

# *In vivo* synergism of free miltefosine or in alginate-based nanocarrier combined with voriconazole on aspergillosis

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**Aim:** To evaluate the activity of miltefosine (MFS), in its free form or loaded-alginate nanoparticles (MFS-AN), alone or combined with voriconazole (VRC) on *Aspergillus fumigatus* and *Aspergillus flavus*. **Materials & methods:** A broth microdilution assay was used for the susceptibility testing of *Aspergillus* isolates, and the antifungal efficacy was assessed using the aspergillosis model in *Galleria mellonella* larvae. **Results:** The *in vitro* synergistic effect of MFS with VRC was observed only against *A. fumigatus*, whereas both combined therapies (MFS + VRC and MFS-AN + VRC) showed synergism in reducing the larval mortality rate and fungal burden in the larvae infected by *A. fumigatus* and *A. flavus*. **Conclusions:** MFS and MFS-AN combined with VRC may be an important strategy for improving antifungal therapy against aspergillosis.

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*Aspergillus* spp. are important human pathogens, and the most common species is *Aspergillus fumigatus* in ~90% of aspergillosis cases, followed by *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans* and *Aspergillus terreus* [1,2]. Despite the higher global prevalence of *A. fumigatus*, there is an increase in the number of clinical cases of aspergillosis by *A. flavus*, mainly in tropical countries [3]. Aspergillosis is often associated with opportunistic infections [4] and the severe form, invasive pulmonary aspergillosis (IPA), leads to a high mortality rate of 50–95% [1].

The first-line treatment used for aspergillosis is voriconazole (VRC), but other antifungals can be employed, including amphotericin B deoxycholate (AMB) and its lipid formulations, itraconazole, posaconazole, isavuconazole and echinocandins [5]. Despite the effectiveness of azoles in IPA treatment, the number of resistant isolates has increased in recent years [6], and *A. flavus* has a known antifungal tolerance profile, with epidemiological cut-off values higher than those observed for *A. fumigatus* [3]. Other limitations are related to the conventional antifungals, such as a narrow spectrum of action, multiple side effects, drug–drug interactions, low bioavailability and the unavailability of some antifungals in many countries [5,7]. In this regard, resistance and antifungal limitations may result in high rates of therapeutic failures, [5] and in view of this, it is necessary to search for alternative strategies for aspergillosis treatment.

Miltefosine (MFS) is currently used in the treatment of skin metastases in patients with breast cancer and leishmaniasis [8]. Previous studies have demonstrated other pharmacological activities of MFS, including antifungal action against *Aspergillus* and other fungal species [8,9] and an *in vitro* synergistic effect when combined with azoles (VRC and posaconazole) against 20 pathogenic filamentous fungi [10–13]. However, MFS presents several toxic effects when orally administered [8], and encapsulation may be used to improve its distribution and reduce side effects [14]. Previously, our research group developed an alginate-based nanocarrier for MFS encapsulation (MFS-AN) that promoted a sustained drug release and reduced its toxicity, while maintaining antifungal efficacy *in vitro* and *in vivo* against *Candida* and *Cryptococcus* spp. [15–17].

The aim of this study was to evaluate the synergistic effect of MFS, in its free form (as solution) and encapsulated in alginate nanoparticles (MFS-AN), against *A. fumigatus* and *A. flavus* *in vitro* and *in vivo* using a *Galleria mellonella* infection model.

## Materials & methods

### Clinical isolates

*Aspergillus fumigatus* ATCC 16913 and other clinical isolates of *A. fumigatus* (n = 10) and *A. flavus* (n = 10) were maintained at -80°C in brain and heart infusion broth (Becton Dickinson and Company, NV, USA) with 20% glycerol, recovered on potato dextrose agar (Becton Dickinson and Company) and subcultured in the same medium at 35°C for 7 days before each assay.

### Drugs

AMB and VRC (both from Sigma-Aldrich, MO, USA) were dissolved in DMSO and MFS (Cayman Chemical Co., MI, USA) in sterile distilled water to obtain stock solutions 100-times concentrated that were maintained at -20°C. MFS-loaded alginate nanoparticles (MFS-AN) were produced as previously described [15], and the lyophilized powder was reconstituted with phosphate-buffered saline (PBS), pH 7.4, for the experiments.

### Antifungal susceptibility assay

The susceptibility of *Aspergillus* spp. clinical isolates to standard antifungals and MFS was determined using the broth microdilution technique [18]. The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration that inhibited 50% of the fungal growth (MIC-2); except for AMB, that was defined as the minimum concentration able to inhibit 90% of fungal growth (MIC-0). The antifungal susceptibility profile of clinical isolates was defined using the epidemiological cut-off values as previously described [19]. In addition, MIC<sub>50</sub> and MIC<sub>90</sub> values were determined and defined as the concentrations that inhibited 50 and 90% of all clinical isolates, respectively [19].

Using an inverted optical microscopy (magnification of 200×, DM750 Leica, São Paulo, Brazil), the minimum effective concentration (MEC) was determined as the lowest concentration that induced morphological alterations as thinner or shorter hyphae and alteration of the branching pattern, and untreated cells were used as a fungal growth control [20]. MEC<sub>50</sub> and MEC<sub>90</sub> values were defined as the concentrations that altered the fungal hyphae of 50 and 90% of all clinical isolates, respectively.

After MIC and MEC readings, a 10-μl aliquot of the inhibitory concentrations was plated on the drug-free potato dextrose agar plates and incubated at 35°C for 24 h to determine the minimum fungicidal concentration (MFC), defined as the lowest concentration that killed >99% of initial inoculum of fungal cells. The effect was considered fungicidal when MFC was  $\leq 4 \times$  MIC; otherwise, the effect was considered fungistatic [21]. Afterward, MFC<sub>50</sub> and MFC<sub>90</sub> were determined and defined as the concentrations that killed the fungal cells of 50 and 90% of all clinical isolates, respectively.

### Checkerboard assay

The *in vitro* combination of VRC and free MFS was evaluated on two representative clinical isolates of *Aspergillus* (*A. fumigatus* 1220 and *A. flavus* 998) using the checkerboard assay that several drug concentrations were combined for MIC determination, defined as the lowest drug concentrations in combination that inhibited 50% of the fungal growth by visual inspection [22]. The fractional inhibitory concentration index (FICI) was then calculated as the sum of fractional inhibitory concentration (FIC) of the VRC (MIC<sub>combined</sub>/MIC<sub>alone</sub>) with FIC of the MFS (MIC<sub>combined</sub>/MIC<sub>alone</sub>). FICI  $\leq 0.5$  indicates a synergistic effect; FICI >0.5 and <4 corresponds to indifference and FICI  $\geq 4$  indicates antagonism [22].

### Antifungal activity of miltefosine using *G. mellonella* model

Larvae of *G. mellonella* (length 2–2.5 cm and weight 150–200 mg) were infected with conidia of *A. fumigatus* 1220 or *A. flavus* 998, and monotherapies with VRC, MFS or MFS-AN and combined therapies with MFS + VRC or MFS-AN + VRC were assessed for evaluation of antifungal efficacy.

For infection, 10 μl of a conidia suspension of  $1 \times 10^7$  CFU/ml was inoculated into the last left proleg of the larvae. After 30 min, the larvae were treated with the following treatment regimens: AMB (20 or 40 mg/kg), VRC (10 or 20 mg/kg), MFS (20 or 40 mg/kg), MFS-AN (100 mg/kg); or the combinations MFS (20 mg/kg) + VRC (10 mg/kg) or MFS-AN (100 mg/kg) + VRC (10 mg/kg). Uninfected (PBS group) and infected and untreated larvae (untreated group) received only PBS and were included in the assay as control groups. The larvae were incubated at 35°C, and daily observations for up to 5 days were performed to determine survival and health index

**Table 1. Susceptibility of *Aspergillus fumigatus* and *Aspergillus flavus* clinical isolates to standard antifungals and to miltefosine (free-form and loaded alginate nanoparticles) by broth microdilution assay.**

Species	Drugs	MIC			MEC			MFC		
		MIC <sub>50</sub>	MIC <sub>90</sub>	Range	MEC <sub>50</sub>	MEC <sub>90</sub>	Range	MFC <sub>50</sub>	MFC <sub>90</sub>	Range
<i>A. fumigatus</i>	AMB	0.12	0.12	0.06–0.25	0.12	0.25	0.06–0.25	0.50	1	0.25–1
	VRC	0.25	0.50	0.12–0.50	1	2	0.50–2	0.50	1	0.25–1
	MFS	2	2	0.50–2	2	2	0.50–>64	2	8	1–8
	MFS-AN	600	600	18.7–600	18.7	37.5	4.7–37.5	>600	>600	>600
<i>A. flavus</i>	AMB	1	1	0.12–1	1	2	0.12–2	8	16	0.25–>16
	VRC	0.25	0.25	0.06–0.25	2	2	0.50–2	4	16	2–>16
	MFS	2	16	2–16	1	16	0.50–16	>64	>64	16–>64
	MFS-AN	600	600	300–600	150	300	18.7–300	>600	>600	>600

Concentration values are displayed in µg/ml. MIC<sub>50</sub>/MEC<sub>50</sub>/MFC<sub>50</sub> and MIC<sub>90</sub>/MEC<sub>90</sub>/MFC<sub>90</sub> values mean the lowest concentrations that inhibited/altered the hyphae/killed 50 and 90% of all isolates, respectively (*A. fumigatus*, n = 11 and *A. flavus*, n = 10). The assays were performed three times, in duplicate.

AMB: Amphotericin B; MEC: Minimum effective concentration; MFC: Minimum fungicidal concentration; MFS: Miltefosine; MFS-AN: Miltefosine encapsulated in alginate nanoparticles; MIC: Minimum inhibitory concentration; VRC: Voriconazole.

as previously described [23]. This assay was performed in three independent replicates and 20 larvae were used for each group.

After 24 h of infection, the fungal burden by determination of the colony-forming unit by g of larvae (CFU/g) and histopathological analysis of the larval tissues were assessed as described previously [15]. The criteria for semiquantitative analysis of histological sections was the number of fungal elements viewed per field, according to the classification: 0 = none, + = limited, ++ = medium, +++ = high, and ++++ = very high [24]. In addition, we observed the spread of fungal structures throughout the tissue, the number of granulomas containing these elements and the presence of asexual reproduction structures (conidiophores, phialides and conidia).

### Statistical analysis

The statistical analysis was performed using the software Prism 8.0 (GraphPad, CA, USA) and p-values <0.05 were considered statistically significant.

## Results

### MFS inhibits *A. fumigatus* & *A. flavus* growth

*A. flavus* clinical isolates exhibited less susceptibility to AMB and VRC compared with *A. fumigatus*, but all isolates were considered susceptible. A similar profile was observed for MFS and MFS-AN, with MICs ranging from 0.5 to 16 µg/ml for MFS, whereas MFS-AN reached MIC values ranging from 18.7 to 600 µg/ml (Table 1 & Supplemental Tables 1 & 2). The hyphae morphological alterations were observed for all treatments and MEC values were similar to MICs when fungi were treated with AMB, VRC and MFS; however, lower MECs were obtained for MFS-AN (4.7–300 µg/ml). The best antifungal was VRC against both *Aspergillus* spp., but interestingly AMB and MFS had similar MIC<sub>50</sub>/MIC<sub>90</sub> and MEC<sub>50</sub>/MEC<sub>90</sub> values (Table 1). MFS and VRC were fungicidal against all *A. fumigatus* isolates (except for strain 1343), whereas AMB were fungicidal for only 50% of the isolates. However, MFS and VRC were fungistatic against *A. flavus*, and AMB was fungicidal for 40% of the isolates (Table 1 & Supplemental Tables 1 & 2). To perform the next tests, *A. fumigatus* 1220 and *A. flavus* 998 isolates were chosen for their susceptibility profiles to represent majority of the clinical isolates tested.

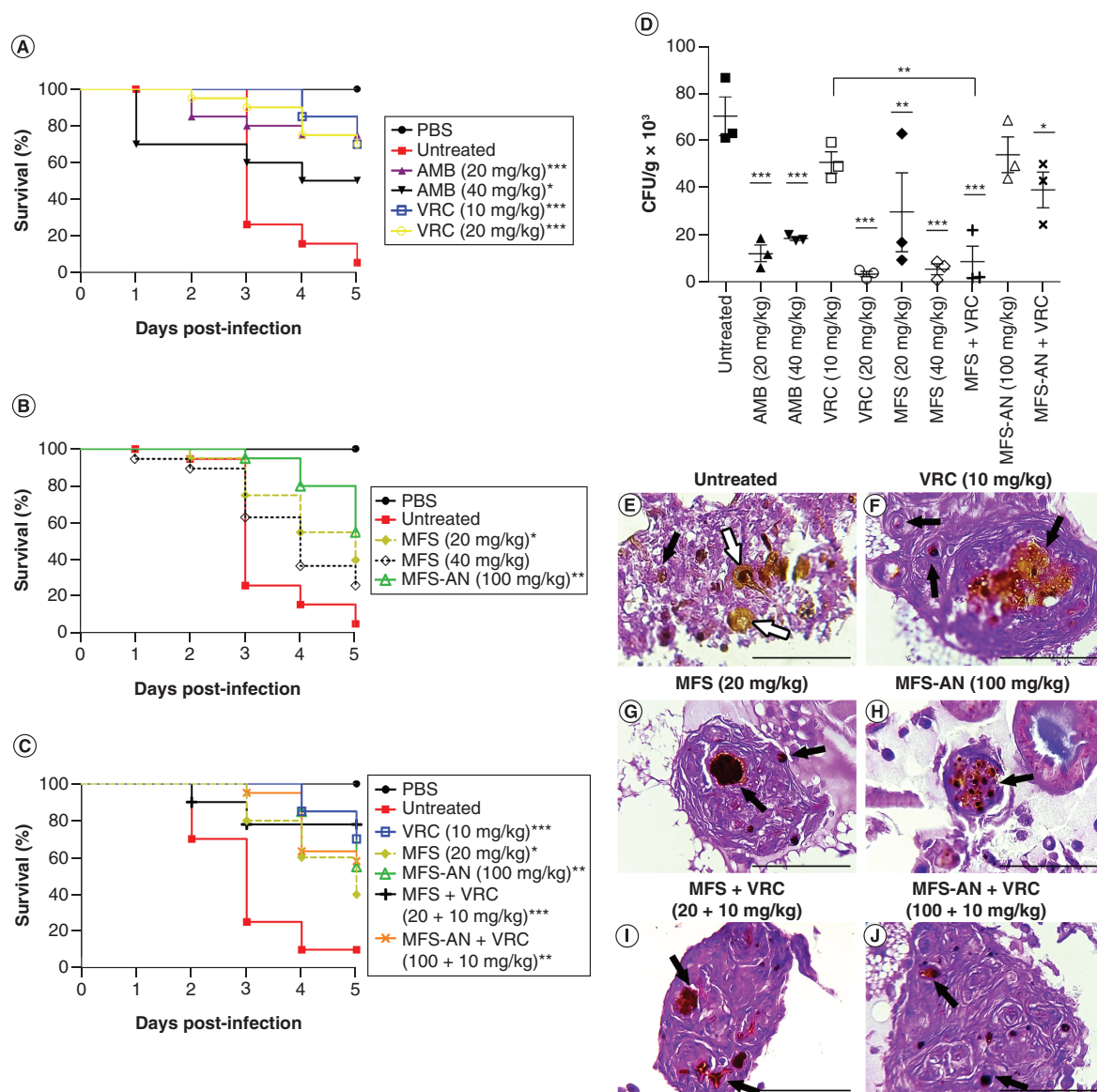
### *In vitro* synergism of MFS combined with VRC on *A. fumigatus*

Synergistic interaction of VRC with free MFS was observed against *A. fumigatus* 1220 strain (FICI = 0.24–0.37) with a reduction of eightfold MICs for both drugs. In contrast, none of combinations tested against *A. flavus* 998 clinical isolate showed synergism (FICI = 0.62–1.25; Supplemental Table 3).

### MFS has antifungal effect & synergism with VRC in an aspergillosis model using *G. mellonella*

#### *A. fumigatus*

The clinical isolate (1220) led to ~100% larval mortality on the fifth day postinfection, whereas all treatments with AMB (20 and 40 mg/kg) and VRC (10 and 20 mg/kg) were statistically efficient to enhance the survival, at



**Figure 1. Antifungal efficacy of miltefosine, in its free form or loaded-alginate nanoparticles (MFS-AN, alone or combined with voriconazole (VRC) on *Galleria mellonella* larvae infected with *Aspergillus fumigatus*.** Survival curves (A–C), fungal burden (D) and histopathological analysis (E–J) of *G. mellonella* larvae infected with  $1 \times 10^5$  conidia/larvae of *A. fumigatus* 1220 clinical isolate and treated with AMB, VRC, free MFS or MFS-AN, and the combinations MFS (20 mg/kg) + VRC (10 mg/kg) or MFS-AN (100 mg/kg) + VRC (10 mg/kg). The brackets indicate statistical analysis between the treatments (one-way analysis of variance followed by the Dunnett’s test). Fungal burden data are represented by the mean  $\pm$  standard deviation. Histological sections of larval tissue were observed at optical microscopy (400 $\times$  magnification). Black arrows indicate fungal cells (hyphae or conidia), and white arrows show asexual reproduction structures. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  compared with the untreated group. AMB: Amphotericin B; Bars: 50  $\mu$ m; VRC: Voriconazole; MFS: Miltefosine; MFS-AN: MFS encapsulated in alginate nanoparticles.

50–75%, of the infected larvae ( $p < 0.05$ ; Figure 1A). MFS at 20 mg/kg led to 40% larval survival on the fifth day postinfection, and MFS-AN resulted in the survival rate of 55% ( $p < 0.05$ ; Figure 1B). In addition, the larvae health index corroborated the survival data, with a statistical difference in the groups treated with AMB and VRC at both doses ( $p < 0.001$ ), MFS at 20 mg/kg ( $p < 0.01$ ) and MFS-AN ( $p < 0.05$ ; Supplemental Figure 1). Both doses of AMB reduced the fungal burden ( $p < 0.001$ ), whereas only at the higher dose VRC (20 mg/kg) was able to significantly reduce the fungal burden ( $p < 0.001$ ; Figure 1D). Treatment with free MFS at both doses also



reduced the fungal burden ( $p < 0.01$ ). Although MFS-AN (100 mg/kg) significantly increased the larval survival, the fungal burden was not significantly different from the untreated group (Figure 1D).

The combination of MFS + VRC significantly increased the survival rate of larvae infected with *A. fumigatus* 1220 compared with the untreated group ( $p < 0.001$ ); although not statistically significant, it was slightly better than MFS monotherapy (survival rates: 70–78% vs 40 %) and similar to the larval survival profile in the VRC monotherapy (Figure 1C). The larvae treated with MFS-AN + VRC displayed a similar survival profile of treatments with MFS-AN or VRC monotherapies (Figure 1C). Moreover, MFS + VRC, at the lowest dose for both, led to a significant reduction of the fungal burden in larvae infected with *A. fumigatus* ( $p < 0.001$ ) as well as for combination of MFS-AN + VRC ( $p < 0.05$ ). The combination MFS + VRC was better than VRC monotherapy ( $p < 0.01$ ; Figure 1D).

The histopathological semiquantitative analysis of the fungal burden in the larval tissue infected with *A. fumigatus* and treated with standard antifungals corroborated the CFU counting data – that is, the higher tested doses (AMB 40 mg/kg or VRC 20 mg/kg) were efficient in reducing the tissue fungal count. There was an evident reduction in fungal burden and granuloma formation in the groups treated with MFS at both doses and MFS-AN (100 mg/kg) compared with the untreated group (Figures 1E–J; Supplemental Table 4). In addition, fungal burden in the larval tissue was similar in the tested combinations (MFS + VRC and MFS-AN + VRC) compared with the monotherapies (Figures 1E–J; Supplemental Table 4).

### *Aspergillus flavus*

Larvae infected with *A. flavus* 998 and treated with AMB or VRC, at all tested doses, had a similar survival rate (~30 %) at the end of the experiment, but neither treatment was statistically different from the untreated group, which reached ~100 % mortality rate on the second day postinfection (Figure 2A). The survival curves of the larvae treated with MFS or MFS-AN were similar to the untreated group, showing that there was no efficacy at any dose used (Figure 2B). Remarkably, only the combination of MFS-AN + VRC was efficient in significantly increasing the survival rate ( $p < 0.001$ ; Figure 2C) and improving the larval health index ( $p < 0.001$ ; Supplemental Figure 1). Interestingly, the combination was significantly more efficient than MFS-AN monotherapy at reducing the larvae mortality ( $p < 0.001$ ; Figure 2C) and slightly better than VRC (10 mg/kg), although there were no statistical differences (Figure 2C).

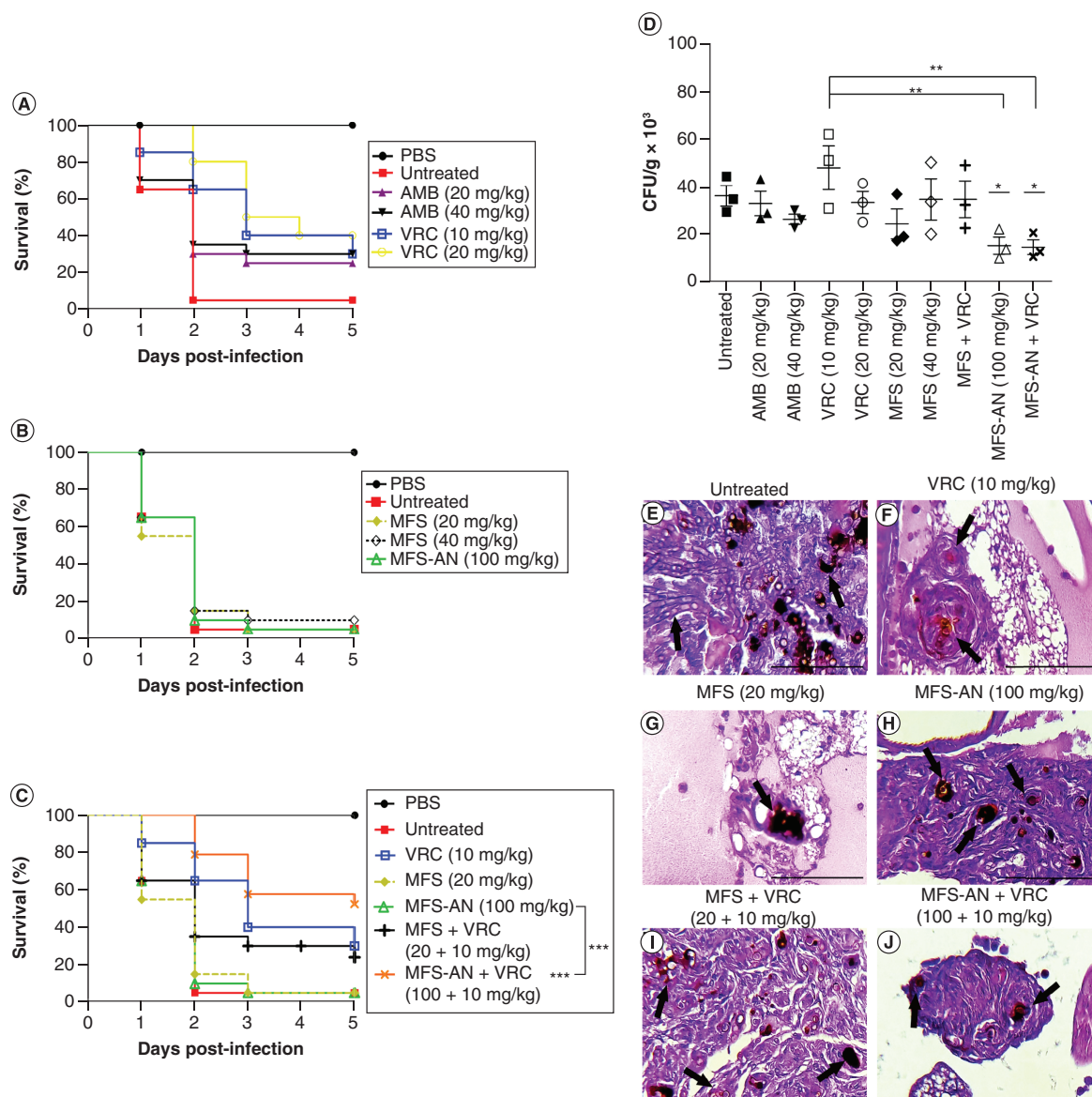
None of conventional antifungal therapies or free MFS significantly reduced the fungal burden of the larval tissues after infection by *A. flavus*, but an important reduction in fungal burden was observed in the groups treated with MFS-AN or MFS-AN + VRC ( $p < 0.05$ ; Figure 2D). The combination MFS-AN + VRC showed a significant reduction in the CFU count compared with VRC (10 mg/kg) ( $p < 0.01$ ; Figure 2D).

Notably, the greatest antifungal efficacy was observed with combined therapy (MFS-AN + VRC) in terms of reduced mortality rate and fungal burden (Figure 2C–D). In the histological analysis, a slight reduction in *A. flavus* fungal elements in the larval tissue in the groups treated with free MFS and MFS-AN (100 mg/kg), combined or not with VRC, were observed compared with the untreated group, corresponding to the CFU count (Figures 2E–J, Supplemental Table 4).

## Discussion

MFS showed inhibitory activity *in vitro* against *A. fumigatus* (MICs: 0.5–2 µg/ml) and *A. flavus* (MICs: 2–16 µg/ml) corroborating the data previously found against both *Aspergillus* species and other filamentous fungi as well as the fungicidal effect [9–11]. The observation of the *in vitro* inhibitory effect of MFS-AN against *Aspergillus* spp. isolates is novel, despite higher MIC values compared with free MFS. This behavior is due to the MFS sustained- and slow-release profile promoted by the nanocarrier system [15]. The great advantage of this alginate-based nanocarrier is the reduction of MFS side effects and frequency of administration, which allows higher dosage and promotes antifungal effects when it is administered in an *in vivo* animal model [15–17], highlighting the relevant potential of this nanocarrier system in the treatment of fungal diseases.

The great potential of MFS or MFS-AN monotherapies has been demonstrated for mycosis treatment. The antifungal efficacy of both free MFS or MFS-AN in a *G. mellonella* model against *Candida albicans*, *Cryptococcus neoformans* and *Cryptococcus gattii* has already been demonstrated, with improvement of survival rates [15]. *In vitro* and *in vivo* activity of free MFS and MFS-AN against *Candida auris* clinical isolates, an emerging and resistant fungal species, was recently described [17]. Here, we highlight that free MFS and MFS-AN increased the survival rate and/or led to a fungal burden reduction in *G. mellonella* larvae infected by *A. fumigatus* and *A. flavus*. Importantly,



**Figure 2.** Antifungal efficacy of miltefosine (MFS), in its free form or loaded-alginate nanoparticles (MFS-AN, alone or combined with voriconazole (VRC) on *Galleria mellonella* larvae infected with *Aspergillus flavus*. Survival curves (A–C), fungal burden (D) and histopathological analysis (E–J) of *G. mellonella* larvae infected with  $1 \times 10^5$  conidia/larvae of *Aspergillus flavus* 998 clinical isolate and treated with AMB, VRC, free MFS or MFS-AN and the combinations MFS (20 mg/kg) + VRC (10 mg/kg) or MFS-AN (100 mg/kg) + VRC (10 mg/kg). Brackets indicate statistical analysis between treatments (one-way analysis of variance followed by the Dunnett's test). Fungal burden data are represented by the mean  $\pm$  standard deviation. Histological sections of larval tissue were observed at optical microscopy (400 $\times$  magnification). Black arrows indicate fungal cells (hyphae or conidia). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  compared with the untreated group. AMB: Amphotericin B; Bars: 50  $\mu\text{m}$ ; MFS: Miltefosine; MFS-AN: MFS encapsulated in alginate nanoparticles VRC: Voriconazole.

in a murine model of vaginal candidiasis, free MFS (2%, once a day for 5 days) and MFS-AN (2% MFS, single dose) were able to control the fungal infection [16] showing the potential use of MFS to treat fungal infections.

We tested combined therapy of MFS, in solution or encapsulated in the alginate nanoparticles, with VRC using a *G. mellonella* invertebrate model of aspergillosis considering the *in vitro* synergistic effects observed in our work and previously described against *A. fumigatus* [11]; we also compared the combined therapies with their respective monotherapies. MFS or MFS-AN, when combined with VRC, led to a reduction of larval mortality and fungal

burden, and this was slightly better than monotherapies against *A. fumigatus*. Remarkably, MFS + VRC and MFS-AN alone or combined with VRC reduced the fungal burden and improved the survival of the larvae infected by *A. flavus*, in contrast to therapeutic failure using MFS or VRC monotherapies. In fact, combined therapy produced the most pronounced increase in survival rate and reduction in fungal burden, indicating a synergistic effect of these drugs.

Three clinical cases have been reported using free MFS as salvage therapy in invasive infections by the multidrug-resistant *Lomentospora prolificans* when combined with VRC and/or terbinafine; there was therapeutic success in all cases [25–27]. To support this idea, the Infectious Diseases Society of America practice guideline for aspergillosis recommends the combination of antifungals (polyenes or azoles with echinocandins) that showed synergistic effects in most preclinical studies, and the combinations can be recommended for IPA treatment in patients with tolerance to monotherapy or salvage therapy [5]. The use of combined therapy has advantages that include a reduction in dosage for a drug that can be associated with considerable side effects and additive activity of drugs to increase efficiency and reduce duration of treatment [28]. In this regard, the use of combined therapy toward resistant or less susceptible isolates can reduce the risk of failure in monotherapy.

## Conclusion

To our knowledge, no previous study has demonstrated antifungal effects of MFS alone or combined with conventional antifungals in an animal infection model by filamentous fungi. Our data show that MFS, in solution or encapsulated in the alginate-based nanocarrier, alone or combined with VRC, has potential as an alternative in the treatment of fungal infections by *Aspergillus* spp., improving therapeutic success.

### Summary points

- Miltefosine, in free solution or loaded alginate nanoparticles, combined with voriconazole showed *in vivo* synergistic effects on the larvae of *Galleria mellonella* infected with *Aspergillus fumigatus* or *Aspergillus flavus*.
- The combined therapy reduced fungal burden and improved larvae health, leading to a significant increase of larval survival rate.
- Combined therapy was more effective in the treatment of aspergillosis than the monotherapies.

### Author contributions

TL Barreto, LB Lopes, ASA Melo and K Ishida were responsible for conceptualization, methodology, validation, formal analysis, writing the original draft and reviewing the manuscript. LB Lopes and K Ishida were responsible by funding acquisition. All authors approved the final version of the manuscript.

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## References

1. Paulussen C, Hallsworth JE, Álvarez-Pérez S *et al*. Ecology of aspergillosis: insights into the pathogenic potency of *Aspergillus fumigatus* and some other *Aspergillus* species. *Microb. Biotechnol.* 10(2), 296–322 (2017).
2. Shishodia SK, Tiwari S, Shankar J. Resistance mechanism and proteins in *Aspergillus* species against antifungal agents. *Mycology.* 10(3), 151–165 (2019).

3. Rudramurthy SM, Paul RA, Chakrabarti A *et al.* Invasive aspergillosis by *Aspergillus flavus*: epidemiology, diagnosis, antifungal resistance, and management. *J. Fungi (Basel)*. 5(3), 55 (2019).
4. Kousha M, Tadi R, Soubani AO. Pulmonary aspergillosis: a clinical review. *Eur. Respir. Rev.* 20(121), 156–174 (2011).
5. Patterson TF, Thompson GR 3rd, Denning DW *et al.* Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* 63(4), e1–e60 (2016).
6. Sanglard D. Emerging threats in antifungal-resistant fungal pathogens. *Front. Med.* 3, 11 (2016).
7. Global Action Fund for Fungal Infections (GAFFI). Antifungal drug maps (2020). [www.gaffi.org/antifungal-drug-maps/](http://www.gaffi.org/antifungal-drug-maps/)
8. Dorlo TPC, Balasegaram M, Beijnen JH, Vries PJ. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *J. Antimicrob. Chemother.* 67(11), 2576–2597 (2012).
9. Widmer F, Wright LC, Obando D *et al.* Hexadecylphosphocholine (miltefosine) has broad-spectrum fungicidal activity and is efficacious in a mouse model of cryptococcosis. *Antimicrob. Agents Chemother.* 50(2), 414–421 (2006).
10. Biswas C, Sorrell TC, Djordjevic JT *et al.* *In vitro* activity of miltefosine as a single agent and in combination with voriconazole or posaconazole against uncommon filamentous fungal pathogens. *J. Antimicrob. Chemother.* 68(12), 2842–2846 (2013).
11. Imbert S, Palous M, Meyer I *et al.* *In vitro* combination of voriconazole and miltefosine against clinically relevant molds. *Antimicrob. Agents Chemother.* 58(11), 6996–6998 (2014).
12. Compain F, Botterel F, Sitterlé E *et al.* *In vitro* activity of miltefosine in combination with voriconazole or amphotericin B against clinical isolates of *Scedosporium* spp. *J. Med. Microbiol.* 64(3), 309–311 (2015).
13. Wu Y, Totten M, Memon W *et al.* *In vitro* antifungal susceptibility of miltefosine alone and in combination with amphotericin B against emerging multidrug resistant *Candida auris*. *Antimicrob. Agents Chemother.* 64(2), e02063–19 (2020).
14. Kavian Z, Alavizadeh SH, Golmohamadzadeh S *et al.* Development of topical liposomes containing miltefosine for the treatment of *Leishmania* major infection in susceptible BALB/c mice. *Acta Trop.* 196, 142–149 (2019).
15. Spadari CC, De Bastiani FWMS, Lopes LB, Ishida K. Alginate nanoparticles as non-toxic delivery system for miltefosine in the treatment of candidiasis and cryptococcosis. *Int. J. Nanomed.* 14, 5187–5199 (2019).
16. de Bastiani FWMS, Spadari CC, de Matos JKR *et al.* Nanocarriers provide sustained antifungal activity for amphotericin B and miltefosine in the topical treatment of murine vaginal candidiasis. *Front. Microbiol.* 10, 2976 (2020).
17. Barreto TL, Rossato L, Freitas ALD *et al.* Miltefosine as an alternative strategy in the treatment of the emerging fungus *Candida auris*. *Int. J. Antimicrob. Agents.* 56, 106049 (2020).
18. CLSI. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. *Clinical and Laboratory Standards Institute* (3rd Edition). 1–62 (2017).
19. CLSI. Epidemiological cut-off values for antifungal susceptibility testing. *Clinical and Laboratory Standards Institute* (2nd Edition). 1–12 (2018).
20. Shalit I, Shadkchan Y, Samra Z, Osherov N. *In vitro* synergy of caspofungin and itraconazole against *Aspergillus* spp.: MIC versus minimum effective concentrations end points. *Antimicrob. Agents Chemother.* 47(4), 1416–1418 (2003).
21. Espinel-Ingroff A, Fothergill A, Peter J *et al.* Testing conditions for determination of minimum fungicidal concentrations of new and established antifungal agents for *Aspergillus* spp.: NCCLS collaborative study. *J. Clin. Microbiol.* 40(9), 3204–3208 (2002).
22. Philip A, Odabasi Z, Rodriguez J *et al.* *In vitro* synergy testing of anidulafungin with itraconazole, voriconazole, and amphotericin B against *Aspergillus* spp. and *Fusarium* spp. *Antimicrob. Agents Chemother.* 49(8), 3572–3574 (2005).
23. Loh JM, Adenwalla N, Wiles S, Proft T. *Galleria mellonella* larvae as an infection model for group A streptococcus. *Virulence.* 4(5), 419–428 (2013).
24. Quintella LP, Passos SR, do Vale AC *et al.* Histopathology of cutaneous sporotrichosis in Rio de Janeiro: a series of 119 consecutive cases. *J. Cutan. Pathol.* 38, 25–32 (2011).
25. Kesson AM, Bellemore MC, O'Mara TJ *et al.* *Scedosporium prolificans* osteomyelitis in an immunocompetent child treated with a novel agent, hexadecylphosphocholine (Miltefosine), in combination with terbinafine and voriconazole: a case report. *Clin. Infect. Dis.* 48(9), 1257–1261 (2009).
26. Trubiano JA, Paratz E, Wolf M *et al.* Disseminated *Scedosporium prolificans* infection in a 'extensive metaboliser': navigating the minefield of drug interactions and pharmacogenomics. *Mycoses.* 57(9), 572–576 (2014).
27. Quaesaet L, Stindel E, Lantermier F *et al.* Miltefosine-based regimen as salvage therapy in *Lomentospora prolificans* bone and joint infection. *Med. Mal. Infect.* 48(1), 63–65 (2018).
28. Livengood SJ, Drew RH, Perfect JR. Combination therapy for invasive fungal infections. *Curr. Fungal Infect. Rep.* 14, 40–49 (2020).