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Fungi scope

Assessment of the in vitro efficacy of CHD-FA on different yeasts and filamentous moulds

Final report for Fulhold Ltd.

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Methods

All tests were performed using CHD-FA (carbohydrate derived fulvic acid) provided by Fulhold Ltd. as a 4% or 8% solution and stored at room temperature in the dark.

Strains
All strains were isolated from cases of invasive human diseases (Fungiscope collection)

1. Zygomycetes

   a) *Rhizopus oryzae*
   b) *Myocladus ramosus (=A. corymbifera)*
   c) *Mucor circinelloides*
   d) *Rhizomucor pusillus*

2. Fusarium

   a) *F. solani*
   b) *F. incarnatum*
   c) *F. verticilloides*

3. Scedosporium

   a) *S. prolificans*
   b) *S. apiospermum*
   c) *S. apiospermum*

4. Yeasts

   a) *T. asahii*
   b) *G. capitatum*
   c) *Rhod. mucilaginosa*

Determination of the minimal inhibitory concentration (MIC)

All moulds were cultured on Sabouraud dextrose agar or oatmeal agar for 2 to 14 days at 30°C until they sporulated readily. Spores were harvested by adding sterile aqua dest. supplemented with 0,1% Tween 20 to the cultures. Yeasts were cultured on Sabouraud dextrose agar or chocolate agar for 24h at 37°C.
Inoculum density was determined in a haemocytometer chamber and adjusted to a final inoculum between $1x10^5$ CFU/mL and $2.5x10^5$ CFU/mL.

MIC was determined following EUCAST recommendations using RPMI 1640 supplemented with glucose to a final concentration of 2% and 3-(Morpholino) propanesulfonic acid (MOPS) at a final concentration of 0.165 mol/L. The pH was adjusted to 7.0.
All tests were performed in duplicates with control of the inoculum.

Assay conditions

Sterile, 96-well flat bottom plastic microtiter plates were used for all tests.

Dilution of CHD-FA
Column 1 contained 200µl of 4% CHD-FA (native pH or pH 7.0), columns 2-7 contained 100µl of distilled water.
100µl of CHD-FA from column 1 were transferred with a multichannel pipette to column 2 and thoroughly mixed, before 100µl were then transferred to the next well, resulting in a 2 fold dilution.
CHD-FA was tested at its native pH of 2.1-2.2 or after the pH was adjusted to 7.0 by addition of 10 M NaOH.

Addition of RPMI 1640
50µl of 4x RPMI 1640 was added to each well.

Addition of spore suspensions
50µl of a spore suspension containing 4 - 10 x 10^5 CFU/ml was added, resulting in a final spore concentration of 1 - 2.5 x 10^5 CFU/ml. Inoculum controls as well as sterility controls were done for each isolate tested.

Incubation and reading of plates
Plates were incubated for 24-48h at 36°C, reading was done visually at complete growth inhibition.

Results

1. Zygomycetes

Isolate	Identification	MIC pH 2.1	MIC pH 7.0
I	*M. circinelloides*	0,5%	1%
II	*R. oryzae*	1%	1%
III	*Rhizom. pusillus*	0,25%	0,5%
IV	*Myocl. ramosus*	0,25%	0,25%
V	*R. oryzae*	0,25%	0,25%
VI	*Myocl. ramosus*	0,5%	0,5%

All strains of zygomycetes tested were inhibited by 1% or less of CHD-FA. Differences between pH 2.1 and pH 7.0 were observed for one strain of *M. circinelloides* and one strain of *R. oryzae*.

2. *Fusarium* spp.

Isolate	Identification	MIC pH 2.1	MIC pH 7.0
I	*F. verticilloides*	0,5%	1%
II	*F. incarnatum*	1%	1%
III	*F. solani*	1%	1%
IV	*F. solani*	2%	2%
V	*F. solani*	0,5%	2%

All strains were inhibited at a concentration of CHD-FA of 2% or less.

4. *Scedosporium* spp.

Isolate	Identification	MIC pH 2.1	MIC pH 7.0
I	*S. prolificans*	0,25%	1%
II	*S. apiospermum*	0,5%	1%
III	*S. apiospermum*	0,5%	1%

The *Scedosporium* spp. isolates tested were all inhibited by 1% CHD-FA at pH 7.0, with lower MICs at pH 2.1.

5. Yeasts

Isolate	Identification	MIC pH 2.1	MIC pH 7.0
I	*T. asahii*	1%	2%
II	*G. capitatum*	0,5%	2%
III	*Rhod. mucilaginosa*	1%	2%
IV	*T. asahii*	1%	1%
V	*T. asahii*	1%	2%

All yeasts were inhibited by CHD-FA 0,5-1 at pH 2.1. At pH 7.0 the MICs increased to 2,0% for almost all isolates; after 48h of incubation, MICs increased to >2% for all isolates.

Effect of human serum on the antifungal activity of CHD-FA
In order to assess the efficacy of CHD-FA in the presence of human serum, 2 isolates were tested. Instead of 50µl 4x RPMI 1640, 25µl human serum and 25µl RPMI 1640 were used.

At pH 2,1 there was no MIC difference when two *Scedosporium* spp. isolates were tested with or without human serum. At pH 7,0 however the MIC increased by the factor 2.

Effect of hydrochloric acid on fungal growth
To exclude the inhibition of growth by the acid alone/the pH, hydrochloric acid at pH 2.1 and pH 5.5 was also tested against a strain of R. oryzae and M. circinelloides. No inhibitory effect of HCl at pH 2.1 and 5.5 was noted. At the same pH, CHD-FA inhibited growth at 1% and 0,5%, respectively. The antifungal effect of CHD-FA

therefore does not seem to be related to pH alone. This is furthermore underlined by the inhibitory effect of CHD-FA at pH 7.0.

Conclusion

CHD-FA was effective *in vitro* against all fungi tested. Its antifungal activity was better at its native pH of 2.1 for most isolates than at pH 7.0.
CHD-FA remained active against *Scedosporium* spp. when tested in presence of 12,5% human serum; however, the MIC rose at pH 7.0 when compared to the results without serum.

Outlook

The results show good antifungal activity of CHD-FA *in vitro* against a broad range of medically important fungi. Depending on the species, the MIC obtained was between 0,25% and 2%. It remains to be established if these levels can also be achieved *in vivo*.
Before a possible use the levels of CHD-FA that can be reached *in vivo* have to be determined. This will be crucial before any approval as a drug will be granted.

If effective levels can be achieved *in vivo*, CHD-FA has to be tested in animal models of fungal disease.

Furthermore, the exact mechanism of CHD-FA's effect on fungi has to be established.

Other tests which should be completed are

- Synergy testing with other antifungal drugs (Amphotericin B, Voriconazole, Caspofungine)

Other promising options are the topical use of CHD-FA in cutaneous fungal infections or in mucositis.