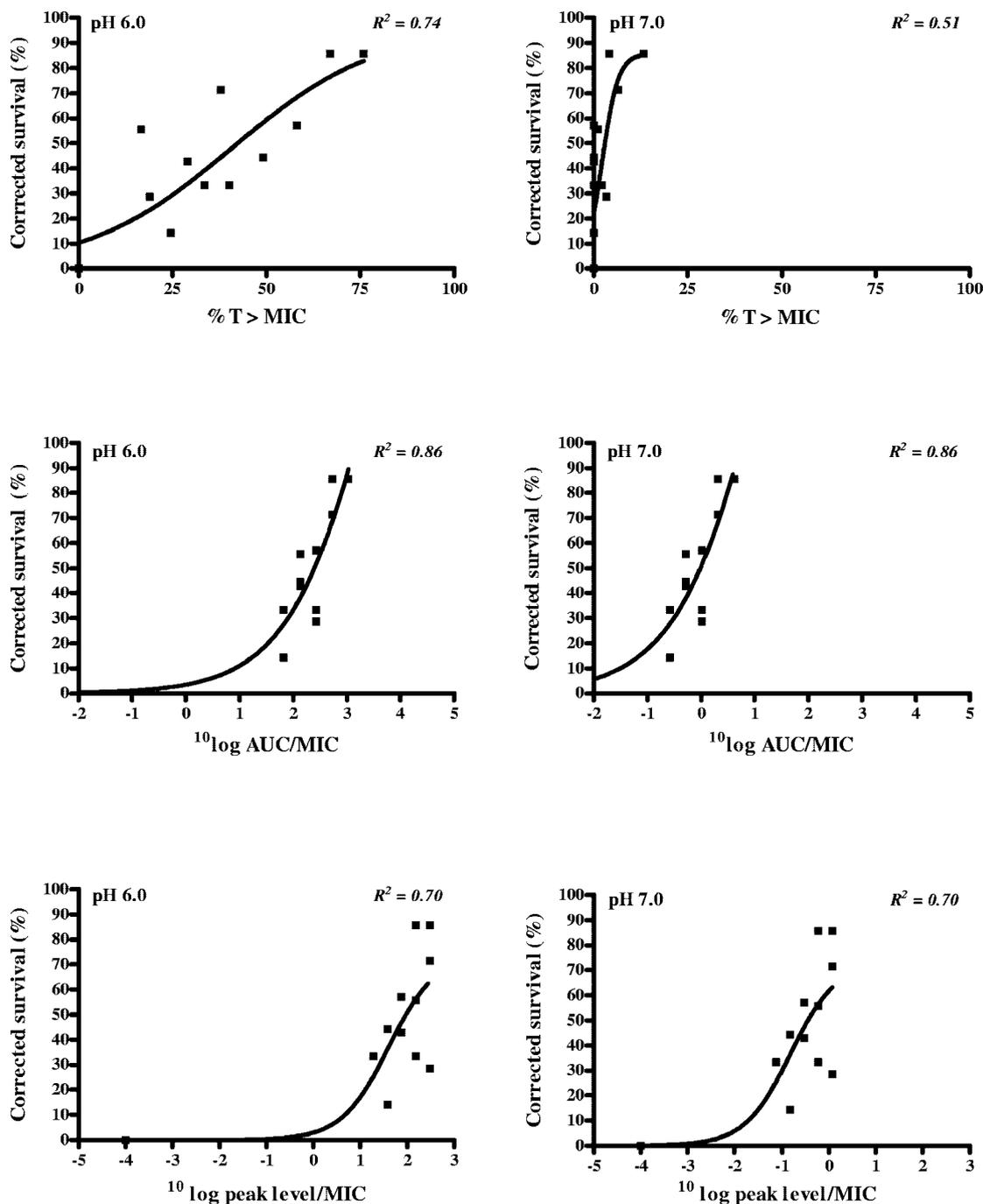
FIG. 5. (Top two panels) Relationship between the percentage of the dosing interval that levels in serum remained above the MIC for *A. fumigatus* AZN 8196 and the corrected survival after 7 days of therapy; (middle two panels) relationship between the AUC/MIC and the corrected survival after 7 days of therapy; (bottom two panels) relationship between the peak level in serum/MIC and the corrected survival after 7 days of therapy.

effective antifungal treatment, since the infection with *A. fumigatus* is acute and is rapidly disseminated into all organs. There may always be animals that do not survive that. In a rat model of invasive pulmonary aspergillosis (8), optimal treatment with liposomal amphotericin B (AmBisome) did not result in 100% survival either.

This in vivo study is one of the few in which the efficacy of

5FC monotherapy against *A. fumigatus* has been determined. Other animal studies that included the effect of 5FC monotherapy were focused mostly on the efficacy of 5FC in combination with another agent. In one of these studies the activity of 5FC was marginal (20). In another, the mortality significantly decreased from 100% to 60% when the mice were treated with 250 mg/kg 5FC (6). Polak et al. (19) showed that

for one *A. fumigatus* isolate mortality decreased from 100% to 80% when high doses of 5FC (100 mg/kg or 200 mg/kg) were given, while for another *A. fumigatus* isolate virtually no activity was seen. In another study with the same model but with a different dosing regimen, they showed that 5FC had definite but slight activity against four *A. fumigatus* isolates (17). George et al. (12) showed that the tissue burden of infection in the livers, lungs, and kidneys of rabbits treated with 5FC at 100 mg/kg was reduced but that 5FC treatment did not decrease mortality. In *Aspergillus* endocarditis, treatment of infected rabbits with 5FC (25 or 50 mg/kg) significantly lowered the number of CFU per gram of vegetation (10). In another endocarditis model, 5FC (35 mg/kg) eradicated *Aspergillus* from the cardiac vegetation in 1 of the 10 rabbits tested, but it had no appreciable effect on mortality or survival time (14). In general, the activity of 5FC monotherapy against *A. fumigatus* in animal models has been marginal.

When we determined the in vitro activity of 5FC against the *A. fumigatus* isolate used in this model by using the M38-A method of the Clinical and Laboratory Standards Institute (16), we found no significant activity (median MIC, 128 μg/ml). However, when we determined the activity at a lower pH we found MICs that were relatively low (median MICs, 0.5 μg/ml at pH 6.0 and 0.031 μg/ml at pH 5.0). It thus appears that for this *A. fumigatus* isolate the in vitro activity at a pH value of 5.0 or 6.0 correlated better with in vivo efficacy than it did at pH 7.0. This stresses the importance of the in vitro method that not only results in good intra- and interlaboratory reproducibilities but, moreover, predicts a drug's efficacy.

In our nonneutropenic murine model of invasive aspergillosis, the efficacy of 5FC monotherapy against *A. fumigatus* was dependent on the total daily dose, and the AUC/MIC was the pharmacodynamic index that most strongly correlated with the efficacy. Andes and van Ogtrop (1) characterized the pharmacodynamic index predictive of efficacy of 5FC monotherapy in a murine model of disseminated *Candida albicans* infection. They found that $T > MIC$ was the best predictor of the outcome, while the AUC/MIC was only slightly less predictive. The differences between our findings and the findings of Andes and van Ogtrop (1) may be explained by the differences in the experimental models used. We used a nonneutropenic murine model of invasive aspergillosis in which groups of 10 mice were treated for 7 days and observed for 8 days after the end of treatment and the efficacy was determined by means of survival, while Andes et al. used a neutropenic murine model of disseminated candidiasis in which groups of two mice each were treated for 24 h and the efficacy was determined by determination of the CFU in the kidneys. The differences between our results and the findings of Andes and van Ogtrop (1) may also be explained by the differences in the microorganism used (*A. fumigatus* versus *Candida albicans*), in the end point used (survival versus CFU quantitation), and perhaps even more by the differences in the length of treatment (7 days versus 24 h). Alternatively, the power of the study may have been too low to detect significant differences in outcomes for total daily doses given either q12h or q6h, and thus, we cannot exclude an effect of dose fractionation. In any case, the relation between AUC/MIC and survival resulted in the best model fit.

We conclude that 5FC monotherapy was efficacious in a nonneutropenic murine model of invasive aspergillosis. The efficacy was dependent on the total daily dose, and the AUC/MIC was the pharmacodynamic index that was the best predictor of the outcome. This observation needs to be confirmed in other experimental models of *Aspergillus* infection, including neutropenic models, and with a number of other *A. fumigatus* isolates.

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