REPORT

ASSESSMENT OF THE IN VIVO EFFICACY OF CHD-FA (CarboHydrate Derived - Fulvic Acid) IN A MURINE MODEL OF DISSEMINATED CANDIDIASIS

FOR

Fulhold Ltd.

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CONFIDENTIAL

1.1 PHYSICAL PROPERTIES OF NARMA ALSO KNOWN AS FULVIC ACID

Euprotec received 1 bottle of CHD-FA reconstituted as a 4% solution. The solution was stored at room temperature in the dark since delivery. The 4% CHD-FA solution is a yellow/brown slightly viscous solution with a strong odour and a pH of 2.1 at 25^{o}C.

2.1 METHODS

2.1.1 Regulatory Issues

All animal experiments were performed under UK Home Office Licence 40/3101 Invasive Fungal Diseases (Licence Holder Dr Peter Warn) and with local ethical committee clearance. All experiments will be performed by technicians that have completed parts 1, 2 and 3 of the Home Office Personal Licence course and hold a current personal licence. All experiments were performed in dedicated Biohazard 2 facilities within the Biological Services Unit of The University of Manchester (this site holds a Certificate of Designation).

2.1.2 Animal Model

Mice used in this study, male CD1 mice (an outbred strain that is very similar to Swiss mice) were supplied by Charles River (Margate UK) and were specific pathogen free (16-18g at delivery). All mice weighed 20-22g at the time of immunosuppression.

Mice were housed in individual ventilated cages (IVCs) that are supplied with HEPA filtered air. Sterile aspen chip bedding was supplied in pre-autoclaved boxes. Sterile water was provided ad libitum using disposable pouches. Standard mouse chow was provided ad libitum (food was moistened into mash if mice demonstrated signs of sepsis).

Mice experienced a 12hour light dark cycle at 22±1^{o}C, 55-60% relative humidity and background noise of <60db.

Animals were treated using either 30G disposable 'insulin' Monojects (for iv or ip administrations) or reusable 19G gavage needles.

All animals were immunosuppressed with a single dose of 200mg/kg cyclophosphamide ((Pharmacia) ip 3 days before infection. This results in a profound state of neutropenia lasting for 3-4 days post infection (detailed data available on request).

2.1.3 Experiment Duration

The experiment was continued till 53 hours post infection.

2.1.3 Animal Group Size

For the combination study animals were treated in groups of 4 mice per treatment group.

2.1.4 Infection

Candida albicans FA7070 demonstrates high level resistance to fluconazole *in vitro* (MIC 128mg/L when tested using CLSI M28A2 and EUCAST methods). *In vivo* the strain demonstrates moderate levels of resistance (compared to fully susceptible strains) and should be considered susceptible-dose-dependent (SDD).

Mice are infected with 0.2ml of a suspension of FA7070 in PBS + 0.05% tween 80 containing 1.5 x 10^5 blastoconidia/ml i.e. 3.0 x 10^4 yeasts per mouse. Following infection all mice were observed at least 4 times daily. Animals exceeding the severity band of the experiment (substantial) were humanely euthanized.

Endpoints observed in this study are listed in Appendix 1 and 2

2.1.5 Antifungal Treatment

Mice were treated 5 hours post infection with either:

a) 0.125ml of 2% CHD-FA administered by gavage (assuming mice are 25g at the time of treatment). CHD-FA was administered twice daily (total of 6 doses administered).
b) 0.125ml of 0.5% CHD-FA administered by gavage (assuming mice are 25g at the time of treatment). CHD-FA was administered twice daily (total of 6 doses administered).
c) 10mg/kg fluconazole administered intravenously in 5% glucose (in 0.25ml)
d) Combination therapy of 0.125ml of 2% CHD-FA administered twice daily orally and 10mg/kg fluconazole administered intravenously in 5% glucose.
e) Combination therapy of 0.125ml of 0.5% CHD-FA administered twice daily orally and 10mg/kg fluconazole administered intravenously in 5% glucose.
f) 0.5mg/kg amphotericin B (diluted in 5% glucose) administered intraperitoneally.
g) Vehicle treated mice will be given 0.125ml of 0.9% saline by gavage administered twice daily and 0.25ml 5% glucose administered intravenously.

2.1.6 End of Animal Experiment

53 hours post infection all animals were euthanized using a schedule 1 procedure. All animals were weighed; kidneys were removed immediately and homogenized in ice cold sterile phosphate buffered saline. Kidney homogenates were quantitatively cultured onto Sabouraud Dextrose agar and incubated at 37^{o}C for up to 4 days and colonies counted.

2.1.7 Statistical Analysis

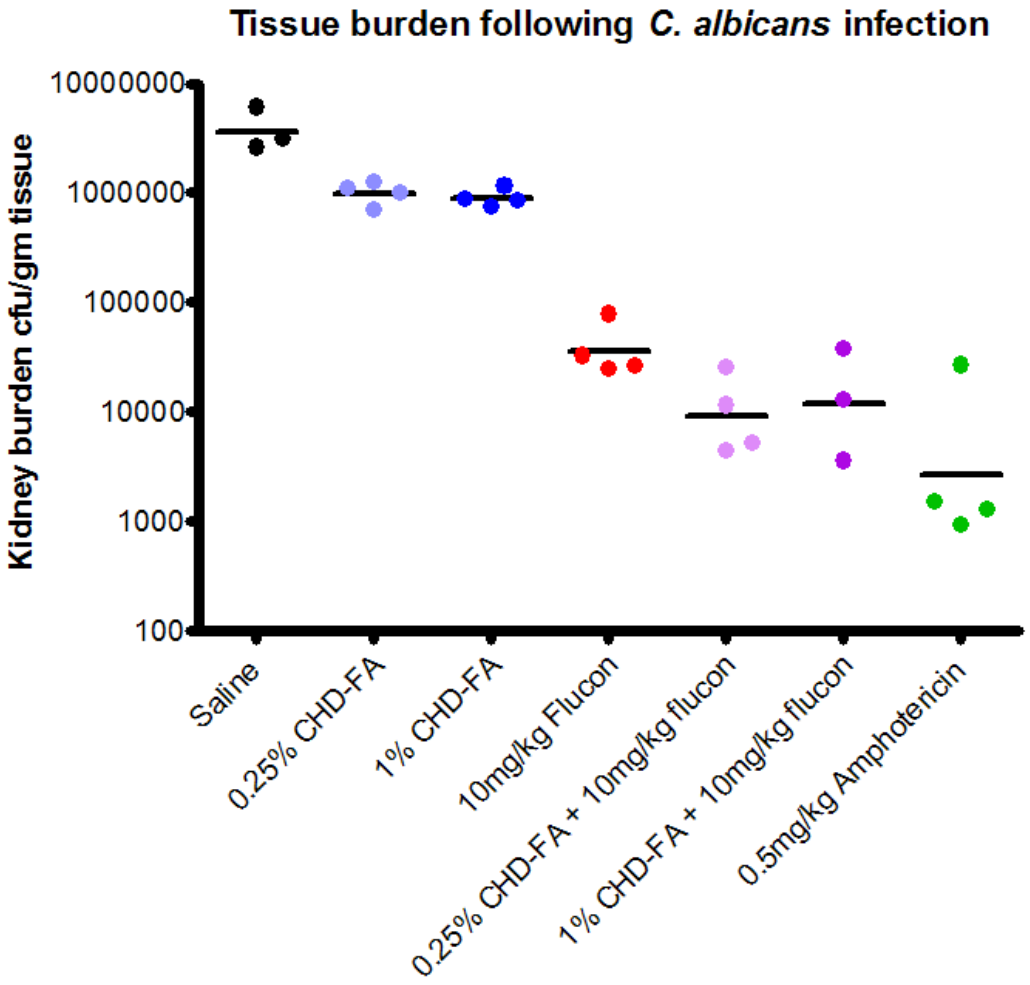
The data from the culture burdens was analyzed by the Kruskal-Wallis test using Stats Direct.

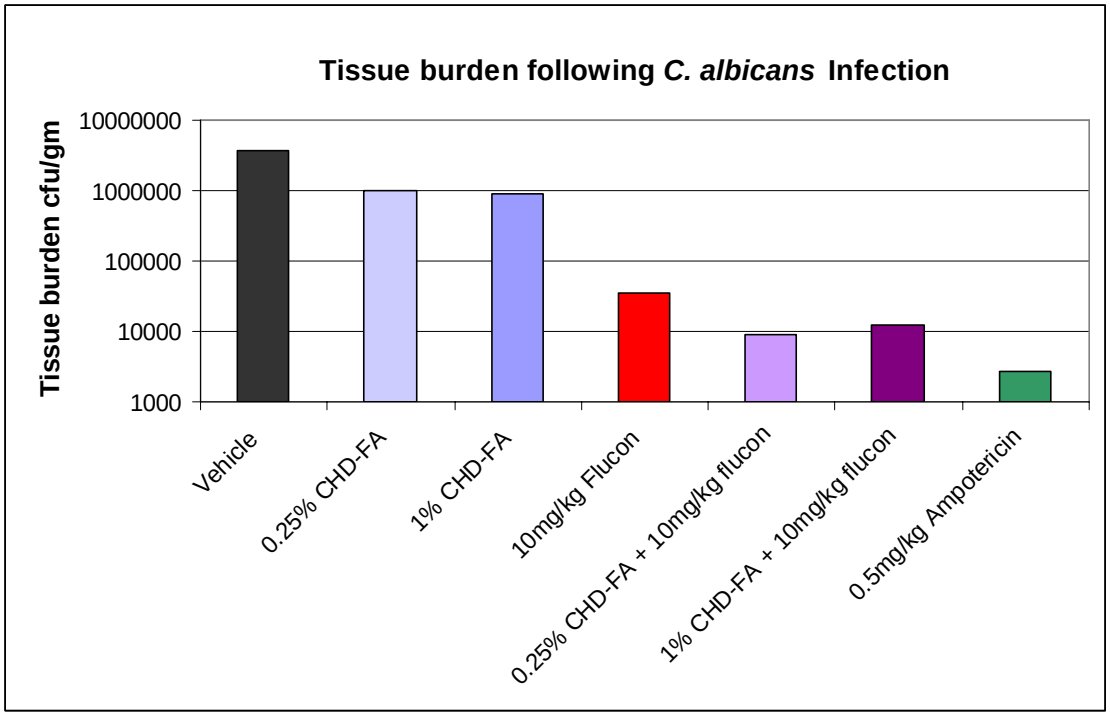
3.1 RESULTS

3.1.1

A summary of the kidney burdens is detailed in figure 1 and 2 and table 1 (full data is in appendix).

In this study there were no side effects noted after treatment with CHD-FA and study was ended at 53 hours post infection due to severe infection in the vehicle treated group.


Tissue burden following C. albicans infection
Kidney burden cfu/gm tissue
10000000
1000000
100000
10000
1000
100
Saline
0.25% CHD-FA
1% CHD-FA
10mg/kg Flucon
0.25% CHD-FA + 10mg/kg flucon
1% CHD-FA + 10mg/kg flucon
0.5mg/kg Amphotericin



Treatment	Vehicle	0.25% CHD-FA	1% CHD-FA	10mg/kg Fluconazole	0.25% CHD-FA + 10mg/kg Fluconazole	1% CHD-FA + 10mg/kg Fluconazole	0.5mg/kg Amphotericin
Kidney Burden (cfu/gm)	3663064	999605	896813	35867	9057	12032	2644
Kidney Burden Log_{10} cfu/g	6.564	6.000	5.953	4.555	3.957	4.080	3.422
Reduction from vehicle	0.000	0.564	0.611	2.009	2.607	2.484	3.142

3.1.3. Statistical Analysis

Kruskal-Wallis: all pairwise comparisons (Conover-Inman)

n.b. due to the small group sizes statistical analysis is difficult but the following were calculated

	Saline	0.25% CHD-FA	1% CHD-FA	10mg/kg Fluconazole	0.25% CHD-FA + 10mg/kg fluconazole	1% CHD-FA + 10mg/kg fluconazole	0.5mg/kg Amphotericin
Saline		NS (0.06)	0.028	<0.0001	<0.0001	<0.0001	<0.0001
0.25% CHD-FA			NS (0.67)	0.0026	<0.0001	<0.0002	<0.0001
1% CHD-FA				0.007	<0.0001	0.006	<0.0001
10mg/kg Fluconazole					0.044	NS (0.19)	0.004
0.25% CHD-FA + 10mg/kg fluconazole						NS (0.51)	NS (0.29)
1% CHD-FA + 10mg/kg fluconazole							NS (0.11)
0.5mg/kg Amphotericin							

NS = not significant

4.1. SUMMARY

- Experiments were established using 0.125ml gavages of 2% and 0.5% CHD-FA twice daily (equivalent to 1% and 0.25% CHD-FA administered at 10ml/kg).

- 5ml/kg of 2% or 0.5% CHD-FA (equivalent to 10ml/kg at 1% and 0.25% CHD-FA) was well tolerated in mice.

- 5ml/kg of 2% or 0.5% CHD-FA (equivalent to 10ml/kg at 1% and 0.25% CHD-FA) was effective at reducing the kidney burden on mice infected with *Candida albicans*. The burden following treatment was significantly lower than vehicle treated mice (~0.6 $\log_{10}$ cfu/gram reduction)

- 5ml/kg of 2% or 0.5% CHD-FA (equivalent to 10ml/kg at 1% and 0.25% CHD-FA) was additive when used in combination with fluconazole. The combined reduction in tissue burden was significantly superior to either treatment used as monotherapy.

Dr Peter Warn

Director Euprotec