# Supplemental Material

## Title

The Added Value of Longitudinal Imaging for Preclinical *In vivo* Efficacy Testing of Therapeutic Compounds against Cerebral Cryptococcosis

## Authors

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# Supplementary methods

## Generation of an E2-Crimson expressing *C. neoformans* KN99α strain

The strategy for generating a codon-optimized E2-Crimson expression construct was similar to that described for the codon-optimized red-shifted firefly luciferase (1). The protein sequence of the far-red fluorescent protein E2-Crimson (AMO27221) was used as a template to generate a codon-optimized gene sequence for expression in *C. neoformans*. The codon usage table and its accompanied di-codon adaptation index (dCAI) was deduced from a set of 40 highly expressed genes from *C. neoformans* H99 (2). The E2-Crimson protein sequence was then back-translated into a codon-optimized DNA sequence. To prevent problems of reduced mRNA accumulation from intron-less gene sequences in *C. neoformans* (3), the coding region was interrupted at position 120 by a 62 base pair (bp) intron sequence deriving from the *C. neoformans* glyceraldehyde-3-phosphate dehydrogenase gene. As this intron causes a frame shift and early termination of translation if not spliced from the transcript, only correctly processed mRNA results in the production of a functional fluorescent protein. To regulate gene expression, the synthetic E2-Crimson gene (GenBank accession number MN242783) was fused by *in vitro* recombination (InFusion HD cloning kit, Takara/Clontech) with a 1167 bp *EF1α* promoter and a 402 bp *TRP1* terminator sequence from *C. neoformans*.

The plasmid pNE562 was constructed by cloning the 2317 bp synthetic construct containing the optimized E2-Crimson encoding gene in the “safe haven” plasmid pSDMA25 (4) at the *SacI* restriction site. After linearization using the *Pac*I restriction enzyme, the pNE562 plasmid was integrated in the *C. neoformans* KN99α genome by biolistic transformation (5). The transformants were selected on nourseothricin containing medium (200 µg/mL). Correct integration at the safe haven site was

confirmed by multiplexed PCR using the primers UQ1768, UQ2962, UQ2963 and UQ3348, as previously described (4).

**Assessment of fluorescence in E2-Crimson-expressing cells**

E2-Crimson expressing *C. neoformans* cells were exposed to the same concentrations of antifungals described for the BLI experiments, in a black 96-well plate (Nunc® Microwell, Thermo Fisher Scientific, Merelbeke, Belgium). At 45 min, 3, 5 and 24 hours after addition of the antifungals, fluorescence was measured using an IVIS Spectrum imaging system with excitation/emission filters 605/660 nm, 30 sec exposure time, F/stop 2, medium binning and a subject height of 1 cm. The total radiant efficiency in a region of interest placed in every well was quantified using Living Image Software (version 4.5.4).

**Testing of potential auto-fluorescence and auto-luminescence of antifungals**

The background fluorescence and luminescence of the antifungal compounds (0.1 mg/ml), SDS (1%) or an equal volume sterile water was measured in sterile water and Sabouraud medium as described previously. Luminescence was measured before and after the addition of D-luciferin.

**Histology**

After a pentobarbital overdose, animals were transcardially perfused with saline and 4% paraformaldehyde (PFA, Sigma-Aldrich, Steinheim, Germany). Isolated brains were post-fixed overnight in 4% PFA. Paraffin sections (5 µm) were cut along the coronal axis. Sections were stained with periodic acid–Schiff (PAS) using orthoperiodic acid (AnalaR NORMAPUR®, VWR Prolabo, Haasrode, Belgium) and Schiff's reagent (Merck, Darmstadt, Germany). Sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). Slides were scanned using a

Mirax desk digital slide scanner (Carl Zeiss, Göttingen, Germany) and analyzed using Pannoramic Viewer (version 1.15.4, 3DHISTECH Ltd., Budapest, Hungary).

## Data availability

The genetic sequence of the synthetic E2-Crimson gene is available via GenBank under accession number MN242783.

## References


1. Vanherp L, Ristani A, Poelmans J, Hillen A, Lagrou K, Janbon G, Brock M, Himmelreich U, Vande Velde G. 2019. Sensitive bioluminescence imaging of fungal dissemination to the brain in mouse models of cryptococcosis. Dis Model Mech 12:dmm039123.

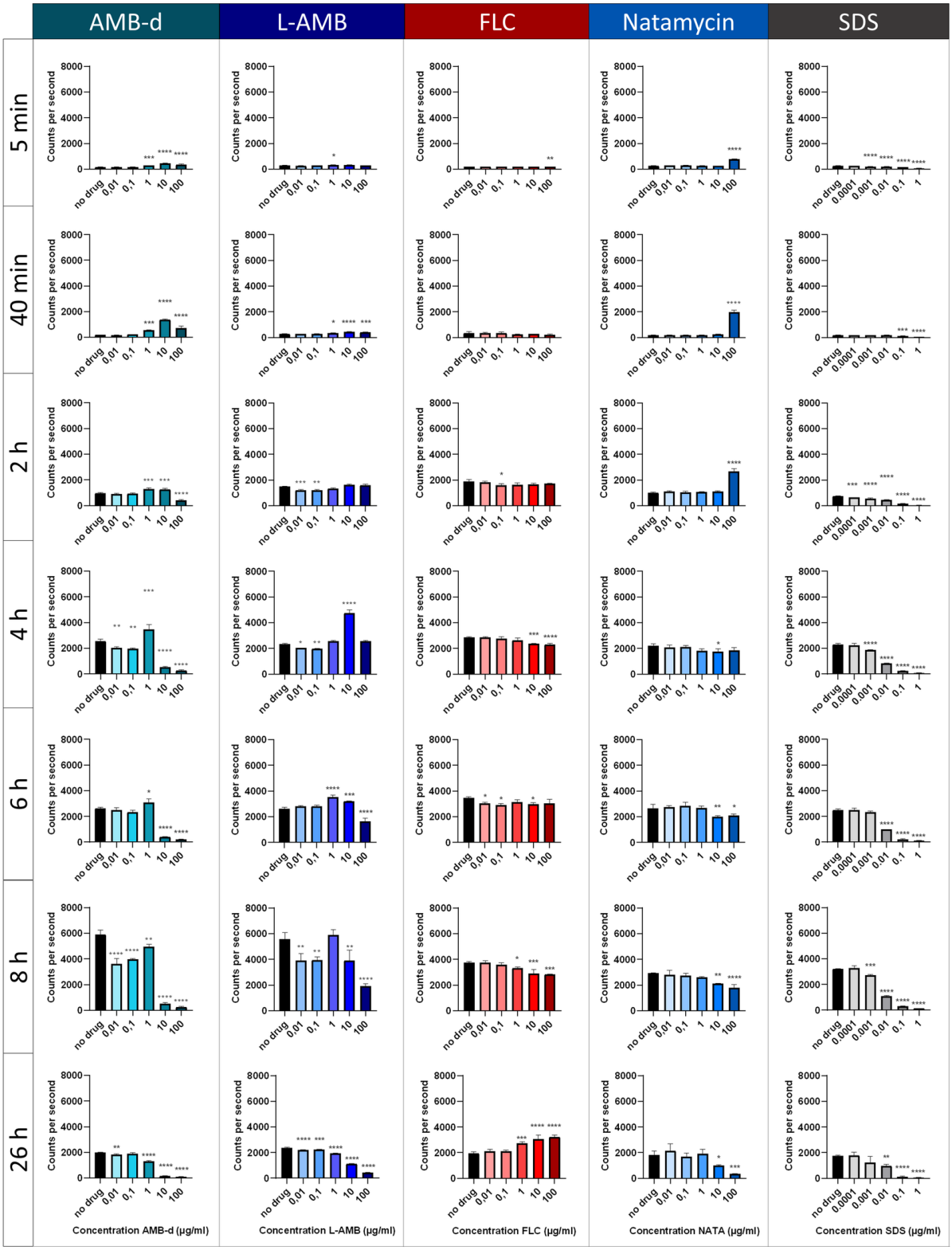
2. Jacobsen ID, Lüttich A, Kurzai O, Hube B, Brock M. 2014. In vivo imaging of disseminated murine *Candida albicans* infection reveals unexpected host sites of fungal persistence during antifungal therapy. J Antimicrob Chemother 69:2785–2796.

3. Goebels C, Thonn A, Gonzalez-Hilarion S, Rolland O, Moyrand F, Beilharz TH, Janbon G. 2013. Introns regulate gene expression in *Cryptococcus neoformans* in a Pab2p dependent pathway. PLoS Genet 9:e1003686.

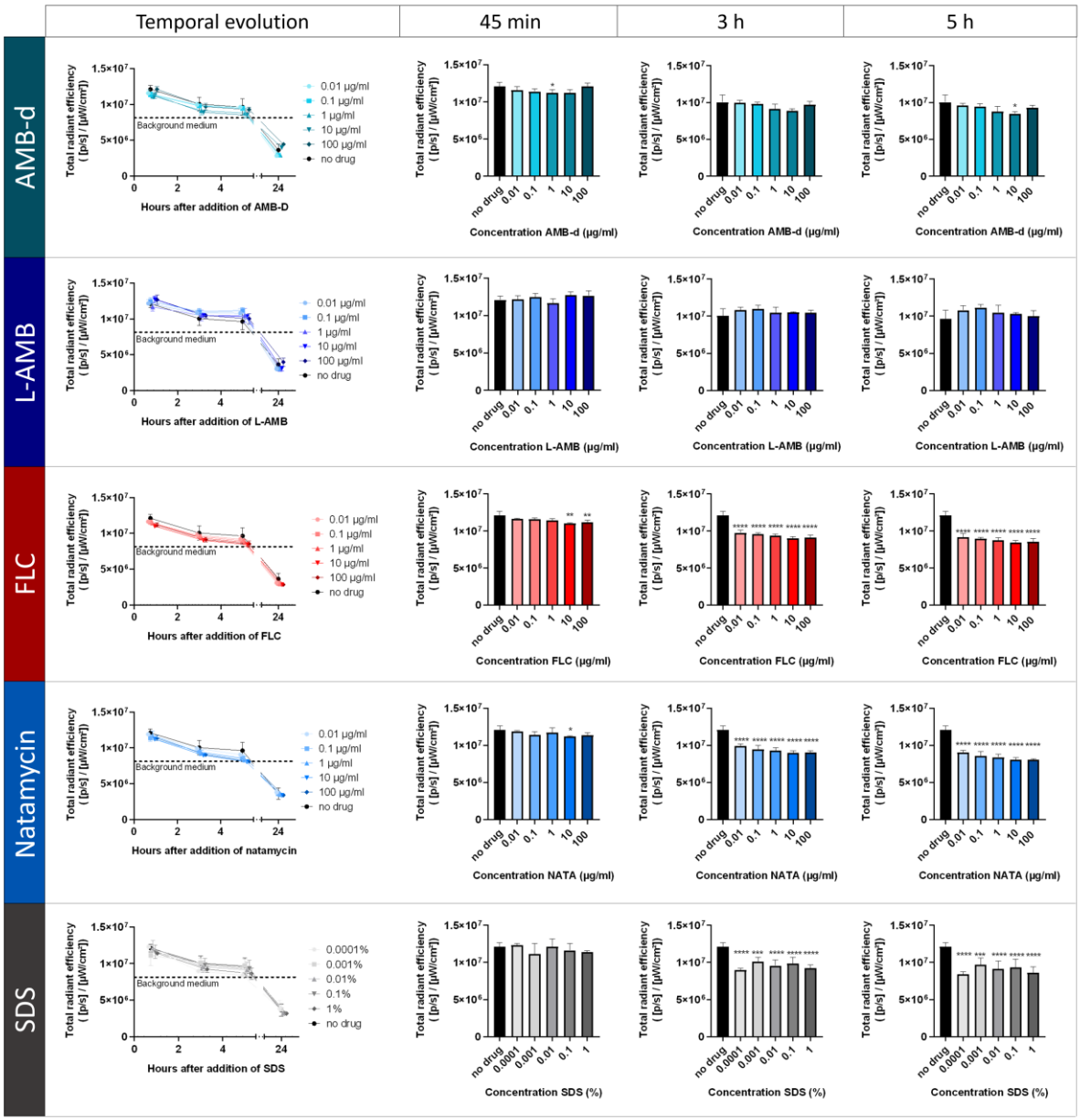
4. Arras SDM, Chitty JL, Blake KL, Schulz BL, Fraser JA. 2015. A genomic safe haven for mutant complementation in *Cryptococcus neoformans*. PLoS One 10:e0122916.

5. Toffaletti DL, Rude TH, Johnston SA, Durack DT, Perfect JR. 1993. Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. J Bacteriol 175:1405–11.

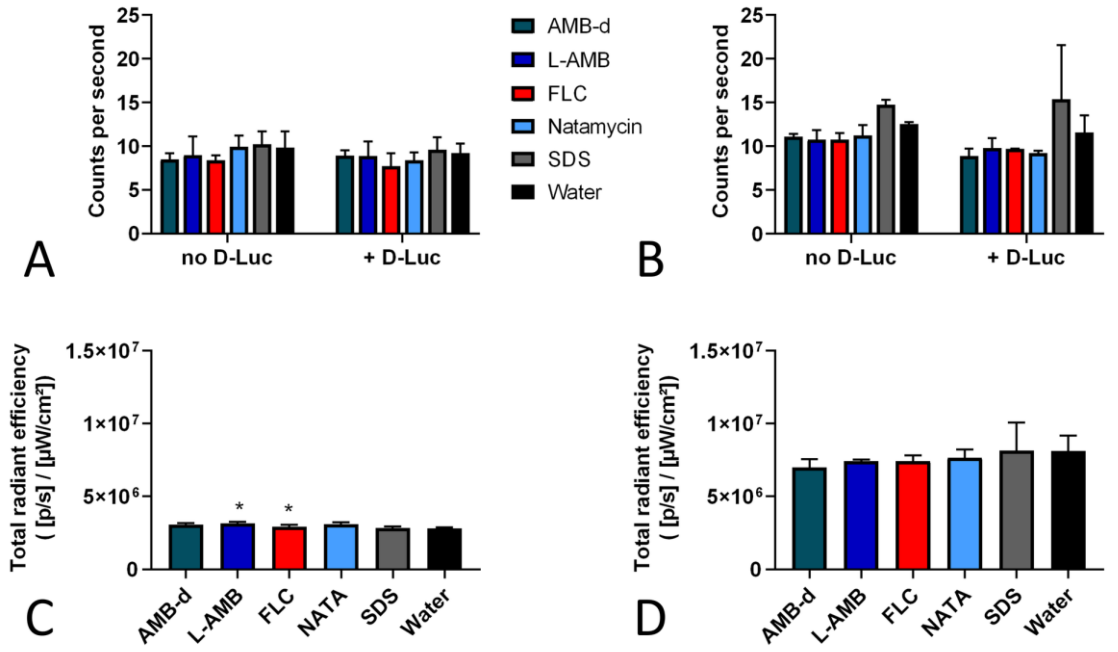
# Supplementary Figures



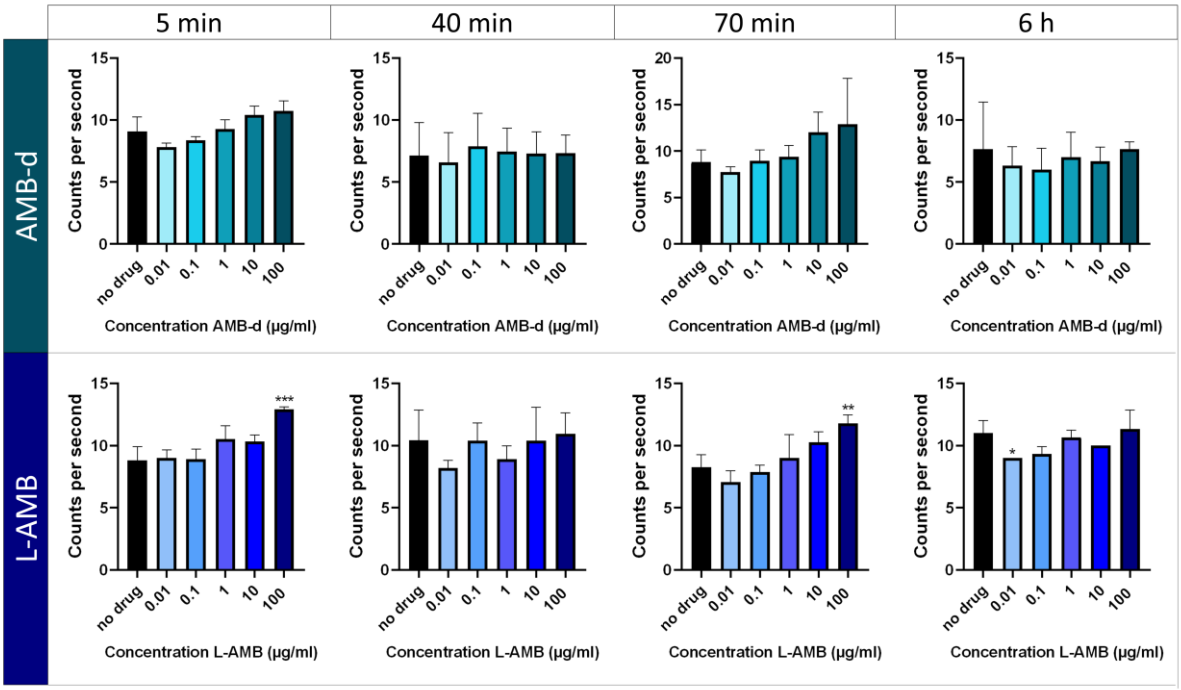
**Supplementary Figure S1. *In vitro* bioluminescence assay of antifungal efficacy at specific time points.** Amphotericin B deoxycholate (AMB-d), liposomal amphotericin B (L-AMB), fluconazole (FLC), natamycin (NATA) or sodium dodecyl sulphate (SDS) was added to liquid cultures and the BLI signal was measured by adding D-luciferin to samples of the culture at 5 min, 40 min, 2, 4, 6, 8 and 26 hours. Graphs show mean + SD, one-way ANOVA with Dunnett's post-test compared to no drug control. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.



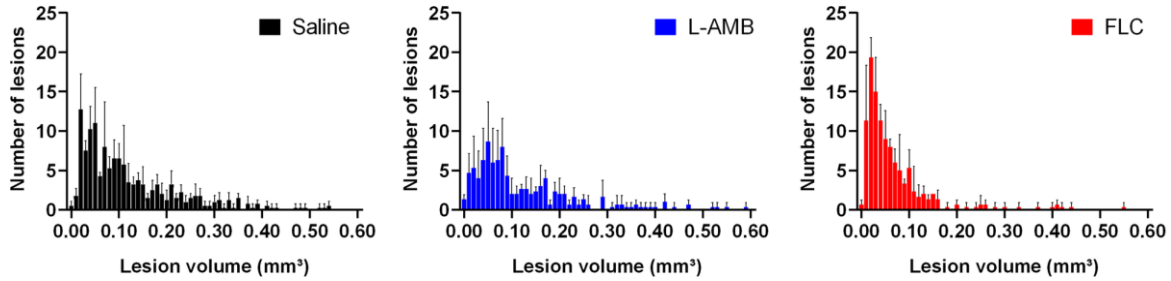

**Supplementary Figure S2. Fluorescence-based *in vitro* testing of antifungal efficacy**. Various concentrations of antifungals were added to *C. neoformans* cells expressing fluorescent E2-Crimson in a black 96-well plate. Fluorescence was measured at 45 min, 3, 5 and 24 hours after addition. Graphs show the temporal evolution in the fluorescence signal and detailed analysis for 45 min, 3 and 5 hours. The dashed line represents the background fluorescence obtained from sterile Sabouraud medium. Graphs show mean + SD, one-way ANOVA with Dunnett's post-test compared to no drug control. *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001.



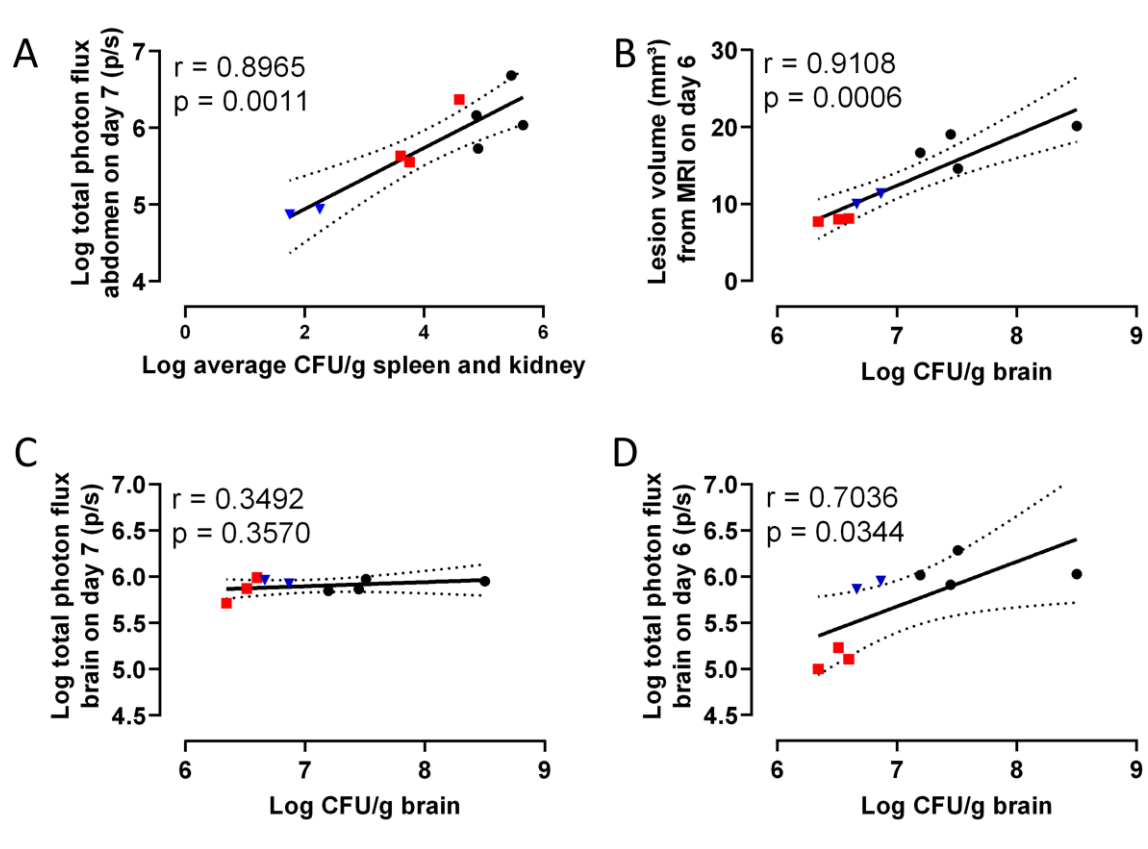

**Supplementary Figure S3. Assessment of potential auto-luminescence and auto-fluorescence of the antifungal compounds.** Liposomal amphotericin B (L-AMB), amphotericin B deoxycholate (AMB-d), fluconazole (FLC), natamycin (NATA), or sodium dodecyl sulphate (SDS) was added to sterile water or Sabouraud medium (0.1 mg/ml antifungal or 1% SDS). A, B) In sterile water (A) or Sabouraud medium (B), the compounds showed no detectable auto-luminescence, both in absence and presence of D-luciferin. C, D) The compounds were not auto-fluorescent in sterile water (C) or Sabouraud medium (D). Graphs show mean + SD, one-way (fluorescence) or two-way (luminescence) ANOVA with Dunnett's post-test, compared to the control condition with sterile water. *: $p<0.05$.



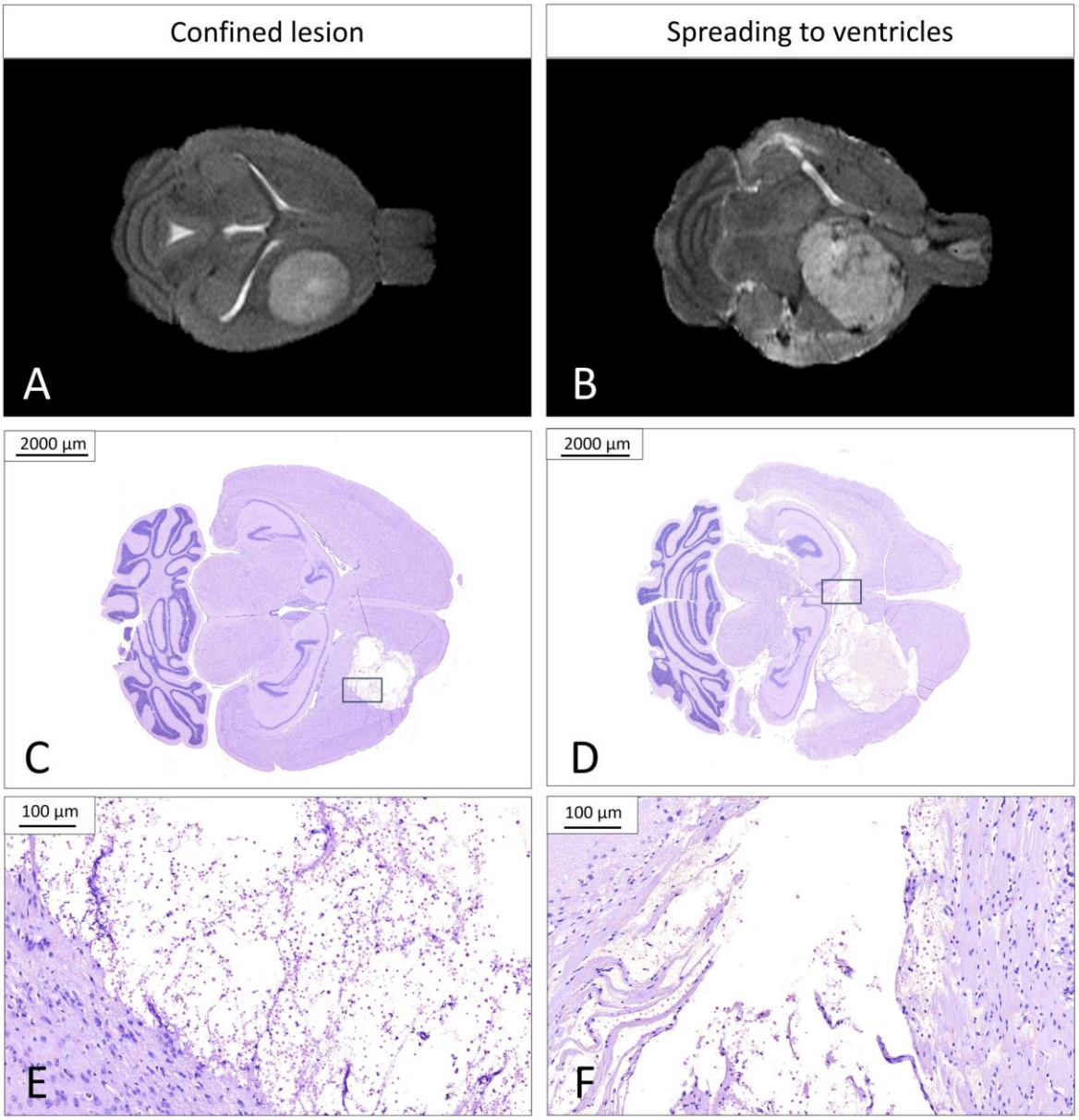

**Supplementary Figure S4. Measurement of potential luminescence of wild-type *C. neoformans* cells exposed to antifungals.** Amphotericin B deoxycholate (AMB-d) and liposomal amphotericin B (L-AMB) were added to liquid cultures of wild-type *C. neoformans* KN99α cells and luminescence was measured after addition of D-luciferin to culture samples. Graphs show mean + SD, one-way ANOVA with Dunnett's post-test, in comparison to the no drug control. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.



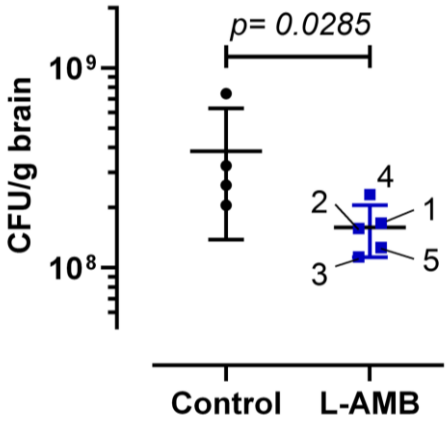

**Supplementary Figure S5. Lesion volume distribution after systemic treatment in the meningoencephalitis model**. After intravenous infection, animals were treated with saline, liposomal amphotericin B (L-AMB) or fluconazole (FLC). The volume of the individual lesions was quantified based on the 3D MRI scans obtained on day 6 p.i.. The histograms (bin size 0.01) were averaged for all animals in a treatment group, showing mean + SD in every bin.




**Supplementary Figure S6: Correlation of imaging findings with the fungal burden in the meningoencephalitis model.** A) *In vivo* BLI results in the abdomen were in good correspondence with the average fungal load in the spleen and kidneys. B) Lesion volume as quantified from MRI on day 6 showed an excellent correlation to brain fungal load. C-D) *In vivo* BLI results on day 7 (C) showed limited variation and no correlation, but BLI results obtained on day 6 (D) correlated well with the CFUs in the brain (assessed on day 7). Linear regression with 95% confidence band and Pearson correlation coefficients. Dots represent individual animals, colors correspond to the treatment groups: saline (black), L-AMB (blue), FLC (red).




**Supplementary Figure S7: Correspondence of *in vivo* MRI with histology of the brain.** Animals were stereotactically injected with GFP-expressing *C. neoformans* H99, scanned using MRI and sacrificed for histology. A-B) $T_2$-weighted 2D MR images for a confined lesion at day 13 p.i. (A) and a lesion spreading into the ventricles at day 10 p.i. (B). (C-D) Corresponding periodic acid-Schiff (PAS) staining of the brains of these animals. E) Magnification of figure C, showing the presence of numerous cryptococci in the cryptococcoma. F) Magnification of figure D, showing the spreading of fungal cells from the cryptococcoma into the ventricle.




**Supplementary Figure S8: CFU counting after intracerebral treatment in the cryptococcoma model.** Brains were isolated and homogenized after the last imaging session or when animals were sacrificed for humane endpoints (n = 4 (controls) or 5 (L-AMB treated animals)). There was a small but significant reduction of the fungal load in animals receiving intracerebral injection with liposomal amphotericin B (L-AMB, 2.5 µl of 0.6 mg/ml). Graphs show individual mice and mean ± SD, unpaired t-test. Numbers correspond to the individual animals in Fig. 9.