F-762

Bifunctional Small Molecule Immunotherapy: C-001 and C-016 Attract Neutrophils (PMNs) to Inhibit *Aspergillus fumigatus* (Af) Growth in Microfluidic Chambers

James M. Balkovec, Ph.D.
Cidara Therapeutics, Inc.
6310 Nancy Ridge Drive, Suite 101
San Diego, CA 92121
jbalkovec@cidara.com

C. Jones,[1] K.M. Forrest,[2] J.K. Judice,[2] J.B. Locke,[2] A. Almaguer,[2] D. Irimia,[1] **J.M. Balkovec**[2]

[1]Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA; [2]Cidara Therapeutics, Inc., San Diego, CA, USA

ABSTRACT (Amended)

Aspergillosis is difficult to treat and carries a high mortality rate in immunocompromised patients. Neutrophils (PMNs) play a crucial role in control of infection but may be diminished in number and function with immunosuppressive therapies.

Our Cloudbreak™ bifunctional small molecule platform utilizes a targeting moiety (TM) that binds to the pathogen and is conjugated to an effector moiety (EM) that attracts and activates immune cells, directing them to the site of infection and priming them for killing. Two compounds in particular, C-001 and C-016, had an Af MEC/MIC of 0.5 and 2 µg/mL, resp. indicating good affinity for their fungal targets. Regarding PMN migration, both had EC_{max} values of 0.015 µg/mL.

Using microfluidic devices, we found that PMNs are weakly attracted to and modestly control the growth of Af hyphae (49%). C-001 produced a high influx of PMNs and reduced hyphal growth to 5%. Wells containing 0.15 µg/mL of C-016 plus PMNs were reduced to <1% growth. In control experiments, drug-free wells devoid of PMNs gave 82% growth, while those containing PMNs were reduced to 30%.

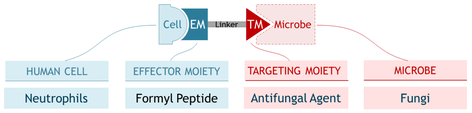
Conclusions: C-001 and C-016 bind to Af and attract and prime PMNs to effect enhanced killing of *A. fumigatus*.

INTRODUCTION

Invasive fungal infections (IFIs), especially aspergillosis, are a serious problem in the immune compromised host and continue to be associated with significant morbidity and mortality, thus requiring a need for new therapy.[1] Host immunity plays a crucial role in clearing the infection while agents that boost immune function have been found to provide additional benefit on top of standard of care therapies.[2]

Using agents that selectively bind to fungi (e.g., known antifungal agents) and chemotactic peptides (e.g., bacterial cell wall formylated peptides), small molecule conjugates were designed that would bind to fungi and attract and activate neutrophils (PMNs), bringing the two in close proximity (Figure 1).

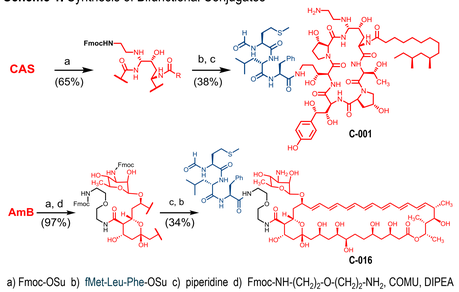
Figure 1. Design of Cloudbreak™ small molecule immunotherapy agents



METHODS

- **Compounds**: C-001 and C-016 were synthesized from caspofungin (CAS) and amphotericin B (AmB), respectively, according to Scheme 1 and had satisfactory NMR and HRMS. fMet-Leu-Phe (fMLF) was obtained commercially.

Scheme 1. Synthesis of Bifunctional Conjugates



a) Fmoc-OSu b) fMet-Leu-Phe-OSu c) piperidine d) Fmoc-NH-$(CH_2)_2$-O-$(CH_2)_2$-NH_2, COMU, DIPEA

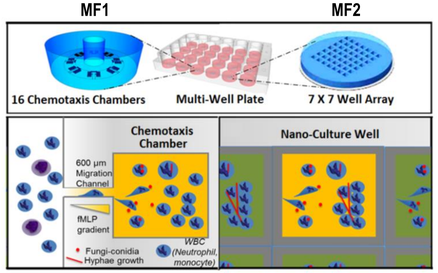
- **MICs/MECs**: Values were determined against *Aspergillus* spp. according to CLSI guidelines including the *A. fumigatus* strain used in the PMN killing studies (MYA-4609) with CAS and AmB as positive controls.
- **Transwell Migration**: Purified, fresh human PMNs (50,000 to 100,000) were placed over 5 µm pore–sized transwell plates in 24-well format with test compounds in the bottom chamber. After incubation at 37ºC for 45 min, migration was confirmed visually and quantitated by ATP detection with ATPlite (PerkinElmer).
- **Microfluidic Devices**: Depicted in Figure 2, were fabricated using standard microfabrication techniques as detailed in the reference.[3]
- **PMN Killing of *A. fumigatus***:

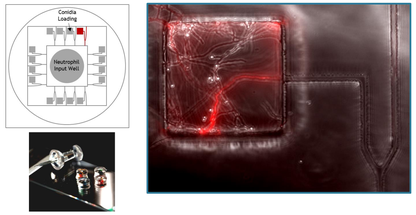
  MF1 Device: Focal chemotactic chambers (FCC) were loaded with *A. fumigatus* (MYA-4609) conidia (~16/chamber) expressing red fluorescent protein and C-001 at 0.0015 µg/mL (1 nM) in PBS. Conidia were allowed to germinate for approximately 7 hr. Next, purified, fresh human PMNs (4000 cells in 2 µL RPMI/10% FBS) were added to the central chamber. PMN migration through the channel toward the FCC started immediately and was recorded using time-lapse imaging on a Nikon TiE microscope (10× magnification) at 37ºC. Image analysis of cell migration counts and fungal growth were analyzed by hand using Image J software and observed for an additional 18 h.[3]

METHODS (cont'd)

MF2 Device: Well arrays (100 µm × 200 µm × 70 µm depth) were loaded with *A. fumigatus* (MYA-4609) conidia (~9 /well), C-016 at 0.15 µg/mL (100 nM) and fresh, purified PMNs (~100 /well). Fungal growth and the interactions with PMNs were monitored for 24 hours as described for the MF1 Device.

Figure 2. Details of microfluidic devices.





RESULTS

- **TM Engagement:** C-001 and C-016 demonstrated potent MEC/MIC values reflecting good affinity of each TM for its respective target, β-1,3-glucan synthase and ergosterol (Table 1).

Table 1. MIC/MEC values (µg/mL)[a] for conjugates and control compounds.

	A. fumigatus			*A. flavus*	*A. niger*
	MYA-4609	MYA-3626	ATCC 13073	MYA-3631	ATCC 16404
C-001	0.5	0.5	0.25	0.25	0.25
CAS	0.06	0.06	0.03	0.03	0.03
C-016	2	ND[b]	2	8	2
AmB	0.25	0.25	0.125	1	0.25

[a] median values (n=3-5) except for MYA-3626 (n=2).
[b] not determined.

RESULTS (cont'd)

- **EM Engagement**: C-001 and C-016 are potent chemotactic agents that activate the formyl peptide receptor and induce migration of human PMNs to a similar degree as fMLF (Figure 3). CAS and AmB were not chemotactic (data not shown).

Figure 3. Chemotaxis of human neutrophils in transwell migration assay.

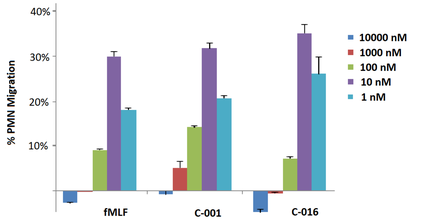
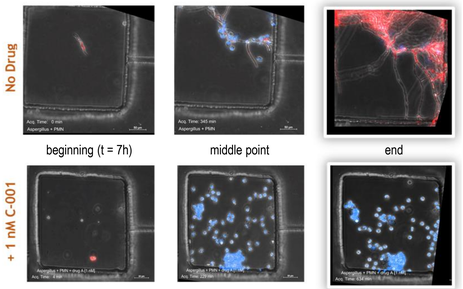
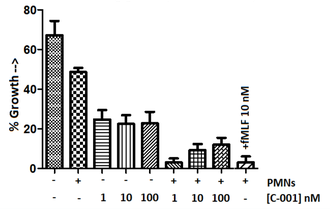


Figure 4. Enhanced killing of *A. fumigatus* (red) by human PMNs (blue) in the presence of C-001 at concentrations >300-fold below its MEC in microfluidic chambers. There is increased PMN infiltration into chambers containing C-001.



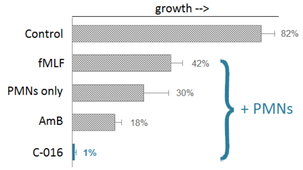
- **Chemotaxis Chamber Results:**

Figure 5. Growth of germinating *A. fumigatus* conidia ± human PMNs and C-001 at 300-, 30- and 3-fold below its MEC in FCCs (**MF1**). 10 nM CAS + PMNs was similar to C-001 alone in inhibiting growth (data not shown).



RESULTS (cont'd)

Figure 6. Growth of germinating *A. fumigatus* conidia in the presence of human PMNs plus C-016 (13-fold below its MIC), its EM (fMLF) and TM (AmB) at 100 nM uniform concentrations in the well array device (**MF2**).



CONCLUSIONS

- C-001 and C-016 demonstrate TM and EM engagement as shown by potent MECs/MICs against *Aspergillus* spp. and potent chemotaxis of human PMNs, respectively.
- C-001 and fMLF enhance the killing of *A. fumigatus* in the presence of PMNs in microfluidic chambers designed to establish a chemical gradient. Increased PMN infiltration correlates with enhanced killing of *A. fumigatus*.
- C-016 establishes a gradient at the fungal cell interface to attract and activate PMNs showing enhanced killing of *A. fumigatus*. C-016 is significantly more effective than fMLF+PMNs, AmB+PMNs or PMNs alone.
- Bifunctional small molecules represent promising immunotherapies for the treatment of aspergillosis and other IFIs. Further study of these agents is warranted.

REFERENCES

1. Denning DW, Bromley MJ. How to bolster the antifungal pipeline. *Science.* 2015;347:1414-6.
2. Segal BH. Aspergillosis. *N Engl J Med.* 2009;360:1870-84.
3. Jones CN, Irimia D, et al. Human neutrophils are primed by chemoattractant gradients for blocking the growth of *Aspergillus fumigatus* [published online August 15 2012]. *J Infect Dis.* 2015. doi: 10.1093/infdis/jiv419.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Drs. Kevin Li and Peter Martensen (Scynexis) for compound synthesis, Dr. Sailaja Battula (Applied Immunology) for Transwell Migration assay measurements, Mr. Grayson Hough (Cidara) for analytical assistance and Drs. H. Shaw Warren (MGH) and Martin Springer for helpful discussions.