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Current Opinion in Pharmacology

Assessment of efficacy of antifungals in experimental models of invasive aspergillosis in an era of emerging resistance: the value of real-time quantitative PCR

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Experimental models of invasive aspergillosis (IA) have been used to explore pharmacokinetic and pharmacodynamic (PK/PD) properties of antifungal agents. Survival is still considered the golden standard effect measure but has the disadvantage that a large number of animals are needed to determine the dose–response relationships and PK/PD of antifungals. The feasibility of using fungal load by real-time quantitative PCR (qPCR) as an effect measure has been explored recently. The majority of studies reported convincingly demonstrate a larger dynamic range for qPCR compared to conventional assays. However interpretation and translating the results to guidance in clinical decision making need further study. It is expected that the use of qPCR will become the primary outcome measure for assessment of PK/PD relationships of antifungals in experimental models of IA.

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Current Opinion in Pharmacology 2011, **11**:486–493

This review comes from a themed issue on
Anti-infectives
Edited by U. Theuretzbacher and J.W. Mouton

Available online 1st September 2011

1471-4892/$ – see front matter
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DOI 10.1016/j.coph.2011.08.001

Introduction

Whereas invasive aspergillosis (IA) remains an infection with significant mortality and morbidity ranging from 30 to 80%, the use of clinically licensed azoles such as voriconazole and posaconazole has improved the outcome of patients [1]. However, optimizing antifungal therapy in patients still needs to be addressed. Clinical trials in humans provide valuable evidence for the use of antifungal agents, but these studies are limited due to ethics, time and cost. Experimental models of IA have become a cornerstone to explore pharmacokinetic and pharmacodynamic (PK/PD) relationships of antifungal agents as well as the comparative utilities of diagnostic markers. In addition, animal models allow predicting the impact of resistance on outcome for IA [2•,3]. This is of particular importance, since resistance, in particular azole resistance in *Aspergillus fumigatus* is increasing [4].

In contrast to investigations evaluating the exposure–response relationships of antibacterials where colony forming units (CFU) have become the mainstay of effect measurements [5•], the most commonly used efficacy measures for antifungals are prolongation of survival and various parameters of reduction in tissue burden [6].

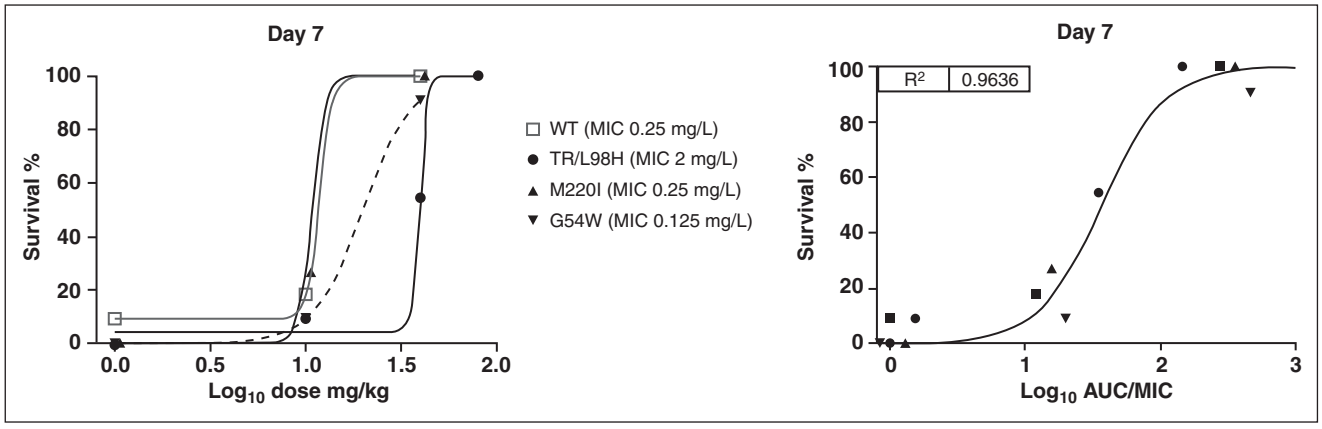
However, measurements of tissue burden in IA suffer from a significant number of problems and non-culture based methods in particular qPCR, are rapidly becoming the new gold standard tool for the diagnosis, detection and evaluation of tissue burden of *A. fumigatus*. We here discuss applications and limitations of qPCR for assessment of therapeutic efficacy of antifungal agents in experimental models of IA.

Benefits and limitations of conventional parameters to monitor therapeutic efficacy in IA

At present survival is considered the most reliable effect measure to assess therapeutic efficacy of antifungals in IA animal models infected by both azole susceptible and resistant *A. fumigatus*. For example, in the recent study of Mavridou et al. in an immunocompetent non-neutropenic murine model of disseminated IA, increased MICs correspond with reduced *in vivo* efficacy [7]. Overall, there was a good relationship between the area under the concentration–time curve (AUC)/MIC ratio and survival (Figure 1). AUC/MIC was a better predictor than dose/MIC because of the non-linear pharmacokinetics of voriconazole resulting in a disproportional increase in AUC by dose. Such PK/PD relationships can subsequently be used to help deducing dosing regimens and clinical breakpoints in humans.

Although survival studies are still considered the gold standard method to assess the efficacy of antifungals in IA, it has the disadvantage that a large number of animals is needed [8].

Figure 1



(a) Voriconazole dose–survival and **(b)** AUC/MIC–survival relationships for four *A. fumigatus* isolates with different MICs. Increased voriconazole exposure was required to obtain maximum efficacy in mice infected by isolates with attenuated susceptibility.
From Ref. [7].

Tissue burden studies can be completed more rapidly than survival studies and thus provide some impetus to the development and indications of drugs and also enable significant reduction in the number of animals required for experimental design [9,10]. However, measurements of tissue burden in IA suffer from a significant number of problems. Choosing the best quantification method has been the major problematic issue. The first parameter used was CFU quantitation in selected organs, mostly in the kidneys, liver, lungs, or brain. However, due to the filamentous nature of *A. fumigatus*, a large fungal mass composed of hundreds of cells may be recorded only as a single unit by the traditional CFU methodology and CFU counts do not accurately reflect the number of viable cells for filamentous fungi such as *A. fumigatus* [11]. $1 \rightarrow 3$, β-D-Glucan has shown promise as a diagnostic adjunct; however, this marker has a limited detection range and more research is required to define the utility of this assay, in particular in non-neutropenic models of IA [12]. The galactomannan (GM) assay has moderate accuracy for diagnosis of invasive aspergillosis [13].

Recently several studies have shown the increased sensitivity and precision of *A. fumigatus* qPCR over CFU and biomarkers measurement that will be discussed in the next section.

Quantitative PCR as an outcome parameter for evaluating antifungal therapy

A number of studies have indicated that a real-time quantitative PCR assay could be used to measure the fungal burden in organs and thus monitor the progression of infection and efficacy of antifungal therapy (Table 1). To that purpose, it is essential that the load of fungal DNA in blood or tissue specimens corresponds to tissue burden and/or survival [14,15,16] in various experimental situations, including acute and chronic infection models. The rationale is that PCR-based quantification of *A. fumigatus* tissue burden can detect every cell in a filamentous fungal mass, and therefore significantly better than CFU counts. Apart from homogenization issues (see below) the dynamic range of CFU determination is too narrow for filamentous fungi [11] and it may underestimate the absolute fungal burden in an established infection compared to qPCR and GM [17]. The majority of studies that have been performed to date indicate an increased sensitivity and precision of real-time qPCR over CFU measurement and biomarker assays. For instance, in a mouse model of invasive aspergillosis treated with amphotericin-B and caspofungin, Bowman et al. observed a 4 $\log_{10}$ decrease in conidial equivalent (CE) counts, while in the same animals a 1 $\log_{10}$ decrease in the number of CFU counts, substantiating the problems stated above. Recovery of CFU did not reflect progression or increasing numbers of *A. fumigatus* in the infected tissues [18•]. Similar results have been shown using qPCR for the assessment of kidney fungal burden in a guinea pig model IA [19]. The performance of 2 quantitative polymerase chain reaction (PCR) assays was compared with quantitative cultures and GM antigen detection in a rabbit model of invasive aspergillosis using blood, serum, lung, and brain specimens [20]. The authors concluded that specific real-time PCR assay is a reliable technique to detect *A. fumigatus* DNA *in vivo* comparable to cultures and GM determination. These results are also in agreement with the observation of Francesconi et al, Osullivan et al. and Petriatis *et al.* [10,21,22]. A similar study was conducted by Sheppard *et al.* [23]. They demonstrated that the method of tissue disruption used herein resulted in consistently higher

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