In vitro Susceptibility of the Endospores of *Rhinosporidium seeberi* to Seven Antimicrobial Agents


**ABSTRACT**

*Rhinosporidium seeberi*, the causative agent of human and animal rhinosporidiosis, has not been cultured in vitro, and experimental rhinosporidiosis has not been established. Hence, there have been no available data on its susceptibility to antimicrobial agents. Based on a previous study regarding the use of 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT)-reduction to a formazan as a test of viability of rhinosporidial endospores, MTT-reduction test was used to determine the in vitro susceptibility of the endospores of *R. seeberi* to seven antimicrobial agents in current clinical use on humans and animals.

In this study, the inhibitory concentrations at the 50-percent end-point (IC$_{50}$) (number of experiments) of amphotericin B, dapsone, ketoconazole, trimethoprim-sulfadiazine, sodium stibogluconate, and berenil, and imizol were 57.1 (8), 29.7 (10), 51 (8), 38.4 (9), 55.7 (7), 12.5 (5), 9 (1) μg/mL, respectively. The drug concentration-dependent effects included deformation, enlargement or fragmentation, and decreased staining with formazan of the intra-endosporial electron dense bodies, without abnormalities of the endospore wall. All agents were non-lytic, endosporostatic, and not endosporicidal. The results with amphotericin B, ketoconazole, and especially dapsone, correlated with the clinical responses of patients with rhinosporidiosis, reported in the literature. No correlation of the IC$_{50}$ with the clinical source of *R. seeberi* strain, was observed. This is the first full report on antimicrobial agents active against *R. seeberi*, in vitro. (*J Infect Dis Antimicrob Agents* 2008; 25:135-43.)

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INTRODUCTION

Rhinosporidium seeberi, the causative agent of human and animal rhinosporidiosis, was discovered in 1900. Routine methods for testing in vitro susceptibility of bacteria and fungi to antimicrobial agents, based on indicators of microbial multiplication, cannot be applied on R. seeberi as in vitro cultures of R. seeberi have not been established. Hence, no data have been available on in vitro susceptibility of this human and animal pathogen.

Previous reports in the literature, indicated that dapsone (4, 4’ diaminodiphenyl-sulphone) was the only agent that was clinically active against R. seeberi (with confirmation from histopathology and ultramicroscopy), and its use prevented the relapse of infection. However, dapsone can aggravate acute hemolytic crisis in patients with rhinosporidiosis who are deficient in glucose-6-phosphate dehydrogenase (G6PD) enzyme. Unfortunately, rhinosporidiosis is endemic in Sri Lanka where some individuals also have G6PD deficiency. Therefore, a search for alternative agents active against R. seeberi is needed. The potent agents are also needed to prevent serious complications of ocular rhinosporidiosis especially staphyloma formation and disseminated rhinosporidiosis. 3-

3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) is reduced by mitochondrial dehydrogenases in mammalian and fungal cells to a purple formazan. Parallelism between the inhibition of MTT-reduction and the absence of growth of mycelial fungi in vitro that were exposed to antifungal agents has been previously demonstrated. Reduced-MTT (formazan) has been found to be capable of staining the cytoplasm and electron dense bodies (EDB) of fresh endospores of R. seeberi, and is regarded as an indicator of endospore viability. It was used in this study to assess the effects on R. seeberi of seven different antibacterial, antifungal, and antiparasitic agents. The rhinosporidial endospores are not comparable with the endospores of bacteria; the former are actively metabolizing entities in the life cycle of R. seeberi, that are currently presumed to be the infective stage in rhinosporidiosis. The effects of antiseptics and disinfectants on the viability of rhinosporidial endospores, determined by the MTT-reduction method, were documented previously.

MATERIALS AND METHODS

Buffers

Sterile 0.2 M phosphate-buffered saline (PBS) at pH 7.4 was used for suspending rhinosporidial endospores, and dilution of the drugs or MTT.

MTT

MTT (Sigma, USA) was dissolved in PBS (0.5 mg/mL), and the solution was used fresh, or stored in the dark at -20°C and used within 2 weeks.

Endospores

Only freshly harvested endospores or those briefly stored at 4°C were used. In the absence of sufficient endospores from a single patient for successive tests, endospores from different patients were individually used. This was to avoid using a pool of endospores from many rhinosporidial patients, in case of strain variation in drug susceptibility.

Rhinosporidial endospores were obtained from surgically resected, freshly homogenized nasal and nasopharyngeal rhinosporidial tissues after sieving as described previously, and then resuspended in PBS to a density of approximately 2 x 10^6 endospores/mL. Suspensions were stored at 4°C for no more than 2 weeks, during which their MTT-reducing ability was retained.
Antimicrobial agents

Drugs for therapy in humans

Drugs in powder formulation: amphotericin B (Bristol Myers Squibb Co, USA), dapsone (Novartis, Switzerland), ketoconazole (Derquimica, Spain), and sodium stibogluconate (GlaxoSmithKline, UK).

Drugs as sterile solutions for injection: injectable trimethoprim-sulfamethoxazole for human use was not available; the preparation used in this study was the veterinary preparation Andine (trimethoprim 40 mg/mL, sulfadiazine 200 mg/mL, Anucop, UK).

Drugs mainly for veterinary therapy

Diminazine aceturate, 70 mg/mL injection (Berenil, Trycip CIPLA Ltd., Mumbai, India) and imidocarb dipropionate (12% (injection) (imizole, Essox Animal Health, Friesothe, Germany).

Drug solutions

Amphotericin B, berenil, sodium stibogluconate, and imizol were dissolved/diluted in sterile PBS. Water-insoluble drugs, dapsone and ketoconazole, were first dissolved in dimethyl sulphoxide (DMSO), and then further diluted in PBS with DMSO not exceeding 0.2 percent (Melanie T. Cushion 2002, personal communication). Freshly prepared solutions of the drug were used.

Concentrations of drugs

Based on reported minimal inhibitory concentration (MIC) or inhibitory concentration at the 50-percent end-point (IC50) values (Table 1) to other susceptible bacteria and fungi, ten-fold or five-fold serial dilutions from initial concentrations of 1,000 or 400 μg/mL were used. A wide range of concentrations was used since no previous data on the drug-susceptibility of R. seeberi, or of the members of the Class Mesomycetozoea to which it belongs, have been available.

Effect of warming of drug solutions on IC50

Since variations in IC50 were obtained with water-insoluble drugs, with incomplete dissolution in PBS-DMSO at room temperature as a possible cause, initial and final solutions of dapsone and ketoconazole in PBS-DMSO were, in later experiments, warmed at 50-60°C to promote solubilization.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Reported MIC or IC50 organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>1 μg/mL Candida spp.11</td>
</tr>
<tr>
<td></td>
<td>0.062-2 μg/mL Cryptococcus neoformans var. neoformans14</td>
</tr>
<tr>
<td>Dapsone</td>
<td>&gt; 32 μg/mL Mycobacterium tuberculosis,12 M. fortuitum12</td>
</tr>
<tr>
<td></td>
<td>8 μg/mL M. avium complex, M. kansasii12</td>
</tr>
<tr>
<td></td>
<td>&lt; 10 ng/mL Mycobacterium leprae in mouse foot pad11</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>2 μg/ml Candida spp.11</td>
</tr>
<tr>
<td></td>
<td>0.125-1 μg/mL C. neoformans var. neoformans14</td>
</tr>
<tr>
<td></td>
<td>0.125-4 μg/mL C. neoformans var. gatti14</td>
</tr>
<tr>
<td>Sodium stibogluconate (Pentostam)</td>
<td>0.04 mg/mL-6.31 mg/mL Leishmania isolates13</td>
</tr>
</tbody>
</table>

MIC: minimal inhibitory concentration, IC50: inhibitory concentration at the 50-percent end-point.
Concentration-dependent inhibition/inactivation of endospores and IC₅₀ determination

These tests were performed with only amphotericin B, ketoconazole, dapsone, trimethoprim-sulfadiazine, pure sodium stibogluconate, berenil, and imizol, since these were the only agents that, on preliminary testing, showed in vitro activity against rhinosporidial endospores concentrations below 100 μg/mL that indicated therapeutic usefulness (Melanie T. Cushion 2000, personal communication).

Drug-endospore mixtures

The basic method for testing of drug actions on endospores was as previously described, with biocides.⁹,¹⁰ The tubes with the drug-endospore mixture were incubated at room temperature (28°C) in the dark for 6 days; a room temperature was chosen to minimize growth of inevitable, contaminant microorganisms from the original rhinosporidial tissue, that might have inactivated the drugs.

The MTT-reduction test of metabolic inactivation of endospores

Acid-propanol extraction of formazan in Mosmann’s method⁴ was replaced by direct microscopy of the MTT-treated endospores. This was because the sites of reduction of the MTT and formazan formation (the cytoplasm and EDBs of the endospores) were clearly visible microscopically at x 1,000 magnification under oil immersion.⁸ In addition, host cells and inevitably present contaminant microorganisms in the original rhinosporidial tissue could have contributed to formazan formation, and spuriously enhanced the spectrophotometric optical density of the extracted formazan. The EDB was the focus of interest as the target of inactivation of the endospore since it is regarded as the ultimate generative unit of R. seeberi.¹,¹⁵,¹⁶ After incubation, the tubes were centrifuged at 400 x g, and the drug solution was removed. Approximately 50 μg of the MTT solution was added, and the pellets were agitated for dispersion. After incubation of the endospore-MTT mixtures at 37°C in air for 3 hours, one drop was placed on a microscope slide under a coverslip for microscopy at x 1,000, under oil. At least 100 endospores in each sample were examined. The percentage inhibition of the test samples minus the percentage loss of viability of the control endospores incubated in parallel, was calculated as the corrected percentage inhibition (CPI). At least 100 endospores from each sample for estimation of the degree of inactivation were used rather than counting of a total of 100 endospores from replicate tubes.

Tests for agent-induced lysis of endospores

The validity of the quantitative, microscopic MTT-reduction method depends on the fact that free rhinosporidial endospores, though in a vegetative state, do not replicate; R. seeberi is propagated only by endosporulation within its mature sporangium. Drug-induced lysis would have invalidated the counts of viable/non-viable endospores. Tests for lysis, in triplicate were thus done with the drugs, at 100 μg/ml. After incubation for 3 days at room temperature, counts of the drug-treated endospores, and endospores in DMSO-PBS, or PBS as untreated controls, were made in an improved Neubauer haemocytometer, for the comparison of means by the student T test.

Indices of inhibition/inactivation of endospores

The normal endospore has approximately 10-16 spherical bodies of which about half that number are the MTT-positive EDBs, while the rest are MTT-negative, lipid-containing bodies.¹ The following features were used in order of priority as indices of inactivation:
Morphological: enlargement with deformation, fragmentation, or loss of the EDBs. Morphological abnormalities were sometimes present despite staining, though decreased, with formazan.

Tinctorial: Decrease or loss of MTT-reducing capability and formazan-staining that was indicated by a pale pink colour rather than a deep purple colour of the formazan in normal endospores.

Indices of potentially useful agents

Proportionality of the CPI with increasing drug concentration, and an IC$_{50}$ of 100 μg/mL or less (Melanie T. Cushion 2000, personal communication) were used to identify potentially useful drugs.

End point of anti-rhinosporidial activity

The concentration of each drug that caused inactivation of 50 percent (IC$_{50}$) of the endospores was read off a log-concentration versus the CPI graph.

Tests for determining whether the agents were endosporicidal or endosporostatic

The endospores in parallel tubes, with the drugs at 100 μg/mL, with drug-free endospores in PBS or with DMSO-PBS as appropriate controls, incubated for 3 days at room temperature were deposited by centrifugation and then washed thrice with sterile PBS, finally resuspended in sterile PBS as recovery medium, and left static at room temperature for more than 4 days after which the endospores were examined for MTT-reducing capability as described above. One set of tubes was used for the MTT-reduction test after drug exposure for measurement of CPI for IC$_{50}$ determination, and replicate tubes were used for the MTT-reduction test after drug removal and reincubation in PBS.

RESULTS

pH of drug solutions

The pH of the initial drug dilutions at the maximum concentration in PBS pH 7.4, were within the range 7.26-7.47.

MTT-reduction by control endospores

Endospores in control samples, in PBS alone or in PBS containing 0.2 percent DMSO, at the end of the incubation period, retained their cytoplasmic and EDB staining with formazan.

General comments on effects of effective anti-rhinosporidial agents

The effects were on their contents, notably the EDBs in their size and morphology, and the intensity of reduction of MTT. The EDBs were deformed or fragmented into small bodies; these effects were drug concentration-dependent. None of the drugs that caused metabolic inhibition/inactivation, produced abnormalities in the walls of the endospores, as caused by biocides.$^9,10$

Lysis of endospores by five agents for human use, with anti-rhinosporidial effects

No evidence of lysis (i.e. no statistically significant fall in counts) of the endospores by amphotericin B, dapsone, ketoconazole, trimethoprim-sulfadiazine, or sodium stibogluconate, tested in triplicate, in duplicate experiments, in comparison with control samples was evident for these five agents for use on humans.

IC$_{50}$ values of the seven agents

The ranges and means ± SD of the IC$_{50}$ of the seven agents are shown in Table 2a; the values after omission of IC$_{50}$ from experiments 4 and 8 are shown in Table 2b.
Table 2a. Inhibitory concentrations at 50 percent (IC₅₀) of the seven agents tested in nine experiments, on rhinosporidial endospores, by the MTT-reduction test for the viability of endospores.

<table>
<thead>
<tr>
<th>Agent</th>
<th>IC₅₀ (μg/mL)</th>
<th>Number, range, mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>4, 4, 9, 20, 30, 46, 148, 196</td>
<td>8, 4-196, 57.1±73.4</td>
</tr>
<tr>
<td>Berenil</td>
<td>5.5, 10, 14, 15, 18</td>
<td>5, 5.5-18, 12.5±4.9</td>
</tr>
<tr>
<td>Dapsone</td>
<td>2, 3, 10, 11, 22, 60, 67, 70, 100</td>
<td>10, 2-100, 29.7±33.7</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>10, 14, 22, 32, 67, 81, 82, 100²</td>
<td>8, 10-100, 51±35.4</td>
</tr>
<tr>
<td>Sodium stibogluconate</td>
<td>1.2, 5.5, 40, 43, 55, 81, 164²</td>
<td>7, 1.2-164, 55.7±55.2</td>
</tr>
<tr>
<td>Trimethoprim-sulfadiazine</td>
<td>3.8, 5.8, 6.1, 15, 18, 37, 49, 196¹</td>
<td>9, 3.8-196, 38.4±60.9</td>
</tr>
<tr>
<td>Imizol</td>
<td>9¹</td>
<td>1, 9</td>
</tr>
</tbody>
</table>

¹nasal strains, ²strain from disseminated rhinosporidiosis, ³nasopharyngeal strains

Table 2b. Inhibitory concentrations at 50 percent (IC₅₀) of the seven agents minus the underscored and bold-type IC₅₀ values in Table 2a.

<table>
<thead>
<tr>
<th>Agent</th>
<th>IC₅₀ (μg/mL)</th>
<th>Number, range, mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>4, 4, 9, 20, 30, 46</td>
<td>6, 4-46, 18.8±16.7</td>
</tr>
<tr>
<td>Berenil</td>
<td>5.5, 10, 14, 15, 18</td>
<td>5, 5.5-18, 12.5±4.8</td>
</tr>
<tr>
<td>Dapsone</td>
<td>2, 3, 10, 11, 11, 22, 60, 67</td>
<td>9, 2-67, 21.9±24.3</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>10, 14, 22, 32, 67, 81, 82</td>
<td>7, 10-82, 44±31.7</td>
</tr>
<tr>
<td>Sodium stibogluconate</td>
<td>1.2, 5.5, 40, 43, 55, 81</td>
<td>6, 1.2-81, 37.6±30.3</td>
</tr>
<tr>
<td>Trimethoprim-sulfadiazine</td>
<td>3.8, 5.8, 6.1, 15, 18, 37, 49</td>
<td>8, 3.8-49, 18.7±16.2</td>
</tr>
<tr>
<td>Imizol</td>
<td>9¹</td>
<td>1, 9</td>
</tr>
</tbody>
</table>

Endosporostatic effects of antimicrobial agents

Single (with amphotericin B, sodium stibogluconate, and trimethoprim-sulfadiazine), triplicate (with berenil, ketoconazole, and trimethoprim-sulfadiazine) and quadruplicate (with amphotericin B, berenil, dapsone, ketoconazole, sodium stibogluconate, and trimethoprim-sulfadiazine) samples of these six agents at 100 μg/mL, in three sets of experiments with an incubation period of 3 days with the agents, and 4 days after drug-removal, in PBS, showed recovery of MTT-reducing capability, as indicated by a significant decrease in the percentage of inhibited/inactivated endospores in the recovery medium, as shown from one experiment as an example, in Table 3.

DISCUSSION

The endospores and sporangia of R. seeberi, unlike endospores of bacteria such as Bacillus spp. and Clostridium spp., are not resting survival stages, but are actively metabolizing entities in the life cycle of R. seeberi, that absorb nutrients from their aqueous environment and are very sensitive to heat and the common biocides.⁶-¹⁰ The chemical nature of the rhinosporidial endospore’s wall is different from that
Susceptibility of endospores of *Rhinosporidium seeberi* to antimicrobials: Arseculeratne S, et al.

of bacterial or fungal cell walls, and none of the agents tested induced malformations of the endospore wall. *R. seeberi* possesses chitin and cellulose that are absent in bacteria while its endosporial and sporangial walls do not have the targets susceptible to the beta-lactams. In contrast, the structural abnormalities observed were restricted to the contents of the endospore, the EDBs, that showed morphological abnormalities such as fragmentation or enlargement, or a decrease or loss of MTT-reduction, indicating that the drug targets were intraendosporially located. Even with the high drug concentrations, the endospores were empty, with their walls morphologically intact. Woodard and Hudson\(^\text{17}\) regarded the failure of drug therapy in rhinosporidiosis as being due to impermeability of the sporangial wall to antimicrobial agents, but in this study the inactivation of all the endospores within a sporangium treated with dapsone is contrary to their observation. Dapsone and sodium stibogluconate have the formula weights of 248 and 902, respectively, and had effectively penetrated the endospore as did antirhinosporidial antibody (molecular weight > 150,000 dalton). Woodard and Hudson’s explanation\(^\text{17}\) is therefore untenable. The resistance to agents described by them was probably due to low susceptibility of *R. seeberi* to the agents.

**Variations of IC\(_{50}\) of each agents**

Amphotericin B, dapsone, ketoconazole, and sodium stibogluconate gave the ranges of IC\(_{50}\) of 49, 50, 10, and 68, respectively. This could be due to variation of the susceptibility of the strains of *R. seeberi* or to a technical problem such as solubility of the agents. Strain variation could not be tested in this study as multiple strains of *R. seeberi* from different patients were not available. The possibility of variation in the solubilization of the agents was indicated by the effect of warming the dapsone solution; the IC\(_{50}\) decreased from 22 with dapsone dissolved at room temperature to 2 \(\mu\)g/mL after warming at 50-60°C, on the same strain of *R. seeberi*, done simultaneously.

In this study, the mean IC\(_{50}\) of dapsone was 29.7 \(\mu\)g/mL, the lowest of the IC\(_{50}\) of the 7 agents used, and the results of the endosporostatic/endosporicidal test, were compatible with previous reports on its efficacy in rhinosporidiosis, and its bacteriostatic action.\(^\text{18-25}\)

This study, antirhinosporidial effects of dapsone were observed after 6 days of direct exposure of *R. seeberi* to the agent. This is in contrast with the

<table>
<thead>
<tr>
<th>Agent</th>
<th>CPI after drug exposure (in quadruplicate)</th>
<th>CPI after drug removal and reincubation in PBS (in quadruplicate)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>33, 44, 45, 47</td>
<td>24, 4, 5, 12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Berenil</td>
<td>70, 76, 68, 70</td>
<td>8, 6, 1, 0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dapsone</td>
<td>46, 61, 48, 46</td>
<td>3, 3, 0, 0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>51, 30, 43, 54</td>
<td>0, 7, 0, 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sodium stibogluconate</td>
<td>32, 40, 39, 54</td>
<td>18, 10, 2, 0</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Trimethoprim-sulfadiazine</td>
<td>57, 50, 56, 55</td>
<td>8, 9, 0, 5, 0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

CPI: corrected percentage inactivation

Table 3. The recovery of MTT-reducing capacity in agent-treated endospores, in quadruplicate, after removal of the drug and re-incubation in agent-free phosphate-buffered saline (PBS).
prolonged duration of several weeks or months of dapsone therapy in patients before microscopic and ultramicroscopic evidence of damage to *R. seeberi* in the tissues was observed.\textsuperscript{18,20} This phenomenon could be related to the in vivo pharmacokinetics of dapsone, its penetration into rhinosporidial polyps with fibrous tissue, and cell infiltrates, while the reported microscopical effects on rhinosporidial bodies occur later than their inactivation. The apparently delayed in vivo effects might yet be compatible with the quicker occurrence of in vitro effects when the pathogen is in direct contact with the drug as we observed.

In conclusion, this is the first full report on antimicrobial agents active against *R. seeberi*, in vitro.

**ACKNOWLEDGEMENTS**

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**References**


