Mycoscience (2011) 52:107–110
DOI 10.1007/s10267-010-0079-y

FULL PAPER

Comparison of in vivo and in vitro inactivation of endospores of *Rhinosporidium seeberi* following dapsone treatment

Sarath N. Arseculeratne · Navaratne B. Eriyagama

Received: 3 August 2010 / Accepted: 4 October 2010 / Published online: 9 November 2010

Abstract Rhinosporidiosis in humans and animals, caused by *Rhinosporidium seeberi*, can now be termed an emerging infective disease worldwide. The pharmacokinetics of dapsone when used as an antimicrobial agent in patients with rhinosporidiosis was compared with its pharmacodynamics in the inactivation of purified rhinosporidial endospores in vitro. A marked discrepancy was noted between the in vitro inactivation, which commenced on day 1 and was a maximum at day 4, whereas earlier reports on the response of endospores in patients under dapsone therapy indicated that the degeneration and disappearance of the endospores was not observed for 18–36 weeks after therapy began. Reasons for this discrepancy that operate in vivo are postulated: (a) impermeability of the barriers of down-growths of squamous epithelium in which the endospore-containing rhinosporidial sporangia are embedded; (b) presence of a mucoid matrix in which the endospores exist within the sporangia. These explanations contribute to resolving the controversial problem of general correlations between in vitro and in vivo results from studies on the action of antimicrobial drugs.

Keywords Pharmacodynamics · Pharmacokinetics

S. N. Arseculeratne (✉) · N. B. Eriyagama
Department of Microbiology, Faculty of Medicine, University of Peradeniya, Peradeniya, Sri Lanka
e-mail: chubby@sltnet.lk

Introduction

Dapsone has been reported by several workers in regions of India that are hyperendemic for rhinosporidiosis to be effective in arresting the progress of rhinosporidiosis with identifiable damage to the morphology of the pathogen *Rhinosporidium seeberi* on light-microscopic histology and ultramicroscopy of the rhinosporidial tissues from treated patients. In view of the absence of a method for both the in vitro culture of *R. seeberi* and a model for the experimental reproduction of the disease, there was no available data until recently, when a new method (Arseculeratne and Atapattu 2004) was developed for assessing the viability of rhinosporidial endospores. With this method, studies were made on responses of rhinosporidial endospores in vitro to antimicrobial drugs, including dapsone, used in human and animal chemotherapy (Arseculeratne et al. 2008). The time course of inactivation of endospores in vitro was determined to compare the pharmacodynamics of the inactivation of rhinosporidial endospores in vitro with the pharmacokinetics of the action of dapsone in patients with rhinosporidiosis, as indicated by changes in the histopathology of dapsone-treated rhinosporidial tissues and the morphology of the pathogen *R. seeberi* described in the literature. Cycloserine, the antituberculous drug; and Berenil, a veterinary antimicrobial formulation of diminazene aceturate; were also investigated in this for pharmacodynamics in vitro for comparison with that of dapsone, because these two drugs have lower IC_{50}s than dapsone. Reasons are postulated for the discrepancy in in vitro and in vivo result durations with dapsone. These explanations provide new perspectives on the general problem of correlations between in vitro and in vivo results of the action of antimicrobial drugs.

Materials and methods

Drugs

Dapsone was kindly supplied by Dr. Peter Heistand (Novartis, Switzerland). Cycloserine and Berenil were preparations used in clinical practice. Dapsone was first dissolved in dimethylsulfoxide (DMSO), which was then diluted with phosphate-buffered saline (PBS, 0.2 M, pH 7.4) with 0.2% DMSO to the required concentration. Cycloserine and Berenil were dissolved in PBS alone. All three drugs were tested at a concentration of 50 μg/ml, which was approximately 2× IC_{50} of dapsone and 3× IC_{50} of Berenil and cycloserine.

Rhinosporidial endospores

Nasal rhinosporidial tissue was homogenized in sterile distilled water (SDW) filtered through nylon sieves (25-μm diameter) to remove tissue fragments, and the endospores were resuspended in SDW at approximately 2 × 10^6 endospores/ml. These suspensions had no sporangia that were presumably ruptured during homogenization.

MTT reduction test for inactivation of endospores

The microscopic adaptation of the 3-[4,5-dimethyl 2-thiazolyl]-2,5diphenyl-2H-tetrazolium bromide (MTT) reduction test for rhinosporidial endospores and the criteria for inactivation, as described earlier (Arseculeratne and Atapattu 2004), were used to assess endospore viability. Endospores in SDW served as controls for their spontaneous inactivation. At the end of the period of incubation (commencing at day 1 for 12 days) at room temperature (28°C ambient), endospores were washed once in SDW, and MTT in PBS (Sigma, USA; 0.5 mg/ml) was added to the centrifuged deposit, which was incubated for 3 h at 37°C before evaluation of inactivation by microscopy at 1,000×. The percentage inactivation of control endospores was subtracted from the crude percentage of drug-treated endospores for the corrected percentage inactivation (CPI) according to the formula:

$$\mathrm{CPI} = \frac{P - C}{V} \times 100$$

where P = crude percentage inactivation, C = percentage inactivation of control endospores, V = percentage of viable endospores in the test suspension.

Time course of inactivation

Control endospores retained their viability over the test period of 12 days (Fig. 1).

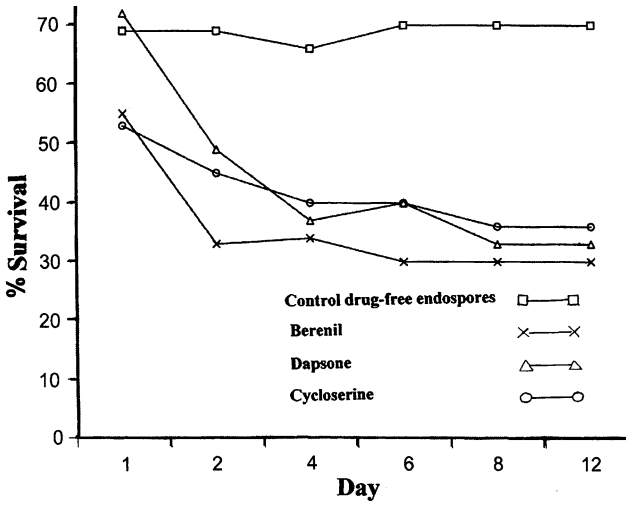

Fig. 1 The time course of the inactivation of the endospores of *Rhinosporidium seeberi* in vitro

The inactivation by dapsone commenced after day 2 and was at a maximum at day 4. In comparison Berenil (diminazene aceturate, the antimicrobial drug for veterinary use) produced the maximum inactivation on day 2, whereas cycloserine produced the maximum inactivation on day 8.

Histopathology of rhinosporidial tissue

Rhinosporidial tissue, irrespective of the patient and site of the disease, showed uniform histopathology. The pathogen *R. seeberi* was present with its diverse ontogenic stages–juvenile, immature, and mature sporangia–with endospores surrounding the pore of the sporangia dispersed mainly in the dermis and surrounded by down-growths and whorls of the squamous epithelium (Fig. 2). As deduced from the appearances of their cells, these down-growths consist of the stratum granulosum and stratum spinosum of the original squamous epithelium enclosing the rhinosporidial sporangia and have been regarded as the earliest stage in the phenomenon of transepidermal elimination of sporangia (Arseculeratne et al. 2001).

Figure 3 shows a mature rhinosporidial sporangium containing mature endospores embedded in an intrasporangial mucoid matrix that prevents the aggregation of endospores.

Ethical aspects

Patients' informed consent for publication of clinical data was obtained. Ethical clearance for the publication was obtained from the Committee on Research and Ethical Review of the University of Peradeniya's Faculty of Medicine on 12 August 2010.

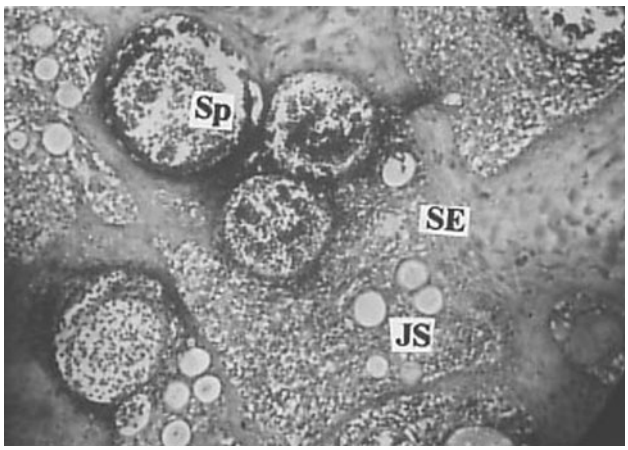

Fig. 2 Histopathology of rhinosporidial tissues showing whorls of down-growths of squamous epithelium (SE) enclosing mature, endospore-containing sporangia (Sp). *JS* degenerate juvenile sporangia. Initial magnification ×400. Hematoxylin and eosin

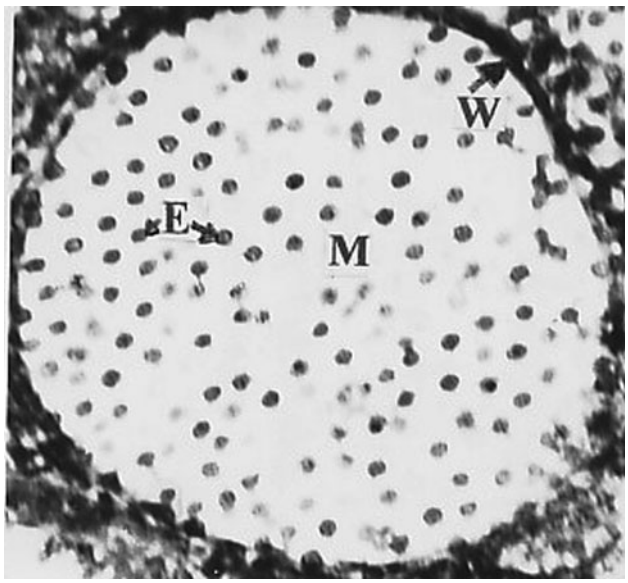

Fig. 3 Mature sporangium of *Rhinosporidium seeberi* in rhinosporidial tissue showing endospores (E) embedded in mucoid matrix (M). *W* wall of sporangium. Initial magnification ×400. Hematoxylin and eosin

Discussion

Clinically, Mahakrisnan et al. (1981) noted complete regression of cutaneous nodules in disseminated rhinosporidiosis, with an accentuated granulomatous host's responses histopathologically and arrest of the maturation and degeneration or absence of endospores that were observed at 18 weeks of dapsone therapy. Job et al. (1993) recorded the time-sequence of the effects of orally administered dapsone in the chemotherapy of rhinosporidiosis in the absence of surgery. Clinically, a reduction in size of the growth at 6 weeks, marked reduction at 36 weeks, and total disappearance after 1 year of treatment, were observed. Histopathologically, at 6 weeks no effect on pathogen was observed, whereas in 18 weeks, degenerative changes and in 36 weeks, reduction in numbers and size of the pathogen, had occurred, with sporangia that had collapsed and been phagocytosed by macrophages. Venkateswaran et al. (1997) observed no effect on the pathogen until the 6th week of therapy and that the patients' tissue responses were augmented after 12 weeks of therapy, with degeneration and disappearance of the pathogen, which was observable after 36 weeks.

These changes in the pathogen that took several weeks to appear in vivo are in marked contrast to the rapidity (within a few days) of degenerative changes and total inactivation that the drug-treated free endospores showed in vitro, which began on the second day and was at a maximum on the eighth day; the in vivo inactivation is reported to have occurred from the 12th to the 36th week of therapy in patients with rhinosporidiosis.

We postulate two possible reasons for the marked differences in the times of occurrence of the in vitro and in vivo antirhinosporidial effects, as relating to the pharmacokinetics of dapsone therapy and pharmacodynamics of its direct action on the pathogen in vitro. The reasons are based on the kinetics of the access of dapsone to the pathogen in vivo—the time taken for absorption after oral dosing, distribution into the tissues, and especially the penetration into the diseased rhinosporidial tissues and access to the endospores within the sporangia, which is in contrast to the immediate exposure of the endospores to dapsone in vitro. The in vitro effects would therefore entirely represent the real time course of inactivation of the free endospores by the drug.

Penetration of rhinosporidial tissues by dapsone

Whereas retardation of the access of dapsone to the pathogen by edema, hemorrhage, cell infiltration, fibrosis, and cystic spaces in the rhinosporidial tissues is likely to occur, the major reason for delayed access of dapsone to the pathogen as endospores that are mainly within sporangia is probably that the latter entities are surrounded by down-growths and whorls of squamous epithelium (Fig. 2). The epithelial whorls consist of the stratum granulosum and stratum spinosum of the original epidermis, which serves as a barrier to the penetration of drugs; in the stratum granulosum are lamellar granules with lipid bilayers, which act as a barrier to penetration by foreign material (Junqueira and Carneiro 2005), and presumably these materials include the antirhinosporidial drugs. In addition, the skin acts as a two-way barrier to prevent the inward or outward passage of water and electrolytes, and it is relatively impermeable to sodium, potassium, and other ions in

aqueous solution (Archer 2004). As with other nonpolar compounds, penetration of antirhinosporidial drugs such as dapsone might be retarded by this barrier function of the skin. The down-growths of squamous epithelium in rhinosporidial tissues are a nonspecific reaction to injury caused by chemicals, diverse mycelial fungi, and irritated seborrheic keratosis (Lever and Lever 1990).

Impermeability of the intrasporangial mucoid matrix

Martos et al. (2007) speculated that the failure of echinocandins in infections by *Cryptococcus neoformans* was possibly due to impenetrability of the drug through the polysaccharide capsule of the yeast. We invoke a similar speculation to explain the difference between the rapidity (2 days) of the in vitro inactivation of *R. seeberi* by dapsone and the delay of several weeks (>18) before the pathogen in rhinosporidial tissues shows degeneration on dapsone therapy. We refer to the presence of a mucoid matrix in which the mature endospores are embedded within the mature sporangium (Fig. 3). Even after the endospores are liberated from the sporangium, they will still be within the down-growths of hyperplastic epithelium or entrapped in the mucoid matrix in the stroma of the rhinosporidial tissue Such interference with the access of drugs in vivo might account for the great difference between the rapidity of the in vitro inactivation and the response of the pathogen in diseased tissues in vivo.

Rex et al. (1993) and Cushion et al. (1997) referred to correlations between the results from in vitro assays and in vivo responses in the testing of antimicrobial drug activity in terms of the effective concentrations of the drugs in vitro and in vivo. The phenomenon of differences in time courses demonstrated for in vitro activity compared with in vivo responses discussed in this paper refer to an additional dimension of this correlation: the pharmacodynamics of drug action in vitro and pharmacokinetics in vivo, rather than to drug concentrations.

Woodard and Hudson (1984) postulated that the failure of antimicrobial drug therapy was due to impermeability of the rhinosporidial sporangia to drugs. The penetration of immunoglobulin (formula weight of >150,000 Da) into the rhinosporidial sporangia during indirect immunofluorescence tests (Atapattu et al.(1999) and dapsone (formula weight 248 Da) into endospores, would render improbable Woodard and Hudson's (1984) explanation for the failure of drug therapy in rhinosporidiosis based on the impenetrability of the sporangia of *R. seeberi* and access of drugs and chemicals of lower formula weights to the pathogen. Such failure is more likely to have been due to insensitivity of *R. seeberi* to the respective drugs in the first place.

No data is available on the temporal sequences of the effects of cycloserine and Berenil in vivo for comparison with our in vitro findings, as these two drugs have not yet been used to treat rhinosporidiosis.

Acknowledgments We thank the National Science Foundation of Sri Lanka for a research grant (NSF/Fellow/2006/01) and Dr. Peter Heistand (Novartis, Switzerland) for the gift of dapsone.

References

Archer CB (2004) Functions of the skin. In: Burns T, Breathnach S, Cox N, Griffiths C (eds) Rook's textbook of dermatology, 7th edn. Blackwell Science, Great Britain

Arseculeratne SN, Atapattu DN (2004) The assessment of the viability of the endospores of *Rhinosporidium seeberi* with MTT (3-[4,5-dimethyl 2-thiazolyl]-2,5diphenyl-2H-tetrazolium bromide). Mycol Res 108(12):1423–1430

Arseculeratne SN, Panabokke RG, Atapattu DN (2001) Lymphadenitis, trans-epidermal elimination and unusual histopathology in human rhinosporidiosis. Mycopathoplogia 153:57–69

Arseculeratne SN, Kumarasiri R, Sumathipala S, Atapattu D, Eriyagama NB, Balasooriya P, Fernando R (2008) In vitro susceptibility of the endospores of *Rhinosporidium seeberi* to seven antimicrobial agents. J Infect Dis Antimicrob Agents 25:135–143

Atapattu DN, Arseculeratne SN, Rajapakse RPVJ, Perera NAND, Eriyagama NB (1999) Purification of the endospores and sporangia of *Rhinosporidium seeberi* on Percoll columns. Mycopathologia 145:113–119

Cushion MT, Franklin C, Kloepfer N (1997) A cytotoxicity assay for evaluation of candidate anti-*Pneumocystis carinii* agents. Antimicrob Agents Chemother 41:379–384

Job A, Venkateswaran S, Mathan M, Krishnaswami H (1993) Medical therapy of rhinosporidiosis with dapsone. J Laryngol Otol 107:809–812

Junqueira LC, Carneiro J (2005) Basic histology: text & atlas, 11th edn. McGraw Hill, New York, p 362 (see also pp 363)

Lever WF, Lever G-S (1990) Histopathology of the skin, 7th edn. Lippincott Co., Philadelphia, p 480

Mahakrisnan A, Rajasekaram V, Pandian PI (1981) Disseminated cutaneous rhinosporidiosis treated with dapsone. Trop Geogr Med 33:189–192

Martos AI, Martin de La Escalera C, Lopez-Oviedo E, Aller AI, Romero A, Serrano C, Valverde A, Martin-Mazuelos E (2007) In vitro activity of echinocandins (caspofungi, micafungin, and anidulafungin) against *Aspergillus* spp. In: 17th European congress of clinical microbiology and infectious diseases. Munich, Germany, pp 1733 (see also pp 1177)

Rex JH, Pfaller MA, Rinaldi MG, Polack A, Galgiani JN (1993) Antifungal susceptibility testing. Clin Microbiol Rev 6(4): 367–381

Venkateswaran S, Date A, Job A, Mathan M (1997) Light and electron microscopic findings in rhinosporidiosis after dapsone therapy. Trop Med Internat Health 12:1328–1332

Woodard B, Hudson J (1984) Rhinosporidiosis: ultrastructural study of an infection in South Carolina. Southern Med J 77:1587–1588