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Development of a novel *in vitro* onychomycosis model for the evaluation of topical antifungal activity.

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ABSTRACT

A novel *in vitro* onychomycosis model was developed to easily predict the topical activity potential of novel antifungal drugs. The model encompasses drug activity and diffusion through bovine hoof slices in a single experimental set-up. Results correspond well with the antifungal susceptibility assay and Franz cell diffusion test.

Keywords: onychomycosis, topical antifungals, drug evaluation

Onychomycoses are fungal nail infections responsible for 50% of all nail dystrophies and mostly caused by dermatophytes, of which *Trichophyton rubrum* and *T. mentagrophytes* are the most important ones. Although rarely life-threatening, the high incidence and associated morbidity make them an important public health concern (Gupta et al., 2000; Sigurgeirsson and Baran, 2013). Treatment can be oral or topical with the latter having the advantage of lack of systemic exposure, avoiding drug interactions and liver toxicity. Unfortunately, topical application is often not satisfactory, particularly due to poor penetration of the antifungal through the nail plate (Gupta et al., 2013). As such, convenient models are necessary to evaluate the biological activity potential of topical antifungals. Since current *in vivo* models with guinea pigs and rabbits are extremely time-consuming, labour-intensive and expensive, they are less suited for early drug testing, making *in vitro* models an interesting alternative (Shimamura et al., 2011; Tatsumi et al., 2002). Currently available *in vitro* models investigate drug activity against onychomycosis in the presence of nail powder, however often without penetration of the compound through a keratin membrane, which is necessary to evaluate topical antifungals (Nowrozi et al., 2008; Osborne et al., 2004; Schaller et al., 2009). Within this respect, we aimed to develop a reliable, fast and inexpensive *in vitro* onychomycosis model to assess the activity of topical drugs upon penetration through the nail plate. Reference compounds with known antifungal activity e.g. terbinafine (Sigma-Aldrich, Belgium), fluconazole (Sigma-Aldrich, Belgium), itraconazole (Janssen, Beerse, Belgium) and amorolfine (TCI Europe, Belgium) were used to evaluate the new onychomycosis model against the standard broth microdilution assay for antifungal susceptibility and the Franz diffusion assay for penetration.

48 The *in vitro* susceptibility was determined using the microdilution assay in
49 accordance to CLSI guidelines M38-A2 (Cos et al., 2006; Espinel-Ingroff and Pfaller,
50 2007). Briefly, a 2-fold serial dilution of all test compounds was made and incubated
51 with a fungal suspension of 5 x 10^3 CFU/ml. After incubation, the minimum inhibitory
52 concentration (MIC) in µg/ml was determined by visual inspection of the wells. Three
53 independent replicates were analysed for each compound. Terbinafine and
54 amorolfine showed the highest activity against *T. mentagrophytes* with a MIC of 0.04
55 µg/ml and 0.32 µg/ml respectively, and against *T. rubrum* with a MIC of 0.11 µg/ml
56 and 0.03 µg/ml. Itraconazole proved to be active against both dermatophytes (MIC <
57 0.75 µg/ml), whereas fluconazole only demonstrated poor activity (MIC > 15 µg/ml).
58 Activities were comparable with those found in literature (de Wit et al., 2010; Jo Siu et
59 al., 2013).

60 To determine the penetration of terbinafine, fluconazole, itraconazole and amorolfine,
61 11.28 mm unjacketed Franz diffusion cell (Permegear inc, Hellertown, Pennsylvania)
62 (Vejnovic et al., 2010) were used in conjunction with bovine hoof slices, an
63 appropriate alternative for human nails (Mertin and Lippold, 1997). Concentrations in
64 the acceptor compartment were measured with LC-MS^2 (Waters Acquity UPLC with
65 XEVO TQ mass spectrometer and Acquity UPLC BEH C18 column). Fluconazole,
66 amorolfine and terbinafine did penetrate the slices with cumulative penetrated
67 amounts after 10 days of 2.14 ± 0.37 µg/cm^2, 0.29 ± 0.37 µg/cm^2 and 0.30 ± 0.67
68 µg/cm^2 respectively (Table 1). Concentrations of itraconazole remained below the
69 detection limit (< 1 ng/ml).

70 Since the actual biological potential of a formulation against onychomycosis is difficult
71 to assess with two independent assays, we developed a new onychomycosis model
72 combining drug activity and penetration (Figure 1). Bovine hoof slices with

approximate thickness of 600 µm were mounted in a polypropylene screw vial cap with an opening of 5 mm diameter. A rubber ring was placed on the surface of the slice to prevent leaks and tightened on a screw vial (Filter Service, Belgium). The vial was cut beneath the neck to create a reservoir to apply compounds onto the hoof slice. Next, the mounted hoof slices were placed on a Sabouraud dextrose agar (SDA), serving as a surrogate for the nail bed. To preload with test compound, 50 µl of 20 mg/ml test compound in DMSO was added on the surface of the hoof slice and daily refreshed for 7 consecutive days. Pure DMSO was used as vehicle control. After preincubation, the vial cap was transferred to a freshly inoculated *T. mentagrophytes* B70554 or *T. rubrum* B68183 (Scientific Institute of Public Health, Brussels, Belgium) (10^5 CFU) SDA and test solutions were applied daily for another 7 days. During the whole experiment, agars were incubated at 27°C. Seven days post-inoculation, the inhibition zone was calculated as the area of inhibition relative to the total area of growth (vehicle) using ImageJ 1.48 software (Java-based freeware for advanced image processing). To identify possible leaks at termination of the experiment, vial caps were transferred to a non-inoculated agar and filled with a methyl blue formulation for another 7 days. Due to the large molecular weight, methyl blue (800 g/mol) will only penetrate the hoof slices in case of leaks, confirmed by a clear blue diffusion area in the agar.

Using the new onychomycosis model, terbinafine and amorolfine demonstrated growth inhibition against *T. mentagrophytes* (33.7 ± 6.4% and 22.4 ± 6.5%) and *T. rubrum* (38.5 ± 6.3% and 72.3 ± 27.3%) (Figure 2). The stronger activity of amorolfine against *T. rubrum* correlated nicely with the higher antifungal potency as determined with the microdilution test. For fluconazole, no inhibition zone could be found, although it penetrated the hoof to higher extend compared to terbinafine and

amorolfine. The low activity of fluconazole (MIC > 15 µg/ml) may explain the observed lack of growth inhibition. Itraconazole did not demonstrate growth inhibition despite its high potency against dermatophytes (MIC < 0.75 µg/ml). This discrepancy can be explained by its inability to penetrate the bovine hoof slices as clearly demonstrated with the Franz diffusion cell. DMSO alone had no influence on dermatophyte growth. No variability could be found between assays ($p > 0.05$, unpaired t-test, 3 replicates x 2 experiments). Only one experiment demonstrated diffusion of methyl blue and was excluded from the results.

To evaluate novel antifungal formulations, Lusiana et al. recently developed an *in vitro* model using infected keratin films of human hair (Lusiana et al., 2013). Although this model demonstrated promising results, no comparison with reference compounds and standard tests for antifungal evaluation were performed.

In conclusion, the present findings support a good correlation between the novel onychomycosis model and the standard susceptibility and Franz diffusion cell assays. Being inexpensive, fast and accurate, the proposed onychomycosis model can be regarded as a useful tool to assess biological activity potential of novel formulations and onychomycosis drugs.

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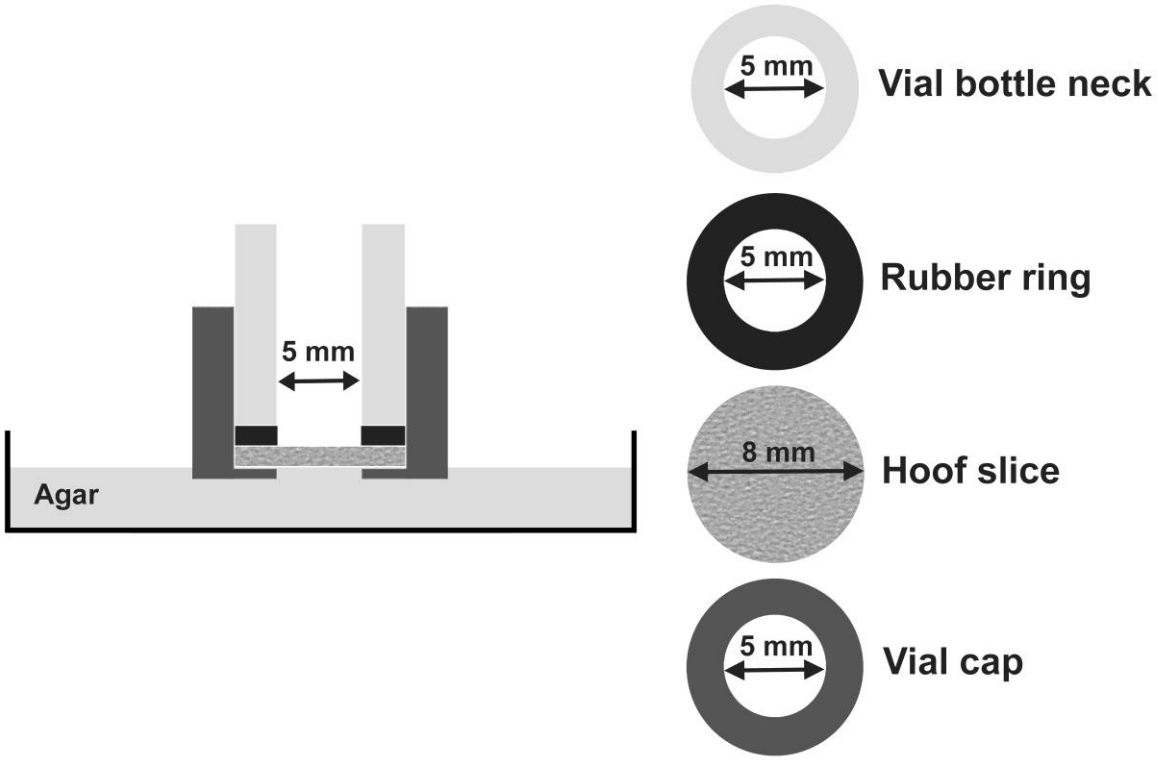
Table 1: Cumulative permeated amount of drug diffused through bovine hoof slices after a single dose of 1 mg/ml terbinafine, itraconazole, fluconazole and amorolfine solution.

Time (days)	Cumulative permeated amount (µg/cm²)			
	Terbinafine	Itraconazole	Fluconazole	Amorolfine
0	ND	ND	ND	ND
1	ND	ND	0.04 ± 0.09	ND
2	ND	ND	0.06 ± 0.13	0.07 ± 0.14
4	ND	ND	0.08 ± 0.19	0.11 ± 0.20
7	0.20 ± 0.45	ND	0.84 ± 1.68	0.18 ± 0.31
10	0.30 ± 0.67	ND	2.14 ± 3.74	0.29 ± 0.37

Values are represented as mean ± SD of 2 independent repeats (n=3). ND: not detected because the concentrations in the acceptor compartment were below the lower limit of detection (<1 ng/ml).



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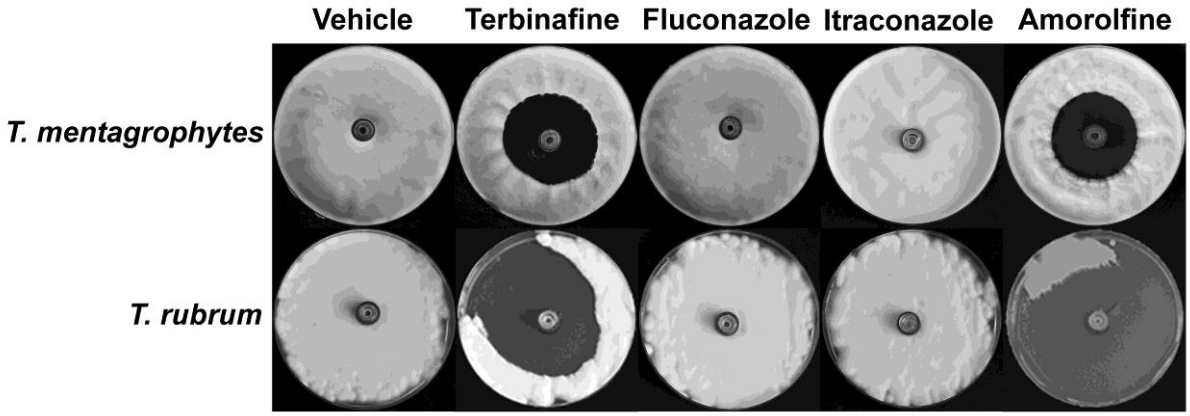
171 Figure 1: Schematic overview of the *in vitro* onychomycosis model on SDA (left) with detailed
172 information of the components of the model (right).



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174 Figure 2: Activity of vehicle (DMSO) and 20 mg/ml solutions of terbinafine, fluconazole, itraconazole
175 and amorolfine against *T. mentagrophytes* and *T. rubrum* on SDA after 7 days of
176 incubation (n=6).