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# *In vitro* activity of a new triazole BAL4815, the active component of BAL8557 (the water-soluble prodrug), against *Aspergillus* spp.

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***Objectives*: BAL4815 is the active component of the antifungal triazole agent BAL8557 (the water-soluble prodrug). We compared the *in vitro* activity of BAL4815 with that of itraconazole, voriconazole, caspofungin and amphotericin B against 118 isolates of *Aspergillus* comprising four different species (*fumigatus*, *terreus*, *flavus* and *niger*); the isolates were pre-selected to include 16 isolates demonstrating *in vitro* resistance to other agents.**

***Methods*: Susceptibilities were determined for BAL4815, amphotericin B, itraconazole and voriconazole using the microdilution plate modification of the NCCLS M38-A method with RPMI 1640 buffered to pH 7.0 with MOPS; for caspofungin the method was modified using incubation in a gas mixture of 1% $O_2$/5% $CO_2$/94% $N_2$ to aid reading. MFCs (≥99% kill) were also determined for all drugs other than caspofungin.**

***Results*: For all isolates, geometric mean (GM) MIC values and ranges (in mg/L) were: BAL4815, 0.620 and 0.125–2.0; itraconazole, 0.399 and 0.063–>8.0; voriconazole, 0.347 and 0.125–8.0; caspofungin, 0.341 and 0.125–4.0; amphotericin B, 0.452 and 0.06–4.0. No significant differences in susceptibility to BAL4815 were seen between species and in contrast to itraconazole no isolates demonstrated MICs >2.0 mg/L. For all isolates, GM MFC values and ranges (in mg/L) were: BAL4815, 1.68 and 0.25–>8.0; itraconazole, 1.78 and 0.06–>8.0; voriconazole, 1.09 and 0.25–>8.0; amphotericin B, 0.98 and 0.25–>4.0.**

***Conclusions*: BAL4815 demonstrated promising antifungal activity against all four *Aspergillus* species *in vitro* including strains resistant to itraconazole, caspofungin or amphotericin B.**

Keywords: antifungal susceptibility, itraconazole, voriconazole, caspofungin, amphotericin B

## Introduction

Despite advances in antifungal therapy, mortality rates following invasive aspergillosis remain unacceptably high.[1] For many years amphotericin B has been the bedrock of systemic antifungal therapy and concerns about its toxicity have been partially addressed by the introduction of lipid formulations, but significant toxicity still remains often leading to withdrawal of therapy.[2] The introduction of the echinocandins has been welcomed as they have an alternative target compared with other antifungal agents but the mechanism of action against *Aspergillus* is predominantly fungistatic and this is less than ideal in neutropenic patients.[3]

Since the discovery of the antifungal activity of the first azoles 60 years ago, enormous advances have been made in the group to reduce toxicity, enhance bioavailability, improve the antifungal spectrum and counteract resistance. It is hoped that with the introduction of the second-generation triazoles many of the shortcomings of earlier azoles have been eliminated. Voriconazole in a large randomized control trial in patients with aspergillosis demonstrated superiority to amphotericin in terms of both response and survival.[4] However, voriconazole still has some drawbacks, including dose-related visual disturbances, non-linear pharmacokinetics and high inter-individual variability of pharmacokinetics. Cyclodextrin in the intravenous formulation also complicates treatment of patients with significant renal dysfunction.[5]

BAL4815 is the active antifungal component of BAL8557 (the water-soluble precursor, suitable for oral and intravenous delivery) and is of the triazole class of agents (Figure 1). We compared the *in vitro* activity of BAL4815 with that of itraconazole, voriconazole, caspofungin and amphotericin B against 118 isolates of *Aspergillus* comprising four different species (*fumigatus*, *terreus*, *flavus* and *niger*); the isolates were pre-selected to include 16 isolates demonstrating resistance to itraconazole, amphotericin B or caspofungin.

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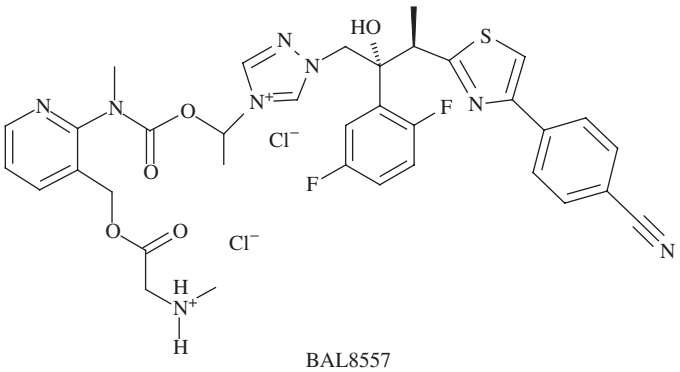
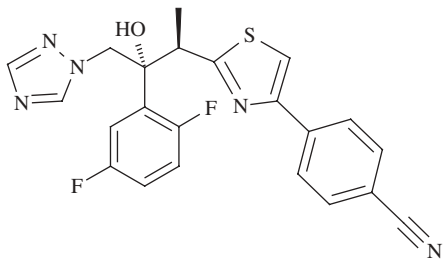



BAL8557




BAL4815

**Figure 1.** Structure of the prodrug BAL8557 and the active component BAL4815.

## Materials and methods

### *Organisms*

Susceptibility tests were performed on 118 clinical *Aspergillus* isolates comprising 62 *Aspergillus fumigatus* isolates, 20 *Aspergillus flavus* isolates and 18 isolates each of *Aspergillus niger* and *Aspergillus terreus*. Sixteen *A. fumigatus* isolates were resistant to itraconazole, caspofungin or amphotericin B (some also had increased MICs of posaconazole).[6] Isolates for which the MICs of itraconazole, voriconazole, caspofungin and amphotericin B are known (*Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019) were included as quality control strains for susceptibility testing.[7] All cultures were cultivated from frozen stock on Sabouraud dextrose agar (SAB) (Oxoid, Basingstoke, UK).

### *Antifungal agents*

BAL4815 (Basilea Pharmaceutica, Basel, Switzerland) was provided as a pure powder by the manufacturer. Itraconazole (Janssen Pharmaceuticals, Beerse, Belgium) and amphotericin B (Sigma, Poole, UK) were obtained as pure compounds. Voriconazole (Pfizer Ltd Sandwich, UK) and caspofungin (Merck Sharp & Dohme Ltd, Hoddesdon UK) were obtained in vials for intravenous administration.

Stock solutions (3200 mg/L) of all drugs were prepared using appropriate solvents—voriconazole and caspofungin (sterile distilled water); BAL4518, itraconazole and amphotericin B (dimethyl sulphoxide)—and adjusted for potency when necessary. Each aliquot was then dispensed in further aliquots and stored in glass vials, protected from the light, at –20°C until required.

### *Susceptibility testing*

Susceptibility tests were performed according to the broth microdilution modified method of the NCCLS M38-A accepted standard using RPMI 1640 medium (Sigma) buffered to pH 7.0 with MOPS (Sigma).[7] In brief, final drug ranges (in mg/L) were 0.0078–8 for BAL4815, voriconazole and itraconazole, and 0.0156–4 for caspofungin and amphotericin B.

Inoculum suspensions were prepared from 5–8 day cultures grown on SAB at 37°C and adjusted using a counting chamber. The final inoculum was between $0.5 \times 10^4$ and $5 \times 10^4$ cfu/mL as demonstrated by quantitative colony counts. Drug-free and cell-free controls were included. BAL4815, itraconazole, voriconazole and amphotericin B microdilution plates were incubated in air; caspofungin microdilution plates were incubated in 1% $O_2$/5% $CO_2$/94% $N_2$ to aid reading.[8] Readings were made after 48 h of incubation at 37°C (the *Candida* control strains were examined at 24 h). The MIC endpoints for BAL4815, itraconazole, voriconazole and amphotericin B were read visually as the lowest drug concentration that prevented any discernible growth. The MIC endpoints for caspofungin were read visually and taken as that which reduced growth by 80% compared with the drug-free control.

Minimum fungicidal concentrations (MFCs) were also determined for all drugs (other than caspofungin). For each isolate, 100 µL was removed from all wells without visible growth. Each aliquot was spot inoculated onto SAB plates, and the liquid was allowed to soak into the agar. When dry, the plate was streaked to separate any conidia and to

**Table 1.** *In vitro* susceptibilities of 118 isolates of *Aspergillus* to amphotericin B, BAL4815, caspofungin, itraconazole and voriconazole

| Species (no. of isolates) | Antifungal agent | MIC (mg/L)[a] | | | |
|---|---|---|---|---|---|
| | | GM[b] | range | 50% | 90% |
| *A. fumigatus* (62) | amphotericin | 0.334 | 0.06 to 0.5 | 0.25 | 0.5 |
| | BAL4815 | 0.578 | 0.125 to 2.0 | 0.5 | 2.0 |
| | caspofungin | 0.437 | 0.25 to 4.0 | 0.5 | 0.5 |
| | itraconazole | 0.676 | 0.125 to >8.0 | 0.25 | >8.0 |
| | voriconazole | 0.334 | 0.125 to 8.0 | 0.25 | 0.5 |
| *A. terreus* (18) | amphotericin | 0.735 | 0.25 to 1.0 | 1.0 | 1.0 |
| | BAL4815 | 0.463 | 0.25 to 0.5 | 0.5 | 0.5 |
| | caspofungin | 0.354 | 0.125 to 0.5 | 0.5 | 0.5 |
| | itraconazole | 0.151 | 0.06 to 0.5 | 0.125 | 0.25 |
| | voriconazole | 0.291 | 0.25 to 0.5 | 0.25 | 0.5 |
| *A. flavus* (20) | amphotericin | 0.785 | 0.5 to 4.0 | 1.0 | 1.0 |
| | BAL4815 | 0.732 | 0.5 to 2.0 | 0.5 | 1.0 |
| | caspofungin | 0.240 | 0.125 to 0.25 | 0.25 | 0.25 |
| | itraconazole | 0.138 | 0.06 to 0.5 | 0.125 | 0.5 |
| | voriconazole | 0.420 | 0.25 to 1.0 | 0.5 | 0.5 |
| *A. niger* (18) | amphotericin | 0.412 | 0.25 to 1.0 | 0.5 | 0.5 |
| | BAL4815 | 0.890 | 0.25 to 2.0 | 0.5 | 2.0 |
| | caspofungin | 0.206 | 0.125 to 0.25 | 0.25 | 0.25 |
| | itraconazole | 0.606 | 0.25 to 4.0 | 0.5 | 2.0 |
| | voriconazole | 0.381 | 0.25 to 1.0 | 0.25 | 1.0 |
| All isolates (118) | amphotericin | 0.452 | 0.06 to 4.0 | 0.5 | 1.0 |
| | BAL4815 | 0.620 | 0.125 to 2.0 | 0.5 | 2.0 |
| | caspofungin | 0.341 | 0.125 to 4.0 | 0.25 | 0.5 |
| | itraconazole | 0.399 | 0.06 to >8.0 | 0.25 | >8.0 |
| | voriconazole | 0.347 | 0.125 to 8.0 | 0.25 | 1.0 |

[a]50% and 90%, MICs at which 50% and 90% of isolates were inhibited, respectively.
[b]In calculation of the GM values, MICs of >8 mg/L were classed as 16 mg/L.

remove them from the drug source. The plates were incubated at 37°C for 48 h. The MFC was defined as the lowest drug concentration that allowed the growth of 50 or fewer colonies (99% kill).

Twenty percent of the isolates (24 of 118) were randomly selected and retested against each drug to assess the reproducibility of the method. In all cases wherein there was a discrepancy tests were repeated a third time before values were assigned.

The differences between species were analysed using the Kruskal–Wallis test for multiple comparisons using the computer package StatsDirect (Ashwell, Herts, UK).

## Results

Summaries of *in vitro* susceptibility values for the 118 isolates are shown in Table 1 and Figure 2. For all isolates, geometric mean (GM) MIC values and ranges (in mg/L) were: BAL4815, 0.620 and 0.125–2.0; itraconazole, 0.399 and 0.063–>8.0; voriconazole, 0.347 and 0.125–8.0; caspofungin, 0.341 and 0.125–4.0; amphotericin B, 0.452 and 0.06–4.0. With respect to MICs, isolates of *A. terreus* were significantly more susceptible to BAL4815 than *A. flavus* and *A. niger* ($P = 0.0082$ and $P = 0.0009$, respectively). No significant differences in susceptibility to BAL4815 were seen between the other species and in contrast to itraconazole no isolates demonstrated MICs >2.0 mg/L. The MICs of all antifungal agents for the quality control isolates were within the prescribed range.

For all isolates, GM MFC values and ranges (in mg/L) were: BAL4815, 1.68 and 0.25–>8.0; itraconazole, 1.78 and 0.125–>8.0; voriconazole, 1.09 and 0.25–>8.0; amphotericin B, 0.98 and 0.25–>4.0. For all isolates BAL4815, itraconazole, voriconazole and amphotericin B were fungicidal in 98.3%, 71.1%, 96.6% and 96.6% of instances, respectively (the $MFC_{50}$s and $MFC_{90}$s for all isolates were: BAL 4815, 1.0 and 4.0; itraconazole, 1.0 and 8.0; voriconazole, 1.0 and 4.0; caspofungin, >8.0 and >8.0; amphotericin B, 1.0 and 4.0). With respect to MFCs, isolates of *A. terreus* were significantly less susceptible to BAL4815 than *A. fumigatus, A. flavus* and *A. niger* ($P < 0.0001$, $P < 0.0002$ and $P = 0.0287$, respectively).

The MFC value was the same as or only one well higher than the MIC for 63%, 51% and 44% of isolates tested using BAL4815, itraconazole and voriconazole, respectively (the strains resistant to itraconazole were not used in the analysis of the itraconazole data for this comparison).

The reproducibility study on 20% of the isolates showed that 87.5%, 87.5%, 87.5%, 91.7% and 95.8% of isolates retested were within one well of the original MIC value of BAL4815, itraconazole, voriconazole, caspofungin and amphotericin B, respectively.

## Discussion

This study demonstrates that *in vitro* testing of BAL4815 is possible with reproducible endpoints using the general recommendations of the NCCLS 38A document.[7] The study further demonstrates that BAL4815 has potent *in vitro* antifungal activity against all four *Aspergillus* species including strains resistant to itraconazole, caspofungin or amphotericin B. The GM and range of MICs of BAL4815 are similar to those of itraconazole and voriconazole and are generally within the achievable range demonstrated in single and multiple dose human pharmacokinetic studies.[9]

This study further demonstrated that BAL4815 showed primary fungicidal activity (MFC within two dilutions of the MIC) against all four *Aspergillus* species and that for 63% of isolates the MFC was within one well of the MIC, superior to itraconazole or voriconazole. Indeed 68% of isolates were killed at ≤2.0 mg/L against BAL4815, compared with 51% against itraconazole and 92% against voriconazole.

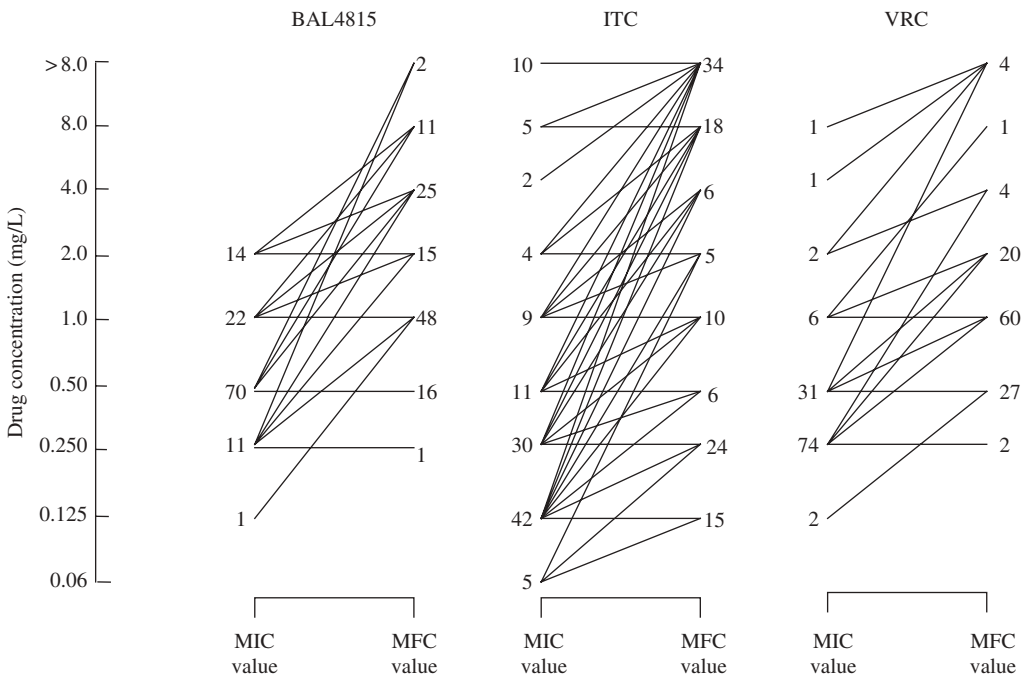



**Figure 2.** Relationship between the MIC and MFC for 118 strains of *Aspergillus* spp. of BAL4815, itraconazole (ITC) and voriconazole (VRC). The slope of the line linking the MIC and the MFC demonstrates the relationship between inhibitory and cidal concentration of the antifungal agents.

In this study 14 isolates were selected with MICs of itraconazole of ≥8 mg/L; the GM MIC and MFC for these strains of BAL4815 were 1.1 and 2.3 mg/L, respectively. Many of these strains had previously also demonstrated increased MICs and MFCs of posaconazole.[6] Although these values are ∼2-fold higher than the GMs for the total data set they are still within the expected therapeutic range of the compound. If the itraconazole-resistant strains had not been pre-selected and only more 'typical' strains included, the GM MICs and MFCs would be 0.56 and 1.57 mg/L, 0.25 and 1.34 mg/L, and 0.33 and 0.97 mg/L for BAL4815, itraconazole and voriconazole, respectively.

BAL8557 is a prodrug, which is rapidly cleaved into the active component (BAL4815) and an inactive prodrug cleavage product (BAL8728) after oral or intravenous administration. The conversion is rapid and complete with very low levels of the cleavage product detectable in the serum after oral or intravenous administration. In humans the serum beta phase half-life is long (85–117 h) and the $AUC_{0-24}$ 14–40 μg · h/mL (50–100 mg/day) demonstrates high levels of drug exposure. With such rapid conversion of a water-soluble prodrug, addition of potentially toxic cyclodextrin to increase/achieve solubility as occurs in itraconazole and voriconazole intravenous solutions is not required for this new azole.

There is currently little data available on the likely pharmacodynamic drivers of azoles against infections caused by *Aspergillus*. Recent murine *Candida* pharmacodynamic models have indicated that the pharmacodynamic driver most likely to predict the outcome of azoles is the AUC/MIC ratio[10] in agreement with a murine model of candidiasis with BAL8557.[11] The GM MIC of BAL4815 for *Aspergillus* strains was 0.62 mg/L with an average AUC/MIC ratio in the range 23.3–66.7 after treatment with 50–100 mg/day. The AUC/MIC ratio required to predict success of therapy has not yet been established for *Aspergillus* infections but in *Candida* infections target ratios of free drug in excess of 20–25 are associated with treatment efficacy (in experimental *in vivo* models).[12] Such large AUC/MIC ratios might not be necessary in *Aspergillus* therapy; preliminary encouraging results from an *in vivo* efficacy study in mice were observed with BAL4815 levels above but close to the MIC.[13] Therefore it seems probable that after treatment with BAL8557 there will be sufficient exposure to BAL4815 to have a favourable outcome.

BAL4815 demonstrates promising antifungal activity *in vitro* warranting further *in vivo* investigation. Additionally at the doses evaluated in Phase I studies, efficacy against invasive aspergillosis is probable after either oral or intravenous administration. BAL4815 is now entering Phase III clinical trials.

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## Transparency declarations

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