Revista Brasileira de Biociências
Brazilian Journal of Biosciences
ISSN 1980-4849 (on-line) / 1679-2343 (print)

SHORT COMMUNICATION

The use of 2-[2'-hydroxy-5'-aminophenyl]benzoxazole (HAMBO), a new fluorochrome for the morphological analysis of *Fonsecaea pedrosoi* ATCC 46428 using a microculture technique

Maria Lúcia Scroferneker[1,2]*, Ivana Pelegrim de Oliveira[3], Mariana Carissimi[4], Valter Stefani[5], Cheila Denise Ottonelli Stopiglia[2,6] and Valeriano Antonio Corbellini[7]

Received: July 22 2010 Received after revision: October 14 2010 Accepted: October 22 2010
Available online at http://www.ufrgs.br/seerbio/ojs/index.php/rbb/article/view/1672

ABSTRACT: (The use of 2-[2'-hydroxy-5'-aminophenyl]benzoxazole (HAMBO), a new fluorochrome for the morphological analysis of Fonsecaea pedrosoi ATCC 46428 using a microculture technique). Fluorochromes have been employed in fungi for many reasons, including the evaluation of cell feasibility, growth, physiological monitoring during stress, antifungal resistance and diagnosis of infections in clinical specimens. This study evaluated the incorporation of 2-[2'-hydroxy-5'-aminophenyl] benzoxazole (HAMBO) into the vegetative and reproductive mycelia of *Fonsecaea pedrosoi*. *Fonsecaea pedrosoi* (strain ATCC 46428) was incubated for 15 days on a microculture of potato dextrose agar containing HAMBO. Slides with fungal growth were analyzed using epifluorescence microscopy. The fluorochrome impregnated the surface of the cellular material. The detailed external definition of the microstructure allowed for the differentiation of hyphae and conidia and for the detection of changes in fluorochrome deposition and membrane thickness in the cellular elements investigated. HAMBO has intrinsic fluorescence, lack of functional groups susceptible to enzymatic hydrolysis, and slow photofading, which make it suitable for microscopic analysis when it is necessary to expose material to UV light for a long period.
Key words: chromoblastomycosis, fluorofore.

RESUMO: (Utilização de 2-[2'-hidroxi-5'-aminofenil]benzoxazol (HAMBO), um novo fluorocromo para análise morfológica de *Fonsecaea pedrosoi* ATCC 46428 na técnica de microcultivo). Fluorocromos tem sido empregados em fungos para a avaliação da viabilidade celular, crescimento, acompanhamento durante o estresse fisiológico, resistência a antifúngicos, diagnóstico de infecção em amostras clínicas, entre outros. Este estudo avaliou a incorporação de 2-[2'-hidroxi-5'-aminofenil]benzoxazol (HAMBO) no micélio vegetativo e reprodutivo de *Fonsecaea pedrosoi*. Cepa de *F. pedrosoi* ATCC 46428 foi incubada em microcultivo em agar batata dextrose contendo (HAMBO) por 15 dias. As lâminas com o crescimento fúngico foram observadas em microscopia de epifluorescência. O fluorocromo impregnou na superfície do material celular. A definição detalhada da microestrutura externa permitiu a diferenciação de hifas e conídios e a detecção de alterações na deposição do fluorocromo e na espessura da membrana nos elementos celulares investigados. A fluorescência intrínseca do HAMBO, a ausência de grupos funcionais suscetíveis à hidrólise enzimática e o desvanecimento lento, o tornam adequado para a análise microscópica quando é necessária longa exposição do material à luz UV.
Palavras-chave: cromoblastomicose, fluoróforo.

INTRODUCTION

Chromoblastomycosis is a subcutaneous fungal disease caused by dematiaceous fungi, such as *Fonsecaea pedrosoi*, which is a major cause of this disease in Brazil being common in tropical and subtropical regions (Lacaz *et al.* 2002). In Brazil, the most frequent etiological agents are *F. pedrosoi, Phialophora verrucosa* and *Cladosporium carrionii* (Minotto *et al.* 2001). Diagnosis is usually made by direct examination of crusts, scales or pus from the lesions, and histopathological examinations of a biopsy or a culture from collected material; however, a delayed--type skin test using antigens produced in synthetic media may be useful for the assessment of primary exposure (Corbellini *et al.* 2006).

Laboratory diagnostic procedures in dermatological mycology are based on direct microscopy and culture of clinical material (Aslanzadeh & Roberts, 1991). In this context, fluorochromes are an important tool, as they can be added to the growth media and incorporated into fungal cells, providing better visualization of fungal structures. Fluorochromes have been employed in fungi for the evaluation of cell feasibility (Correa *et al.* 1986, Lopes *et al.* 2002), growth (Cohen *et al.* 1987), physiological monitoring during stress (Thrane *et al.* 1999), antifungal resistance (Yang *et al.* 2001), diagnosis of in-

1. Department of Microbiology, Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul. CEP 90050-170, Porto Alegre, RS, Brazil.
2. Graduate Program in Medicine, Medical Sciences, Universidade Federal do Rio Grande do Sul. CEP 90035-003, Porto Alegre, RS, Brazil.
3. School of Pharmacy, Universidade Federal do Rio Grande do Sul. CEP 90610-000, Porto Alegre, Rio Grande do Sul, Brazil.
4. Local Department of Environmental Protection, City Hall of Caxias do Sul, Caxias do Sul, Brazil.
5. Department of Organic Chemistry, Universidade Federal do Rio Grande do Sul. Porto Alegre, RS, Brazil.
6. CAPES Researcher and 7Department of Physics and Chemistry, Universidade de Santa Cruz do Sul, CEP 96815-900, Santa Cruz do Sul, RS, Brazil.
*Author for correspondence. E-mail: scrofern@ufrgs.br

fections in clinical specimens (Holler *et al.* 2002, Rüschel *et al.* 2001) and interaction of these dyes with structural components of cells, such as membranes (Brasch *et al.* 2003) and walls (Watanabe *et al.* 2005).

2-(2'-hydroxyphenyl)benzoxazoles are highly fluorescent molecules when irradiated with ultraviolet light by means of an intramolecular proton-transfer mechanism in the excited state (ESIPT) (Campo *et al.* 2000). 2-(2'-hydroxyphenyl)benzoxazole derivatives are well known for their photochemical properties (Stefani *et al.* 1992, Pla-Dalmau 1995, Costela *et al.* 1998, Campo *et al.* 2000, Fournier *et al.* 2000, Holler *et al.* 2002, Rodembusch *et al.* 2005), but their interaction with biological systems has been poorly investigated (Daboit *et al.* 2009). This article describes the use of 2-(2'-hydroxy-5'-aminophenyl)benzoxazole (HAMBO) for this purpose, and focuses on its incorporation into *F. pedrosoi* and its staining capacity of the cell structures of this species using a microculture technique.

MATERIALS AND METHODS

2-(2'-hydroxy-5'-aminophenyl)benzoxazole (Fig. 1) was synthesized as described in Holler *et al.* (2002) and Stefani *et al.* (2003). *Fonsecaea pedrosoi* (strain ATCC 46428) was purchased from the Department of Microbiology, at the Universidade Federal de Minas Gerais. The fungal strain was maintained in Sabouraud agar slants at 36.5ºC. HAMBO was diluted in dimethyl sulfoxide at 8×10^{-2} molL^{-1}, added to sterile melted (40ºC) potato dextrose agar at 1:100 and poured into plates (20 mL per plate). The medium was cut into cubes and placed on microscope slides in triplicate. The strain was inoculated onto each cube and the microculture was maintained at 36.5ºC for 15 days under constant moisture. Slides with fungal growth were observed using epifluorescence microscopy, with an Optiphot-2/Nikon. The HAMBO was analyzed using the 400 nm wavelength.

RESULTS AND DISCUSSION

The fluorochrome was capable of impregnating the fungal wall and cells after its incorporation. The slide stained with HAMBO shows fungal hyphae with a green outline (Fig. 2), and the thickness of the wall with sites showing a bilaminar structure.

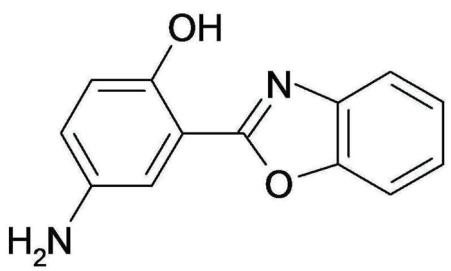

Figure 1: Chemical structure of 2-(5'-amino-2'-hydroxyphenyl) benzoxazole (HAMBO).

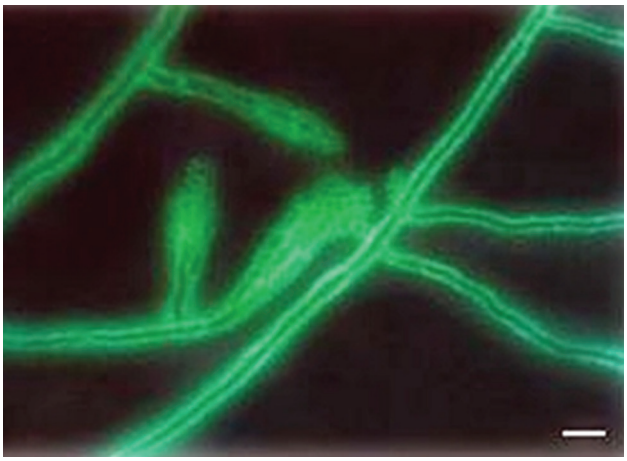

Figure 2: Epifluorescence microscopy of a slide containing the fungal growth of a *F. pedrosoi* microculture with a medium containing HAMBO. 200x. Magnification bar: 50µm.

This fluorochrome, when used for the detection of morphological structures, had a singular staining pattern on the fungal hyphae, and the slides stained with HAMBO allowed the structure of the cell wall (Fig. 2) to be visualized. Hyperchromia on terminal conidia, septal regions and hyphal tips visible from the HAMBO impregnation can be associated with lipid accumulation in these areas (Gomes & Resende 1991) because this fluorochrome has an affinity for a hydrophobic environment and can be observed by the increase in the fluorescence quantum yield (Douhal *et al.* 1994). Other fluorochromes become visible in young cells and conidia, which relates to the fact that these structures have a high cellular metabolism (Correa *et al.* 1986), where the fluorochromes are enzymatically hydrolyzed. Thus, only living and actively metabolizing microorganisms become visible when stained with these dyes. Unlike these fluorochromes, HAMBO has intrinsic fluorescence and lacks functional groups susceptible to enzymatic hydrolysis.

Another advantage is the slow photofading of this molecule, explained by photoemission and photoexcitation (Campo *et al.* 2000), which makes it suitable for microscopic analysis when long exposure of material to UV light is necessary. Conversely, fluorescein diacetate has rapid photofading in large visual fields (Horobin & Kiernan 2002), limiting the time available for observation, which also makes it difficult to photograph material (Correa *et al.* 1986, Jensen & Lysek, 1995).

HAMBO has proved to have good image resolution and to make a clearer distinction between the vegetative and reproductive mycelia of *F. pedrosoi* when incorporated into agar. Further investigations should be carried out using this new fluorochrome for the characterization of the cell wall and the determination of its possible effects on fungi of clinical interest, as well as to clarify its uptake kinetics.

ACKNOWLEDGMENTS

We would like to express our thanks to CAPES (Co-

ordenação de Aperfeiçoamento de Pessoal de Nível Superior) and FAPERGS (Fundação de Amparo à Pesquisa do Rio Grande do Sul) for their financial support.

REFERENCES

ASLANZADEH, J. & ROBERTS, G.D. 1991. Direct microscopic examination of clinical specimens for the laboratory diagnosis of fungal infections. *Clin Microbiol News, 13*: 185-192.

BRASCH, J., KREISELMAIER, I. & CHRISTOPHERS, E. 2003. Inhibition of dermatophytes by optical brighteners. *Mycoses, 46*: 120-125.

CAMPO, L.F., CORREA, D.S., ARAUJO, M.A. & STEFANI, V. 2000. New fluorescent monomers and polymers displaying an intramolecular proton-transfer mechanism in the electronically excited state (ESIPT), 1. Synthesis of benzazolylvinylene derivatives and its copolymerization with methyl methacrylate (MMA). *Macromol Rapid Commun, 21*: 832-836.

COHEN, Y., PETER, S., BALASS, O. & COFFEY, M.D. 1987. A fluorescent technique for studying growth of *Peronospora tabacina* on leaf surfaces. *Phytopathol, 77*: 201-204.

CORBELLINI, V.A., SCROFERNEKER, M.L., CARISSIMI, M. & SANTOLIN, L.D. 2006. Delayed-type hypersensitivity response to crude and fractionated antigens from *Fonsecaea pedrosoi* CMMI 1 grown in different culture media. *Mycopathol, 162*: 51–55.

CORREA, B., PURCHIO, A., PAULA, C.R. & GAMBALE, W. 1986. Evaluation of a fluorescent method (fluorescein diacetate and ethidium bromide solution) in the study of the viability of *Cryptococcus neoformans* strains. *Mycopathol, 96*: 91-96.

COSTELA, A., GARCÍA-MORENO, I., MALLAVIA, R., AMAT-GUERRI, F., BARROSO, J. & SASTRE, R. 1998. Proton-transfer lasers based on solid copolymers of modified 2-(2'-hydroxyphenyl)benzimidazoles with methacrylate monomers. *Opt Commun, 152*: 89-95.

DABOIT, T.C., STOPIGLIA, C.D.O., CARISSIMI, M., CORBELLINI, V.A., STEFANI, V. & SCROFERNEKER, M.L. 2009. *In vitro* antifungal activity of 2-(2'-hydroxy-5'-aminophenyl)benzoxazole in *Candida* spp. strains. *Mycoses*, 52: 507-510.

DOUHAL, A., AMAT-GUERRI, F., LILLO, M.P. & ACUÑA, A.U. 1994. Proton transfer spectroscopy of 2-(2'-hidroxyphenyl)imidazole and 2-(2'-hidroxyphenyl)benzimidazole dyes. *J Photochem Photobiol A. Chemistry, 78*: 127-138.

FOURNIER, T., POMMERET, S., MIALOCQ, J.C., DEFLANDRE, A. & ROZOT, R. 2000. Femtosecond laser studies of excited state intramolecular proton transfer in an ultraviolet-filter molecule. *Chem Phys Lett, 325*: 171-175.

GOMES, M.H.R. & RESENDE, M.A. 1991. *Fonsecaea pedrosoi*: Lipid composition and determination of susceptibility to amphotericin B. *Can J Microbiol, 38*: 209-214.

HOLLER, M.G., CAMPO, L.F., BRANDELLI, A. & STEFANI, V. 2002. Synthesis and spectroscopic characterisation of 2-(2'-hydroxyphenyl) benzazole isothiocianates as new fluorescent probes for proteins. *J Photochem Photobiol A: Chemistry, 149*: 217-225.

HOROBIN, R.W. & KIERNAN, J.A (Ed.) 2002. *CONN'S Biological Stains. A Handbook of Dyes, Stains and Fluorochromes for Use in Biology and Medicine.,* Oxford: BIOS Scientific Publishers Ltda. 555 p.

JENSEN, C. & LYSEK, G. 1995. Fluorescence microscopy of fungi in native soil – improvements by additional substances. *Microsc Anal, 49*: 7-9.

LACAZ, C.S., PORTO, E., MARTINS, J.E.C., HEINS-VACCARI, E.M. & MELO, N.T. (Ed.) 2002. *Tratado de Micologia Medica*. São Paulo: Sarvier.

LOPES, M.A., FISCHMAN, O., GAMBALE, W. & CORRÊA, B. 2002. Fluorescent method for studying the morphogenesis and viability of dermatophytes cells. *Mycopathol, 156*: 61-66.

MINOTTO, R., BERNARDI, C.D., MALLMANN, L.F., EDELWEISS, M.I. & SCROFERNEKER, M.L. 2001. Chromoblastomycosis: a review of 100 cases in the state of Rio Grande do Sul, Brasil. *J Am Acad Dermatol, 44*: 585-592.

PLA-DALMAU, A. 1995. 2-(2'-Hydroxyphenyl) benzotiazoles, -benzoxazoles, and -benzimidazoles for plastic scintillation applications. *J Org Chem, 60*: 5468-5473.

RODEMBUSCH, F.S., MEDINA, L.F., BRANDELLI, A. & STEFANI, V. 2005. Synthesis and spectroscopic characterisation of new ESIPT fluorescent protein probes. *Photochem Photobiol Sci*, 4: 254-259.

RÜSCHEL, R., BEHE, M., TORP, B. & LAATSCH, H. 2001. Usefulness of optical brighteners in medicals mycology. *Rev Iberoam Micol, 18*: 147-149.

STEFANI, V., SCROFERNEKER, M.L., CORBELLINI, V.A., GEHLEN, G., BOBLEGA, H.G., OLIVEIRA, I.P. & ELENA, M.A. 2003. *Métodos para Corar Microrganismos Utilizando Fluorocromos da Família das Benzazolas e Compostos Relacionados*. Brazilian Patent requested (PI0307854-0) (in portuguese).

STEFANI, V., SOUTO, A.A., ACUNA, A.U. & AMAT-GUERRI, F. 1992. Synthesis of proton-transfer fluorescent dyes: 2,5-bis(2'-benzazolyl)hydroquinones and related compounds. *Dyes and Pigments, 20*: 97-107.

THRANE, C., OLSSON, S., NIELSEN, T.H. & SØRENSEN, J. 1999. Vital fluorescent stain for detection of stress in *Pythium ultimum* and *Rhizoctonia solani* challenged with viscosinamide from *Pseudomonas fluorescens* DR54. *FEMS Microbiol Ecol, 30*: 11-23.

WATANABE, H., AZUMA, M., IGARASHI, K. & OOSHIMA, H. 2005. Analysis of chitin at the hyphal tip of *Candida albicans* using calcofluor white. *Biosci Biotechnol Biochem, 69*: 1798-1801.

YANG, H.C., MIKAMI, Y., IMAI, T., TAGUCHI, H., NISHIMURA, K., MIYAJI, M. & BRANCHINI, M.L.M. 2001. Extrusion of fluorescein diacetate by multidrug-resistant *Candida albicans*. *Mycoses, 44*: 368-374.