Pharmacodynamics of Flubendazole for Cryptococcal Meningoencephalitis: Repurposing and Reformulation of an Anti-Parasitic Agent for a Neglected Fungal Disease

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Potential Conflicts of Interest

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Current therapeutic options for cryptococcal meningitis are limited by toxicity, global supply and emergence of resistance. There is an urgent need to develop additional antifungal agents that are fungicidal within the central nervous system and preferably orally bioavailable. The benzimidazoles have broad-spectrum anti-parasitic activity, but also have in vitro antifungal activity that includes Cryptococcus neoformans. Flubendazole (a benzimidazole) has been reformulated by Janssen Pharmaceutica as an amorphous solid drug nanodispersion to develop an orally bioavailable medicine for the treatment of neglected tropical diseases such as onchocerciasis. We investigated the in vitro activity, the structure-activity-relationships and both in vitro and in vivo pharmacodynamics of flubendazole for cryptococcal meningitis. Flubendazole has potent in vitro activity against Cryptococcus neoformans with a modal MIC of 0.125 mg/L using European Committee for Antimicrobial Susceptibility Testing (EUCAST) methodology. Computer models provided an insight into the residues responsible for the binding of flubendazole to cryptococcal β-tubulin. Rapid fungicidal activity was evident in a hollow fiber infection model of cryptococcal meningitis. The solid drug nanodispersion was orally bioavailable in mice with higher drug exposure in the cerebrum. The maximal dose of flubendazole (12 mg/kg/day) orally resulted in a ~2 log_{10} CFU/g reduction in fungal burden compared with vehicle-treated controls. Flubendazole was orally bioavailable in rabbits, but there were no quantifiable drug concentrations in the CSF or cerebrum and no antifungal activity was demonstrated in either CSF or cerebrum. These studies provide evidence for the further study and development of the benzimidazole scaffold for the treatment of cryptococcal meningitis.
INTRODUCTION

Cryptococcal meningoencephalitis (herein meningitis) is a common and lethal disease in immunosuppressed patients (1, 2). This disease is predominately associated with advanced HIV infection and has the highest incidence in low to middle income countries (1). The number of effective agents is despairingly small (3). All available induction and maintenance regimens are constructed with three antifungal agents: amphotericin B (AmB), flucytosine (5FC) and fluconazole (4). Each of these compounds has significant adverse effects that include infusional toxicity (AmB), nephrotoxicity (AmB (5)), bone marrow suppression (AmB and 5FC (5, 6)) and hepatotoxicity (fluconazole and 5FC (7)). Moreover, there are significant inherent limitations that include fungistatic effects (fluconazole; (8)) and the potential emergence of drug resistance (fluconazole and 5FC; (9–11)). Thus, there is an urgent imperative to develop new agents. Orally bioavailable agents are particularly important given the predominance of this disease in resource constrained settings.

During the process of screening a compound library against fungal pathogens, it was noted by us (M.T. & I.C.) that flubendazole has potent in vitro activity against Cryptococcus neoformans. A literature search revealed other members of the benzimidazole class (e.g. albendazole and mebendazole) of anti-parasitic agents had previously been demonstrated to have potent in vitro activity against Cryptococcus neoformans with minimum inhibitory concentrations (MICs) of 0.16-0.45 mg/L (12, 13). The pharmacological target of the benzimidazoles against Cryptococcus neoformans is β-tubulin (14). The antifungal activity of parenterally administered flubendazole in a murine model of cryptococcal meningitis was...
confirmed by us in a series of preliminary experiments. Concurrently, we became aware of the efforts by Janssen Pharmaceutica to develop a new orally bioavailable formulation of flubendazole that may be active against filariasis and onchocerciasis. The potential value of this new formulation as an oral medicine for the treatment of cryptococcal meningitis in resource poor healthcare settings was therefore evident.

Herein, we describe the in vitro activity, putative structure-activity relationships, and the in vivo pharmacokinetic-pharmacodynamic relationships of flubendazole against Cryptococcus neoformans. A hollow fiber infection model of cryptococcal meningitis was developed as a first step for exploring dose-exposure-response relationships. Subsequently, two extensively used and well-characterized laboratory animal models of cryptococcal meningitis were used to provide the experimental foundation for the potential use of oral formulations of flubendazole or its congeners for the treatment of a neglected infection of global significance.
RESULTS

In vitro studies

Flubendazole displayed potent in vitro activity (MIC 0.06-0.25 mg/L; Table 1) against C. neoformans. The MICs were comparable when EUCAST and CLSI methodology was used.

The flubendazole IC$_{50}$ against porcine tubulin was 2.38 µM. Other known tubulin inhibitors display similar efficacy in this assay (e.g. colchicine IC$_{50}$ = 1.15 µM (unpublished data), paclitaxel IC$_{50}$ = 3.9 µM (15) and vinblastine IC$_{50}$ = 5.3 µM (15). These data are consistent with the known mechanism of action of flubendazole.

In vitro DMPK assessment of commercially available flubendazole powder confirmed a favorable logD7.4 of 2.9. Plasma protein binding was 90.6% and there was low metabolic turnover (Hu Mic Clint = 44 µl/min/mg and Rat Hep Clint = 39 µl/min/10$^6$ cells). However, aqueous solubility was poor (0.8 µM), which is characteristic of the benzimidazoles. This in vitro DMPK assessment was consistent with subsequent in vivo observations (see below). Poor aqueous solubility limits absorption through the gut, but once in the bloodstream the drug has favorable pharmacokinetic properties (e.g. ability to pass through cell membranes, low metabolism, and high concentrations of free drug) that enable it to reach the effect site.

Docking Studies

There were two principal non-covalent binding interactions between flubendazole and the homology model of C. neoformans β-tubulin. First, the hydroxyl group of Serine 350 acts as a hydrogen bond donor and binds the ketone oxygen of flubendazole (Figure 1A). Second,
asparagine (Asn) 247 acted as a hydrogen bond donor via the primary amide with the ketone of the carbamate on flubendazole, but also acted as a hydrogen bond acceptor through the primary carbonyl group of Asn247 and the N-H on the benzimidazole core. There were also several hydrophobic interactions deeper in the binding pocket that involved the benzene ring and the fluorine of flubendazole.

Docking studies of flubendazole and human β-tubulin (Fig 1B) showed that both the N-H of the benzimidazole core and the N-H of the carbamate are hydrogen bond donors (Figure 1B) to the primary amide of the side chain of Asn247. As for the C. neoformans interaction, there were hydrophobic interactions present from the para-substituted benzene and the hydrophobic binding pocket. There was a lack of a hydrogen bond acceptor role from the ketone oxygen. This is due to the replacement of Ser350 from the C. neoformans active site with Lys350 in humans.

**Hollow Fiber Infection Model of Cryptococcal Meningoencephalitis**

Rapid fungicidal activity was observed in the hollow fiber infection models. Controls grew from an initial density of approximately log_{10} CFU/mL 6 to log_{10} CFU/mL 8-9. Following the administration of flubendazole there was a progressive decline in the fungal density in the hollow fibre in all arms. There was an exposure-dependent decline in fungal burden.

**Preliminary Studies to Demonstrate In vivo Efficacy of Flubendazole**

There was no demonstrable antifungal effect of orally administered flubendazole as pure compound when formulated with sterile distilled water, 0.05% polysorbate 80 in PBS, 5% DMSO,
Antifungal activity could only be established when pure flubendazole formulated with polysorbate 80 (Tween 80) and injected s.c. to form a depot. Presumably, formulation with polysorbate 80 solubilized flubendazole to an extent that enabled it to become systemically bioavailable. However, this was only observed when flubendazole was administered s.c. This parenteral regimen resulted in a modest reduction in fungal burden of 1-2 log\textsubscript{10}CFU/g compared with vehicle-treated controls (data not shown). A limited PK study with concentrations measured at a single time-point the end of the experiment also confirmed flubendazole concentrations were quantifiable in plasma and the cerebrum of mice (data not shown).

These preliminary pharmacokinetic and pharmacodynamic data provided the impetus for further detailed experiments examining the pharmacodynamics of a new orally bioavailable solid drug nano-dispersion against Cryptococcus neoformans developed by Janssen.

Pharmacokinetic and Pharmacodynamic Studies of the Flubendazole Nanoformulation in Mice

When flubendazole was formulated as a solid drug nano-dispersion, it was rapidly absorbed after oral dosing and plasma concentrations were readily quantifiable at the first sampling point (i.e. 0.5 hrs. post dose; Figure 3). The pharmacokinetics were linear, with bi-exponential clearance from the bloodstream with a mean and median value of 0.039 and 0.026 L/h, respectively (Figure 3). The pharmacokinetic parameters are summarized in Table 2. There was rapid and extensive distribution of drug to the cerebrum of mice and concentrations of
Flubendazole were consistently higher than those observed in plasma. The AUCserum:

\[ \text{AUC}_{\text{serum}}: \text{AUC}_{\text{cerebrum}} = 1.44 \]

Flubendazole had a significant and discernible antifungal effect in mice. Use of the highest dosage in this study (12 mg/kg) resulted in approximately a 2-3 log reduction in fungal burden relative to controls (Figure 4). This regimen was limited by maximum permissible volumes for oral administration for mice (i.e. 20 mL/kg). In a single experiment in which the effect of 6 mg/kg q12h (i.e. 12 mg/kg/day) was compared to 12 mg/kg/day there was no difference in antifungal effect (data not shown). This is preliminary evidence that the AUC is likely to be the dynamically linked index for flubendazole against *Cryptococcus neoformans*.

**Pharmacokinetic and Pharmacodynamic Studies in Rabbits**

The PK in rabbits was linear with a similar concentration-time profile to that observed in mice. The plasma concentration-time profiles in rabbits had a similar shape to those of mice, but were lower for the dosages used in this study. Despite readily quantifiable plasma concentrations, there was no quantifiable drug concentrations in either the CSF or the cerebrum of rabbits at the time of sacrifice.

There was no demonstrable antifungal effect in rabbits receiving 6 mg/kg/day. There may be some effect in rabbits receiving 22.5 mg/kg q24h, but if present the effect was small and these assessments were limited by few animals. There were no statistically significant differences in the area under the \( \log_{10} \text{CFU/g}-\text{time} \) curve for each regimen even though this may be a relatively insensitive test of antifungal effect. Furthermore, there was no difference in the
fungal burden in the cerebrum at the end of the experiment for any of the groups of rabbits used in this study.
DISCUSSION

When given subcutaneously, flubendazole has striking activity in laboratory animal models of filarial diseases such as onchocerciasis and lymphatic filariasis (16). Janssen developed a novel amorphous solid drug nanodispersion to provide a potential new therapeutic option for patients with these neglected tropical diseases. The systemic drug exposure that was enabled by the new formulation mandated GLP toxicology studies before progression to early phase clinical studies. It was already known that flubendazole is clastogenic (i.e. induces chromosomal breakages) and aneugenic (i.e. induces aneuploidy), as well as embryotoxic (17). GLP toxicology studies were performed by Janssen in the rat (5, 15 and 30 mg/kg/day in male rats and 2.5, 5 and 10 mg/kg/day in female rats) and in the dog (20, 40 and 100 mg/kg/day) for 2 weeks. These experiments showed evidence of toxicity related to the pharmacological activity of flubendazole in the gastrointestinal tract, lymphoid system and the bone marrow, as well as testicular toxicity in both rat and dog. In the dog, liver toxicity was also observed. As a result, the development program was stopped based on an unacceptable risk/benefit profile in humans. This also halted our own efforts to develop flubendazole for cryptococcal meningitis.

Flubendazole has striking in vitro activity against Cryptococcus neoformans that was evident in the MIC testing and the pharmacodynamic studies in the hollow fiber infection model. There was modest antifungal activity in the murine model, which is not as prominent as that previously described by us for fluconazole, amphotericin B deoxycholate or liposomal amphotericin B (8, 18, 19). There was no unequivocal antifungal activity in the rabbit model of cryptococcal meningoencephalitis, which is largely explained by the absence of detectable...
Flubendazole concentrations in the cerebrum or CSF (despite readily quantifiable plasma concentrations). The in vitro susceptibility testing and data from the hollow fiber model suggests that flubendazole is highly potent and fungicidal if able to reach its fungal target in sufficient concentrations. The diminished activity in the mouse (relative to historical controls) and absence of effect in the rabbit (with non-quantifiable concentrations in the cerebrum and CSF) further support this conclusion. Hence, successful exploitation of the benzimidazole backbone requires careful attention to physiochemical properties that promote absorption across the gut and the ability to partition into sub-compartmentsof the CNS.

Flubendazole did not display a comparable degree of in vivo activity to other first-line agents for cryptococcal meningitis (i.e. fluconazole and amphotericin B formulations). Even if the safety profile was not problematic, there is insufficient prima facie evidence from either the murine or rabbit models to further study flubendazole as monotherapy for induction therapy in phase II clinical studies. Nevertheless, additional approaches such as the combination with other antifungal agents for induction therapy and/or used as longer-term consolidation and maintenance therapy may have been possible.

The potential of derivatives of flubendazole to be useful human medicines depends on the differential activity between cryptococcal and human proteins. Characterization of the β-tubulin genes of C. neoformans has been undertaken and two C. neoformans β-tubulin genes (TUB1 and TUB2) have been identified. TUB1 was identified as the primary target of the benzimidazole class of compounds through gene characterization and expression (14). There is 90% homology between fungal TUB1 and human β-tubulin, although the former has not been crystallized and this has prevented definitive structure-activity-relationship docking studies. The
ability to develop new agents based on a benzimidazole scaffold or to further exploit β-tubulin as a pharmacological target will depend on the degree of differential activity of a benzimidazole with these proteins. The differential binding identified through the docking and homology modelling of both human β-tubulin (20) and C. neoformans var. grubii serotype A (strain H99) β-tubulin (14) to the Bos Taurus 1SA0 β-tubulin crystal structure implies an increased number of binding interactions with C. neoformans β-tubulin. This may provide the potential to exploit this differential binding to establish a favorable therapeutic index. It is also worth emphasizing that the benzimidazoles may have additional targets beyond β-tubulin that have the further potential to provide differential activity between human and fungal proteins, but this requires further investigation (21–24).

The potential utility of congeners of flubendazole now rests with medicinal chemistry programs. Compounds must be synthesized that exhibit differential activity against cryptococcal and human tubulin (if that is possible) so that there is an acceptable safety margin and toxicity profile. Furthermore, the compound must be able to traverse the gut (compounds that are not orally bioavailable will be less clinically valuable) and then the blood-brain-barrier to achieve concentrations that are ideally fungicidal. The latter will be promoted by new molecules that low molecular weight lipophilic compounds that are not substrates for active pumps such as P-glycoprotein. This will undoubtedly also require the use of novel formulation technologies to ensure compounds that are poorly soluble to become useful agents for disseminated infections.
METHODS

Drug

Flubendazole that was used for determination of MICs, hollow fibre experiments and preliminary murine experiments was purchased from Sigma. Subsequently, definitive pharmacokinetic-pharmacodynamic experiments that were performed with orally administered flubendazole used a solid drug nano-dispersion formulation of flubendazole developed by Janssen Pharmaceuticals (batch BREC-1113-070, Janssen Pharmaceuticals). The stability of this formulation in liquid and solid phases was confirmed for 1 and 6 months, respectively.

A 10 mg/mL methylcellulose 4000cps stock solution (100 mL batch) was prepared from a dispersion of 1 g methylcellulose 4000cps with stirring into 70 mL demineralized water heated to 70°C-80°C. The solution was stirred for at least 15 minutes followed by the addition of 20 mL of demineralized water. The mixture was stirred until it reached room temperature and was then made up to 100 mL with demineralized water. A total of 50 mL of 6 mg/mL spray dried powder suspension was then prepared (this corresponds with 0.6 mg/mL of flubendazole as the active dose in 0.5% methylcellulose). A total of 24.70 g of demineralized water was added to a 50-mL clear glass vial. A total of 0.30 g of spray dried drug was added to the vial which was then closed with a stopper. The vial was vortexed and then homogenized using a polytron disperser. A 25-mL stock solution of methylcellulose 4000cps was added and the vial was vortexed. The suspension was refrigerated at 5°C until dosing for a maximum of 14 days. Prior to dosing the suspension was vortexed.
Strains

The initial in vitro susceptibility testing was performed with H99 (ATCC 208821). An additional 49 clinical isolates were obtained from the National Centre for Microbiology Instituto de Salud Carlos III, Madrid, Spain (courtesy Ana Alastruey-Izquierdo and Manual Cuenca-Estrella). These isolates were identified to species level using standard microbiological techniques.

Minimum Inhibitory Concentrations

The minimum inhibitory concentration of flubendazole against H99 (ATCC 208821) and the 49 isolates was estimated using methodology of the European Committee on Antimicrobial Susceptibility Testing (EUCAST; (25)) and Clinical Sciences Laboratory Institute (CLSI; (26)). The endpoint for MIC determination using EUCAST and CLSI was 50% for both methods. MICs were performed in triplicate.

Porcine Tubulin Polymerization Assay

Porcine tubulin is generally used as a surrogate for human tubulin because of its high degree of homology (95%) (27). In the studies described herein, this assay was used to determine the extent of interaction between flubendazole and its putative target as also occurs for assessment of the binding of antineoplastic agents (28, 29). The commercially available porcine tubulin assay (BK011P, Cytoskeleton, Inc. Denver, USA) quantifies the time-dependent polymerization of tubulin to microtubules and thus the ability of tubulin inhibitors to disrupt this process.

The porcine tubulin assay was performed according to the manufacturer’s instructions. Briefly, the 96-well assay plate was pre-warmed to 37°C prior to use. Five μL of test
compound(s) and controls at 0, 1.25, 2.5, 5, 10 μM were aliquoted into each well and pre-warmed for 1 min. Colchicine and DMSO were used as positive and negative controls, respectively. Polymerization was initiated by mixing 45 μL of reaction buffer that contained 2 mg/mL of purified porcine brain tubulin, 10 μM fluorescent reporter, PEM buffer (80 mM PIPES, 0.5 mM EGTA, 2 mM MgCl$_2$, pH 6.9), 1mM GTP and 20.3% glycerol. Tubulin polymerization was followed by an increase in fluorescence intensity due to the incorporation of a fluorescence reporter into microtubules as polymerization occurred. The change in fluorescence was measured using an excitation and emission wavelength of 360 nm and 450 nm, respectively every 1-min for 1-hr. at 37°C using a Varioskan multimode plate reader (Thermo scientific Inc.). All data points were acquired in triplicate and IC$_{50}$ values were calculated with GraphPad Prism. The IC$_{50}$ value was defined as the drug concentration required to inhibit tubulin polymerization by 50% compared with negative control.

**Homology Modeling and Docking Studies**

While the amino acid sequence of cryptococcal β-tubulin is known (74% homology with human β-tubulin), the protein has not been crystallized. A homology model was therefore developed to investigate differential binding modes of flubendazole within *C. neoformans* and human β-tubulin. Molecular modelling (Modeller version 9.14, [https://salilab.org/modeller/](https://salilab.org/modeller/)) of both human β-tubulin (20) and *C. neoformans* var. grubii serotype A (strain H99) β-tubulin (14) was undertaken using the Bos Taurus 1SA0 β-tubulin crystal structure (identity: 364/447 (81.4%); similarity: 405/447 (90.6%)).
Virtual flubendazole was built in the molecular modelling software Spartan (Wavefunction Inc., Irvine, USA) and energy minimized. Flubendazole was then subjected to a piecewise linear potential (ChemPLP) docking protocol (a scoring function to provide confidence in the docking pose adopted by the molecule), consisting of 10 genetic algorithm (GA) runs before visualization using the molecular visualization system PyMOL with the top scoring compound depicted in Figure 1. The active site binding interactions were selected by identifying those amino acid residues within 4Å of flubendazole when docked into the β- tubulin binding site. Polar contacts between flubendazole and the surrounding amino acids were identified, which aided in the identification of hydrogen bonding interactions that are key in determining the efficacy of a drug against its pharmacological target.

Finally, hydrogen bond donor interactions, as well as hydrophobic interactions were identified using the pharmacophore (i.e. an abstract description of molecular features that in this case are necessary for molecular recognition of flubendazole by β-tubulin) search software Zinc Pharmer (https://academic.oup.com/nar/article-lookup/doi/10.1093/nar/gks378) at 4Å for hydrogen bonding interactions and 6Å for hydrophobic interactions.

Hollow Fiber Model of Cryptococcal Meningoencephalitis

A new hollow fiber infection model (HFIM) was developed to investigate the in vitro pharmacodynamics of flubendazole against C. neoformans. The same cartridges (FiberCell Systems, Frederick, MD, USA) and configuration as previously described for bacterial pathogens was used (see for example (30)). The extra-capillary space of each cartridge was inoculated with...
40 mL of a suspension containing log$_{10}$CFU/mL 6 of *C. neoformans* var. *grubii* (ATCC 208821; H99). Yeast-extract-peptone-dextrose (YPD) medium was pumped from the central compartment through the cartridge and back again using a peristaltic pump (205 U; Watson-Marlow, United Kingdom). The HFIM was incubated at 37°C in ambient air. The time-course of fungal growth was determined by removing 1 mL from the extra-capillary space of the cartridge and plating serial 10-fold dilutions to YPD agar.

The relationship between flubendazole drug exposure and its effect was explored using a range of drug exposures. Since there is no information on the pharmacokinetics of flubendazole in humans, we attempted to produce AUCs that were comparable to those observed in mice. Various dosages of flubendazole were administered q24h by infusion over 1 hour for 8 days to the central compartment using a programmable syringe driver (Aladdin pump; World Precision Instruments, United Kingdom). There was a 24-hour delay in the initiation of flubendazole therapy post inoculation. To generate first-order pharmacokinetics, fresh YPD medium was pumped into the central compartment, and the same volume of drug-containing medium was simultaneously removed and discarded. Positive controls of currently licensed agents were not studied in these experiments.

*Murine model of cryptococcal meningoencephalitis*

A previously described (31) and well-characterized murine model of cryptococcal meningitis was used to investigate the pharmacodynamics of flubendazole. All laboratory animal experiments were performed under UK Home Office project license PPL40/3630 and were
approved by the University of Liverpool’s Animal Welfare Ethics Review Board. Male CD1 mice were purchased from Charles River and were 20-30 grams at the time of experimentation. An inoculum of $3 \times 10^8$ CFU in 0.25 mL was used for each mouse. Groups of mice (n=3) were serially sacrificed throughout the experimental period. The brains were removed and homogenized. Serial 10-fold dilutions were plated to YPD agar supplemented with chloramphenicol to enumerate the total fungal burden. Plates were incubated in air at 30°C for at least 48 hours.

Pharmacokinetic and Pharmacodynamic Studies of Flubendazole in Mice

Preliminary evidence for the efficacy of flubendazole was obtained by dissolving pure compound in a variety of excipients that included cyclodextrin (F2G, Eccles, UK), DMSO [5%] and polysorbate 80 [10%] and injecting it subcutaneously q24h. Ultimately, only s.c. injection with Tween80 showed any effect. This experiment provided the impetus to further examine the orally bioavailable formulation developed by Janssen (see above).

The pharmacokinetics of oral flubendazole was determined with two independently conducted experiments. Treatment was initiated 24 hrs. post-inoculation. Dosages of 2-12 mg/kg were used. Only the first dosing interval was studied. A serial sacrifice design was used with groups of n=3 mice that were sacrificed at 0.5, 1, 2, 8 and 24 hrs. post-inoculation.

The pharmacodynamics of oral flubendazole was estimated over the course of three separate independently conducted experiments. Groups of n=3 mice were sacrificed at time = 2, 24, 48, 96, 144 and 168 hours post inoculation. Dose finding studies were performed using flubendazole 2, 4, 6, 8, and 12 mg/kg q24h orally. The upper dosage was limited by the volume...
A fourth experiment compared 12 mg/kg q24 with 6 mg/kg q12h to examine whether more fractionated regimens provided any additional antifungal effect.

**Rabbit model of cryptococcal meningitis**

A previously described and well-characterized rabbit model of cryptococcal meningoencephalitis (32) was used to further investigate the pharmacodynamics of flubendazole. Male New Zealand White rabbits were purchased from Harlan. Rabbits weighed 2.5-3 kg at the time of experimentation. Rabbits were immunosuppressed intramuscularly with hydrocortisone 10mg/kg day -1 relative to infection and then daily throughout the experiment. Cryptococcal meningoencephalitis was induced with the intra-cisternal inoculation of 0.25 mL of a suspension containing $3.8 \times 10^8$ CFU/mL under general anesthesia (induced with medetomidine and ketamine). This inoculum results in progressive infection that manifests as an increase in fungal burden in the CSF and reproducible encephalitis. There is minimal clinical disease with no demonstrable neurological signs in the experimental period. Mortality always occurred in the context of cisternal tapping and repeated anesthesia rather than from progressive infection.

**Pharmacodynamic and Pharmacokinetic studies in Rabbits**

PK-PD relationships in the rabbit were estimated in two independently conducted experiments consisting of 6 rabbits in each experiment. Rabbits were placed under general anesthesia for removal of CSF via intra-cisternal tapping at 48 hour intervals. Over the course of the two experiments there were n=3 controls (1 rabbit died after being tapped), flubendazole 6
mg/kg q24h (n=6) and 22.5 mg/kg q24h (n=6). The maximum dosage that was used was limited by the formulation provided by Janssen and the limits of oral gavage in rabbit (15 mL/kg/day). Treatment was initiated 48 hrs. post-inoculation, and continued for 10 days, after which time all rabbits were sacrificed. Thus, the total duration of the experiment was 288 hours.

**Measurement of flubendazole concentrations using LC/MS/MS**

Flubendazole concentrations in all matrices were measured using a validated ultrahigh-performance liquid chromatography tandem mass spectrometry implemented on an Agilent 6420 Triple Quad Mass spectrometer and an Agilent 1290 infinity LC system (Agilent Technologies UK Ltd, Cheshire, UK). Flubendazole was extracted by protein precipitation by adding 300 µL of a 50:50 mix of acetonitrile:methanol that contained the internal standard (6,7-Dimethyl-2,3-di(2-pyridyl) quinoxaline; Sigma Aldrich, Dorset, UK) at a final concentration of 1 mg/L to 30µL of each matrix.

The extraction was performed in 96-well Sirocco protein precipitation plates (Waters, UK). Samples were then shaken for 2 mins and then extracted using a 96-positive pressure manifold (Waters, UK). A total of 200 µL of the supernatant was removed and placed in a 96 well plate. One µL was injected on an Agilent a Zorbax Eclipse Plus C18 column (2.1 by 50 mm, 1.8-m particle size; Agilent Technologies UK Ltd, Cheshire, UK). Chromatographic separation was achieved using a gradient with the starting conditions of a 60:40 mix of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The ratio of A:B changed to 20:80 over 2 minutes and then returned to the starting conditions (60:40) for 1 minute of equilibration.
The mass spectrometer was operated in multiple reaction monitoring (MRM) scan mode in positive polarity. The precursor ion for flubendazole and internal standard was 314.1 m/z, and 313.15 m/z, respectively. The product ion for flubendazole and internal standard was 282.1 m/z and 284.1 m/z, respectively. The source parameters were set as 4000 V for capillary voltage, 350°C for gas temperature, and 60 lb/in² for the nebulizer gas.

The standard curve for flubendazole encompassed the concentration range of 0.0005-8 mg/L and was constructed using the respective blank matrix. The limit of quantitation was 0.0005 mg/L and the CV% was 12.7% over the concentration range 0.0005-8 mg/L and the intra and inter-day variation was <12% for all matrices.

**Mathematical modeling**

The pharmacokinetic and pharmacodynamic datasets from mice were modelled using the program Pmetrics (33) and the following five inhomogeneous differential equations:

\[
\begin{align*}
\text{Eq. 1} & \quad X_P(1) = B(1) - K_a \cdot X(1) \\
\text{Eq. 2} & \quad X_P(2) = \frac{K_a \cdot X(1)}{\left(\frac{SCL}{V}\right)} \cdot X(2) - Kcp \cdot X(2) + Kpc \cdot X(3) - Kcb \cdot X(2) + Kbc \cdot X(5) \\
\text{Eq. 3} & \quad X_P(3) = Kcp \cdot X(2) - Kpc \cdot X(3) \\
\text{Eq. 4} & \quad X_P(4) = Kcp \cdot X(2) - Kpc \cdot X(4) \\
\text{Eq. 5} & \quad X_P(5) = K_{gmax} \cdot \left(1 - \left(\frac{X(4)}{C50gHg + \left(\frac{X(4)}{V}\right)^Hg}\right)\right) \cdot \left(1 - \left(\frac{X(5)}{popmax}\right)\right) \cdot X(5)
\end{align*}
\]
The system parameters and their units are as follows: \( B(1) \) (mg) represents the bolus input of flubendazole into the gut. \( K_a (h^{-1}) \) is the first order rate constant collecting the gut and the central compartment; \( SCL \) (L/h) is the clearance of flubendazole from the central compartment; \( V \) (L) is the volume of the central compartment; \( K_p (h^{-1}) \) and \( K_{pc} (h^{-1}) \) are the first-order inter-compartmental rate constants. \( K_{gmax} (\log_{10} CFU/g/h) \) and \( k_{killmax} \) (\( \log_{10} CFU/g/h \)) are the maximal rates of cryptococcal growth and flubendazole-induced kill, respectively. \( \text{POPMAX} \) (CFU/g) is the maximum theoretical fungal density. \( C_{50g} \) (mg/L) and \( C_{50k} \) (mg/L) are the concentrations of flubendazole that induce half-maximal effects on growth and kill, respectively. \( H_g \) and \( H_k \) are the respective slope functions for growth and kill. The initial condition (CFU/g; not shown in the equations) is the fungal density immediately following inoculation, and is estimated along with other parameters.

Equations 1, 2, 3 and 4 are pharmacokinetic equations that describe the movement of drug from the gut, throughout the body and into the brain. Equation 1 describes the movement of drug from the gut. Equation 2 describes the rate of change of flubendazole in the central compartment (plasma) with first-order clearance and movement of drug to and from both a peripheral (unmeasured) compartment and the cerebrum. Equations 3 and 4 describe the rate of change of drug in the peripheral and cerebral compartments, respectively. The pharmacodynamics of flubendazole against \( \text{Cryptococcus neoformans} \) is described by Equation 5, which has terms that describe the capacity limited growth of \( \text{Cryptococcus} \), flubendazole-induced suppression of growth and drug-induced fungal killing. The antifungal activity in the cerebrum is primarily related to concentrations in the cerebrum.
A similar model was used to model the PK-PD data from rabbits, but there were some differences. Firstly, no drug was detectable in the brain or the CSF. Therefore, we let plasma concentrations of drug drive the antifungal effect and did not attempt to model the concentration of drug in the central nervous system (as was the case for mice). We directly linked plasma concentrations with the antifungal effect.
REFERENCES

1. Rajasingham R, Smith RM, Park BJ, Jarvis JN, Govender NP, Chiller TM, Denning DW, Loyse A, Boulware DR. 2017. Global burden of disease of HIV-associated cryptococcal meningitis: An updated analysis. Lancet Infect Dis 309:1–9.

2. Jarvis JN, Bicanic T, Loyse A, Namarika D, Jackson A, Nussbaum JC, Longley N, Muzoora C, Phulusa J, Taseera K, Kanyembe C, Wilson D, Hosseinipour MC, Brouwer AE, Limmathurotsakul D, White N, Van Der Horst C, Wood R, Meintjes G, Bradley J, Jaffar S, Harrison T. 2014. Determinants of mortality in a combined cohort of 501 patients with HIV-associated cryptococcal meningitis: Implications for improving outcomes. Clin Infect Dis 58:736–745.

3. Denning DW, Hope WW. 2010. Therapy for fungal diseases: Opportunities and priorities. Trends Microbiol 18.

4. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, Harrison TS, Larsen RA, Lortholary O, Nguyen MH, Pappas PG, Powderly WG, Singh N, Sobel JD, Sorrell TC. 2010. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of america. Clin Infect Dis 50:291–322.

5. Bicanic T, Bottomley C, Loyse A, Brouwer AE, Muzoora C, Taseera K, Jackson A, Phulusa J, Hosseinipour MC, Van Der Horst C, Limmathurotsakul D, White NJ, Wilson D, Wood R, Meintjes G, Harrison TS, Jarvis JN. 2015. Toxicity of amphotericin B deoxycholate-based induction therapy in patients with HIV-associated cryptococcal meningitis. Antimicrob Agents Chemother 59:7224–7231.
6. Stamm AM, Diasio RB, Dismukes WE, Shadomy S, Cloud GA, Bowles CA, Karam GH, Espinel-Ingroff A. 1987. Toxicity of amphotericin B plus flucytosine in 194 patients with cryptococcal meningitis. Am J Med 83:236–242.

7. Milefchik E, Leal MA, Haubrich R, Bozette S a, Tilles JG, Leedom JM, McCutchan JA, Larsen R a. 2008. Fluconazole alone or combined with flucytosine for the treatment of AIDS-associated cryptococcal meningitis. Med Mycol 46:393–5.

8. Sudan A, Livermore J, Howard SJ, Al-Nakeeb Z, Sharp A, Goodwin J, Gregson L, Warn PA, Felton TW, Perfect JR, others, Harrison TS, Hope WW. 2013. Pharmacokinetics and pharmacodynamics of fluconazole for cryptococcal meningoencephalitis: implications for antifungal therapy and in vitro susceptibility breakpoints. Antimicrob Agents Chemother 57:2793–2800.

9. Bicanic T, Harrison T, Niepieklo A, Dyakopu N, Meintjes G. 2006. Symptomatic relapse of HIV-associated cryptococcal meningitis after initial fluconazole monotherapy: the role of fluconazole resistance and immune reconstitution. Clin Infect Dis 43:1069–1073.

10. Scholer HJ. 1980. Flucytosine Antifungal Chemotherapy. John Wiley & Sons.

11. Polak A, Scholer HJ, Wall M. 1982. Combination therapy of experimental candidiasis, cryptococcosis and aspergillosis in mice. Chemotherapy 28:461–479.

12. Cruz MC, Bartlett MS, Edlind TD. 1994. In vitro susceptibility of the opportunistic fungus Cryptococcus neoformans to anthelmintic benzimidazoles. Antimicrob Agents Chemother 38:378–380.
13. Joffe LS, Schneider R, Lopes W, Azevedo R, Staats CC, Kmetzsch L, Schrank A, Poeta M Del, Vainstein MH, Rodrigues ML. 2017. The anti-helminthic compound mebendazole has multiple antifungal effects against Cryptococcus neoformans. Front Microbiol 8:1–14.

14. Cruz MC, Edlind T. 1997. β-Tubulin genes and the basis for benzimidazole sensitivity of the opportunistic fungus Cryptococcus neoformans. Microbiology 143:2003–2008.

15. Gertsch J, Meier S, Tschopp N, Altmann K-H. 2007. New Tubulin Inhibitors from Plants – A Critical Assessment. Chim Int J Chem 61:368–372.

16. Mackenzie CD, Geary TG. 2011. Flubendazole: a candidate macrofilaricide for lymphatic filariasis and onchocerciasis field programs. Expert Rev Anti Infect Ther 9:497–501.

17. Tweats DJ, Johnson GE, Scandale I, Whitwell J, Evans DB. 2016. Genotoxicity of flubendazole and its metabolites in vitro and the impact of a new formulation on in vivo aneugenicity. Mutagenesis 31:309–321.

18. Livermore J, Howard SJ, Sharp AD, Goodwin J, Gregson L, Felton T, Schwartz JA, Walker C, Moser B, Müller W, Harrison TS, Perfect JR, Hope WW, Muller W. 2013. Efficacy of an abbreviated induction regimen of amphotericin B deoxycholate for cryptococcal meningoencephalitis: 3 days of therapy is equivalent to 14 days. MBio 5:e00725-13.

19. Lestner J, McEntee L, Johnson A, Livermore J, Whalley S, Schwartz J, Perfect JR, Harrison T, Hope W. 2017. Experimental Models of Short Courses of Liposomal Amphotericin B for Induction Therapy for Cryptococcal Meningitis.

20. Ravelli RBG, Gigant B, Curmi PA, Jourdain I, Lachkar S, Sobel A, Knossow M. 2004. Insight...
into tubulin regulation from a complex with colchicine and a stathmin-like domain. Nature 428:198–202.

21. Janupally R, Jeankumar VU, Bobesh KA, Soni V, Devi PB, Pulla VK, Suryadevara P, Chennubhotla KS, Kulkarni P, Yogeeswar P, Sriram D. 2014. Structure-guided design and development of novel benzimidazole class of compounds targeting DNA gyraseB enzyme of Staphylococcus aureus. Bioorganic Med Chem 22:5970–5987.

22. Kaur G, Kaur M, Silakari O. 2014. Benzimidazoles: An Ideal Privileged Drug Scaffold for the Design of Multi-targeted Anti-inflammatory Ligands. Mini-Reviews Med Chem 14:747–767.

23. Li Y, Tan C, Gao C, Zhang C, Luan X, Chen X, Liu H, Chen Y, Jiang Y. 2011. Discovery of benzimidazole derivatives as novel multi-target EGFR, VEGFR-2 and PDGFR kinase inhibitors. Bioorganic Med Chem 19:4529–4535.

24. Matsumoto Y, Kakuda S, Koizumi M, Mizuno T, Muroga Y, Kawamura T, Takimoto-Kamimura M. 2013. Crystal structure of a complex of human chymase with its benzimidazole derived inhibitor. J Synchrotron Radiat 20:914–918.

25. Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope W. 2012. EUCAST technical note on the EUCAST definitive document EDef 7.2: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST). Clin Microbiol Infect 18:E246–E247.

26. National NC for CLS. 1997. Reference method for broth dilution antifungal susceptibility.
testing of yeasts. Approved standard M27-A2. NCCLS, Wayne, PA.

27. Hall JL, Dudley L, Dobner PR, Lewis S a, Cowan NJ. 1983. Identification of two human beta-tubulin isotypes. Mol Cell Biol 3:854–62.

28. Brunner M, Albertini S, Würgler FE. 1991. Effects of 10 known or suspected spindle poisons in the in vitro porcine brain tubulin assembly assay. Mutagenesis 6:65–70.

29. Owellen RJ, Hartke CA, Dickerson RM, Hains FO. 1976. Inhibition of Tubulin-Microtubule Polymerization by Drugs of the Vinca Alkaloid Class. Cancer Res 36:1499–1502.

30. Docobo-Pérez F, Drusano GL, Johnson A, Goodwin J, Whalley S, Ramos-Martín V, Ballestero-Tellez M, Rodriguez-Martinez JM, Conejo MC, Van Guilder M, Rodríguez-Baño J, Pascual A, Hope WW. 2015. Pharmacodynamics of fosfomycin: Insights into clinical use for antimicrobial resistance. Antimicrob Agents Chemother 59:5602–5610.

31. Sudan A, Livermore J, Howard SJ, Al-Nakeeb Z, Sharp A, Goodwin J, Gregson L, Warn PA, Felton TW, Perfect JR, Harrison TS, Hope WW. 2013. Pharmacokinetics and pharmacodynamics of fluconazole for cryptococcal meningoencephalitis: Implications for antifungal therapy and in Vitro susceptibility breakpoints. Antimicrob Agents Chemother 57.

32. Perfect JR, Lang SD, Durack DT. 1980. Chronic cryptococcal meningitis: a new experimental model in rabbits. Am J Pathol 101:177–194.

33. Neely MN, van Guilder MG, Yamada WM, Schumitzky A, Jelliffe RW. 2012. Accurate detection of outliers and subpopulations with Pmetrics, a nonparametric and parametric
pharmacometric modeling and simulation package for R. Ther Drug Monit 34:467–76.
Table 1. MIC distributions of flubendazole against *C. neoformans* isolates using CLSI and EUCAST methodologies.

| Methodology | Number of strains | Number of isolates with MIC (mg/L) of: |
|-------------|------------------|---------------------------------------|
|             |                  | 0.03 | 0.06 | 0.125 | 0.25 | 0.5 |
| EUCAST\(^a\) | 50               | 1    | 19   | 25    | 5    | 0   |
| CLSI\(^b\)  | 50               | 2    | 40   | 8     | 0    | 0   |

\(^a\) European Committee for Antimicrobial Susceptibility Testing

\(^b\) Clinical Laboratory Sciences Institute
Table 2. Parameter Values from the PK-PD model fitted to mice

| Parameter (Units) | Mean     | Median   | Standard Deviation |
|-------------------|----------|----------|--------------------|
| Ka (h⁻¹)          | 11.312   | 14.895   | 6.594              |
| SCL/F (L/h)       | 0.039    | 0.026    | 0.031              |
| Vc/F (L)          | 0.051    | 0.069    | 0.033              |
| Kcp (h⁻¹)         | 15.741   | 15.404   | 6.806              |
| Kpc (h⁻¹)         | 16.997   | 16.915   | 5.962              |
| Kcb (h⁻¹)         | 3.446    | 0.594    | 4.709              |
| Kbc (h⁻¹)         | 0.056    | 0.056    | 0.030              |
| Kgmax (log₁₀CFU/g/h) | 0.107  | 0.098    | 0.025              |
| Hg                | 10.338   | 5.096    | 9.782              |
| C₅₀g (L/h)        | 2.036    | 1.681    | 1.517              |
| POPMAX (CFU/g)    | 982934669.178 | 427055621.187 | 2281967059.602 |
| IC (CFU/g)        | 102.255  | 116.462  | 60.966             |
| Vb/F (L)          | 0.277    | 0.146    | 0.335              |
Ka (h^{-1}) is the first order rate constant collecting the gut and the central compartment; SCL/F (L/h) is the apparent clearance of flubendazole from the central compartment; V/F and Vb/F (L) are the apparent volumes of the central compartment and brain, respectively; Kcp (h^{-1}) and Kpc (h^{-1}) are the first-order inter-compartmental rate constants. Kgmax (log_{10}CFU/g/h) and kkillmax (log_{10}CFU/g/h) are the maximal rates of cryptococcal growth and flubendazole-induced kill, respectively. POPMAX (CFU/g) is the maximum theoretical fungal density. C50g (mg/L) and C50k (mg/L) are the concentrations of flubendazole that induce half-maximal effects on growth and kill, respectively. Hg and Hk are the respective slope functions for growth and kill.
Figure 1. Homology model of flubendazole docked with both C. neoformans and human β-tubulin. The colours are as follows: red sphere: hydrogen bond donors; blue sphere: hydrogen bond acceptors; yellow sphere: hydrophobic interactions. The docking pose is visualized with PyMOL. Protein is shown as a surface representation coloured 40% transparent light blue.

Flubendazole is represented as sticks composed of carbon (light blue); hydrogen (white); nitrogen (dark blue); oxygen (red); and fluorine (cyan). Binding site residues selected around 4 Å are represented as sticks with carbon (green); nitrogen (blue); oxygen (red); and sulfur (yellow).
Figure 2. Hollow fiber infection model of cryptococcal meningitis. A, pharmacokinetics of flubendazole with the three arms with intended peak concentrations of 1.25, 2.5 and 10 mg/L; and B, pharmacodynamics in response to flubendazole administered at various dosages q24h. Therapy was initiated 24 hrs. post inoculation after which time Cryptococcus had grown from ~6 log$_{10}$CFU/mL to 8 log$_{10}$CFU/mL.
Figure 3. Flubendazole pharmacokinetics in mice and rabbits. A, mouse plasma concentration-time profiles following the administration of flubendazole 2, 4, 6, 8 and 12 mg/kg; B, mouse concentration-time profiles in the brain following the administration of flubendazole 2, 4, 6, 8 and 12 mg/kg. Data are mean ± standard deviation of n=3 mice. C, plasma pharmacokinetics in the serum for individual rabbits receiving 6 mg/kg/day (broken lines, solid triangles) and 22.5 mg/kg (solid lines, solid squares).
Flubendazole is administered orally once daily. Data (open squares) are mean ± standard deviation from n=3 mice. The solid line is the fit of the population predicted pharmacokinetic-pharmacodynamic model. The maximally administered dose in this study (12 mg/kg/day) slowed, but did not prevent fungal growth in the brain.
Figure 5. Pharmacodynamics of flubendazole in a rabbit model of cryptococcal meningitis. A, time-course of fungal burden in the CSF of untreated controls; B, time-course of fungal burden in the CSF rabbits treated with flubendazole 6 mg/kg q24h orally; C, time-course of fungal burden in the CSF rabbits treated with flubendazole 22.5 mg/kg q24h orally; D, the fungal burden in the cerebrum of rabbits at the end of the experiment (time = 288 hrs. post inoculation and after 10 days of treatment with flubendazole). There are no differences in the three groups (p=0.464, ANOVA).