Zeamatin, clotrimazole and nikkomycin Z in therapy of a
Candida vaginitis model

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Objective: To study the interaction of antifungal drugs in topical therapy.

Materials and methods: Local therapy of Candida vaginitis with drugs alone and in combination
was examined in a murine model. Zeamatin, a natural plant-derived antifungal protein, was
tested alone and in combination with an azole, clotrimazole or nikkomycin Z, a chitin synthase
inhibitor.

Results: Whereas alone, zeamatin was ineffective, nikkomycin Z was effective only when dosed
multiple times per day, and clotrimazole efficacy was variable when administered in exper-
imental vehicles (unlike the complex and undefined commercial preparation), zeamatin enhanced
the efficacy of either of the other two drugs when they were given in combination.

Conclusion: Drug interactions between novel drugs with unique mechanisms of action should
be explored further, and may lead to more potent regimens.

Introduction

Zeamatin is a 22 kDa protein abundantly produced in corn,
which apparently protects the plant against fungal patho-
gens.1 It appears to act via a unique antifungal mechanism,
which may involve the link of the cell wall and membrane,
binding to the former and disrupting the latter by a non-
enzymatic method, resulting in osmotic rupture of the cyto-
plasm.1 Nikkomycin Z (nZ),2 an inhibitor of the synthesis of
chitin, which is a cell wall component, is known to interact
synergically in vitro with zeamatin,3 and recently zeamatin
synergy with azole drugs, which block synthesis of membrane sterol, was shown in systemic therapy against murine candidiasis.1 The present studies were undertaken to explore drug interactions in topical therapy against candidiasis.

Materials and methods

In vivo studies

All protocols were approved by the California Institute for
Medical Research Animal Care and Use Committee. Five-
week-old BALB/c mice (Charles River Labs, Wilmington,
MA, USA) (16–18 g) were used in these studies. Ten mice per
experimental or control group were used. Preliminary studies
indicated no diminution in this strain in vaginal infection
between day 1 and day 6 after challenge, in contrast to outbred
CD-1 mice (Charles River Labs).4 Three days prior to infection
and on day 4 post-infection, mice were given 0.5 mg of estra-
diol valerate subcutaneously to induce and maintain pseudo-
estrus. On day 0, mice were anaesthetized with 80 mg/kg
eketamine hydrochloride, intraperitoneally, then inoculated

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intravaginally with 20 µL of a suspension of *Candida albicans*, 2.5 × 10⁶/mL, in RPMI-1640 medium with streptomycin and penicillin. Two *C. albicans* isolates were used in these studies, isolates 5 and SC9172; this was carried out to confirm that any activity seen was not confined to one isolate.

The drugs were received as pure powders: clotrimazole (Alza Corp., Palo Alto, CA, USA), nZ (Shaman Pharmaceuticals, South San Francisco, CA, USA), itraconazole (Janssen Pharmaceutica, Beerse, Belgium), fluconazole (Pfizer Corp., Groton, CT, USA) and D0870 (Zeneca Pharmaceuticals, Macclesfield, UK). Zeamatin was purified from corn. Those agents used for intravaginal studies were suspended directly in high-viscosity carboxy-methyl cellulose (CMC), 15 mg/mL in 20 mM NaCl (vehicle) at the desired concentration. The 20 mM NaCl component is required for expression of zeamatin activity.

In preliminary experiments, vaginas were swabbed 1 day later, prior to treatment, to assure infection was evenly distributed between groups. Each alginate swab was placed in 0.4 mL sterile PBS to dissolve the swab, serial 10-fold dilutions made, and 50 µL plated in duplicates onto Sabouraud Dextrose Agar plates with chloramphenicol to quantify the cfu/mL. Typically, the log₁₀ geometric mean cfu/mL at this time was between 4 and 6. Except where otherwise specified, treatments were given by intravaginal instillation of 20 µL of drug or combination, in the vehicle, for five consecutive days. Initial experiments established that treatment with this vehicle alone produced the same results as no treatment (the number of cfu/mL on day 6 was not different from on day 1); subsequent comparisons were made with the vehicle as the control. On day 6, 24 h after the last treatment, the infection was quantified by swabbing, as described above. Such methods of quantifying infection correlate with assessing whole-organ infection by excision and grinding.

**In vitro studies**

Macrodilution determinations of the MIC and minimum fungicidal concentration (MFC) were performed in broth (RPMI-1640) as described previously. Qualitative drug interaction studies using carrot extract medium were performed as described previously.

**Statistics**

Comparisons were performed using the Mann–Whitney *U*-test, with significance set at *P* < 0.05.

**Results**

**Synergy of nZ and zeamatin**

In experiments with isolate SC9172, synergy was demonstrated between nZ and zeamatin. Two such experiments are shown in Table 1. In Experiment 1, given once daily, nZ alone, and particularly zeamatin alone, produced a slight diminution in vaginal infection, but this was insignificant; however, the combination produced a significant result. The infection after single drug regimens was 2.5–4.4 times greater than after combination therapy.

In Experiment 2, both 10 and 5 mg/mL nZ, when combined with 32.3 mg/mL zeamatin, produced significant diminution of infection compared with the control, whereas neither 10 nor 5 mg/mL nZ, nor zeamatin alone, produced a significant result. In additional experiments, the lack of efficacy of nZ 30 or 10 mg/mL alone once daily, or zeamatin alone, was confirmed.

However, when nZ was administered three times a day, it was effective (isolate 5) alone, and in that circumstance potentiation by zeamatin as above could not be shown. At 20 mg/mL nZ alone, infection was reduced compared with controls, and while the combination regimen was effective (*P* = 0.04 versus control), it was not significantly different from nZ alone. Zeamatin alone was ineffective, even when given three times a day. Zeamatin (or the diluent alone) cleared the infection in no animals, nZ alone cleared one, and the combination cleared three of 10.

**Synergy of clotrimazole and zeamatin**

Clotrimazole also acted synergically with zeamatin. In a study with isolate SC9172, clotrimazole given once daily had a modest effect in reduction of the number of cfu at 20 mg/mL (*P* < 0.05) but had no effect at 5 mg/mL, and zeamatin (32.3 mg/mL) given once daily had a slight but non-significant effect. However, clotrimazole 20 or 5 mg/mL plus nZ produced significant diminution in vaginal infection, but this was insignificant; however, the combination produced a significant result. The infection after single drug regimens was 2.5–4.4 times greater than after combination therapy.

**Table 1. Synergy of nZ and zeamatin, given daily**

<table>
<thead>
<tr>
<th></th>
<th>Day 6 vaginal log₁₀</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>geometric mean cfu/mL</td>
<td>95% CI</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control (diluent)</td>
<td>5.3</td>
<td>4.6–6</td>
</tr>
<tr>
<td>nZ 20 mg/mL</td>
<td>5.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.4–5.7</td>
</tr>
<tr>
<td>zeamatin 32.3 mg/mL</td>
<td>4.82&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.1–5.5</td>
</tr>
<tr>
<td>nZ and zeamatin</td>
<td>4.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.7–5.1</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control (diluent)</td>
<td>5.59</td>
<td>5–6.2</td>
</tr>
<tr>
<td>nZ 10 mg/mL</td>
<td>5.19&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.9–5.5</td>
</tr>
<tr>
<td>zeamatin 32.3 mg/mL</td>
<td>5.38&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.1–5.7</td>
</tr>
<tr>
<td>nZ 5 mg/mL and zeamatin</td>
<td>5.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.8–5.4</td>
</tr>
<tr>
<td>nZ 10 mg/mL and zeamatin</td>
<td>4.98&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>4.7–5.3</td>
</tr>
</tbody>
</table>

CI, confidence interval.

<sup>a</sup>Not significantly different from control (*P* > 0.05).

<sup>b</sup>Significantly better than control (*P* = 0.02) and than nZ alone (*P* = 0.03).

<sup>c</sup>Significantly better than control (*P* = 0.02).

<sup>d</sup>Significantly better than control (*P* = 0.002) and than zeamatin alone (*P* = 0.02).
Zeomatin Candida vaginitis drug interactions

Zeomatin was significantly effective, reducing infection 2.47 or 0.7 log₁₀ geometric mean cfu (P = 0.0003 and 0.007), respectively.

An experiment using isolate 5, where the drugs were administered three times a day, is detailed in Table 2. Clotrimazole and zeomatin (a second batch, prepared at 21 mg/mL) alone were ineffective in reducing the mean number of cfu compared with controls, clearing no animals of infection, whereas the combination was effective, and cleared the infection in three of 10 animals.

Although the possibility that the synergy was related to a non-specific effect of a protein, such as by causing better adherence of a drug, seemed unlikely due to consideration of the total protein already present in vaginal secretions or the increased amounts in candidal vaginitis, this possibility was addressed by studying the effects of intravaginal administration of BSA (25 mg/mL) alone and in combination with clotrimazole 20 mg/mL, given three times a day. The albumin alone had no effect on the course of Candida vaginitis, and no effect on the efficacy of clotrimazole in this experiment (clotrimazole alone, 0.70 log₁₀ mean cfu reduction, plus albumin, 1.08 log₁₀ mean cfu reduction, both P < 0.02).

Clotrimazole and other comparative treatments

Of note in these experiments was the inconstant efficacy of clotrimazole, an effective vaginal antifungal in humans. In another experiment, clotrimazole 20 mg/mL given three times a day against isolate 5 was effective, with a 1.97 log₁₀ mean cfu reduction (P = 0.0009). In a fourth experiment, this was reproduced against isolate SC9172, with a 1.97 log₁₀ mean cfu reduction (P = 0.0001), whereas 10 mg/mL clotrimazole produced results of borderline significance. However, in a fifth experiment of similar design, 20 mg/mL clotrimazole produced results of borderline significance against both isolate 5 and isolate SC9172. When given once a day (against isolate SC9172), clotrimazole 5 and 10 mg/mL gave results of borderline significance in one experiment and were ineffective in another, and at 20 mg/mL was effective in one experiment (P = 0.003), but produced results of borderline significance in three additional experiments. Inferior results were obtained with itraconazole 20 mg/mL.

To give a perspective on the combination studies presented above, the results of concurrent studies of the model itself are presented. The following studies all used isolate SC9172. Alternative diluents were explored to assess the effect on clotrimazole efficacy. Clotrimazole 20 mg/mL in Aquaphor (Beiersdorf, Inc., Wilton, CT, USA), a petrolatum-based ointment, gave the same results as with the standard vehicle (15 mg/mL CMC), and in 10 mg/mL dimethylsulfoxide in CMC, the clotrimazole results were markedly inferior. For comparison, the commercial vaginal antifungal preparation of clotrimazole, Gyne-Lotromin (Schering-Plough, Kenilworth, NJ, USA) (10 mg/mL) was tested. Given two or three times a day, the preparation was highly effective (reduction from 5.92 log₁₀ geometric mean cfu/mL to 2.12 or 2.34, P = 0.0004 and 0.0002, and sterilizing five of 10 and four of 10 animals, respectively). This was confirmed in a second experiment (three times a day treatment, P = 0.0001).

For reference, systemic therapy with azoles was compared. Itraconazole 100 mg/kg (in cyclohexatin), fluconazole 100 mg/kg (in water) and D0870 (in 0.5% Tween 80/saline) each given orally once daily, were all highly effective (reductions of 2.44–4.11 log₁₀ mean cfu, P = 0.0001, 0.0001 and 0.0002, respectively).

In vitro studies

The MIC and MFC, respectively, for both C. albicans isolates of clotrimazole were 1 and >16 mg/L, and of zeomatin >50 and >50 mg/L. The MICs of nZ for isolates 5 and SC9172 were 2048 and >2048 mg/L, respectively. Qualitative drug interaction studies confirmed the previously described nZ–zeomatin synergy, but the clotrimazole–zeomatin interaction showed only a weak synergic or indifferent reaction in vitro.

Discussion

These in vivo studies show a synergic interaction between zeomatin and nZ or clotrimazole. Synergy between nZ and azoles in vaginal candidiasis has been shown previously. The top dose of zeomatin studied was limited by drug supply, and it is possible that higher doses might have demonstrated activity of zeomatin alone.

The predictability of classical MIC testing in defined media to outcome in topical therapy, where local administration may greatly exceed the MIC, is unknown. To define this, future studies would require use of pathogens susceptible and resistant in in vitro testing to the drugs to be used in vivo. The qualitative drug interaction studies with zeomatin in undefined medium are also thus far of uncertain predictability.
to in vivo outcome. The results of combination therapy with azole-resistant organisms would also be of interest.

The relative inefficacy of clotrimazole and nizoldalone, and even the combination regimens, is put into perspective by the efficacy of the commercial human preparation of clotrimazole in this model. CMC was used as the vehicle throughout our studies because it could be used to formulate all the drugs, and the complex excipient used in the commercial preparation is not available by itself. CMC lacks intrinsic antifungal activity and does not antagonize the drugs in vitro. It is possible that the components of the commercial vehicle enhance the activity of clotrimazole, explaining the efficacy differences from our own formulation of the same strength clotrimazole. This enhancement could occur via a direct interaction with the active drug, or by enhancing adherence or penetration, or retarding systemic absorption, of the active component. However, we believe it is most likely that the advantage of the commercial excipient is retention of the drug in the vagina; we noted, even after optimizing the volume of drug delivered and the concentration of CMC, that considerable amounts of the preparation would leak from the vagina after application. Thus, it is possible that results could have improved with all the agents and combinations tested if such commercial vehicles were separately available and used.

The positive control results with systemic therapy also emphasize how formidable the obstacles to topical therapy are in this model. The contrast with the ease of topical therapy in human vaginitis is explained by the ascent of the infection to the rodent uterine horns, a locus not reached by topical therapy and which can reinfect the vagina, a situation that does not occur in the human disease. Despite these obstacles, these studies show that zeamatin can potentiate other agents in topical therapy, and moreover, as zeamatin is the first of a class of related plant proteins to be studied in detail as an antifungal, further studies are warranted.

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References