BRAZILIAN JOURNAL OF MICROBIOLOGY

http://www.bjmicrobiol.com.br/

Biotechnology and Industrial Microbiology

Biotransformation of bromhexine by *Cunninghamella elegans*, *C. echinulata* and *C. blakesleeana*

*Aman K. Dube, Maushmi S. Kumar**

Narsee Monjee Institute of Management Studies, Shobhaben Pratapbhai Patel School of Pharmacy & Technology Management, Vile Parle (W), Mumbai, Maharashtra, India

ARTICLE INFO

Article history:
Received 3 July 2015
Accepted 1 July 2016
Available online 5 December 2016
Associate Editor: Solange Ines Mussatto

Keywords:
Cunninghamella sp.
Bromhexine
RLM
CYP3A4
Inhibitor
Metabolism

ABSTRACT

Fungi is a well-known model used to study drug metabolism and its production in *in vitro* condition. We aim to screen the most efficient strain of *Cunninghamella* sp. among *C. elegans*, *C. echinulata* and *C. blakesleeana* for bromhexine metabolites production. We characterized the metabolites produced using various analytical tools and compared them with mammalian metabolites in Rat liver microsomes (RLM). The metabolites were collected by two-stage fermentation of bromhexine with different strains of *Cunninghamella* sp. followed by extraction. Analysis was done by thin layer chromatography, high performance thin layer chromatography, Fourier transform infrared spectroscopy, high performance liquid chromatography and Liquid chromatography–mass spectrometry. The role of Cytochrome P3A4 (CYP3A4) enzymes in bromhexine metabolism was studied. Fungal incubates were spiked with reference standard – clarithromycin to confirm the role of CYP3A4 enzyme in bromhexine metabolism. Three metabolites appeared at 4.7, 5.5 and 6.4 min retention time in HPLC. Metabolites produced by *C. elegans* and RLM were concluded to be similar based on their retention time, peak area and peak response of 30.05%, 21.06%, 1.34%, and 47.66% of three metabolites and bromhexine in HPLC. The role of CYP3A4 enzyme in metabolism of bromhexine and the presence of these enzymes in *Cunninghamella* species was confirmed due to absence of peaks at 4.7, 5.4 and 6.7 min when RLM were incubated with a CYP3A4 enzyme inhibitor – clarithromycin.

Introduction

The approval and usage of drugs for human consumption need extensive pharmacokinetic and pharmacodynamics studies to determine its safety and efficacy. Drug metabolism plays an important role in the development of new drug entities to be further evaluated for pharmacological/toxicological activities. The formation of metabolite and its role in the body before excretion is important to understand drug's safety and

* *Corresponding author.*
E-mail: maushmiskumar@gmail.com (M.S. Kumar).
http://dx.doi.org/10.1016/j.bjm.2016.11.003

toxicity profile. Cytochrome P450 enzyme plays an important role in the metabolism of the drugs. To signify CYP450 and its isoforms using *in vitro* methods the pharmacokinetic profile of potential drugs in human beings has rapidly progressed.[1] Conventionally drug metabolism studies are performed on tissue culture, *in vitro* enzyme systems, small animal models, perfused organs, *in vitro* cell cultures and liver microsomal preparations. The quality and yield of the microsomes in the preparations is judged by the NADPH-dependent N- and p-hydroxylation of N-ethylaniline, protein content, and cyanide-sensitive respiration.[2] Synthesis of metabolites in laboratories is tedious process therefore microorganisms can be used as a resourceful alternative to produce these metabolites. Cytochrome P450s represent the most important class of enzymes involved in phase I metabolism, being involved in 75–80% of metabolism of marketed drugs. Phase I reactions involve hydroxylation, epimerization, oxidation and reduction.[3] These enzymatically catalyzed reactions alter aromatic amino functional groups. This increases the polarity and/or makes aromatic amino acid structures more accessible to phase II metabolizing enzymes. The primary superfamily of phase I enzymes are Cytochrome P450, which hold catalytic versatility.[1] A relatively novel perspective in modern drug development is the use of pharmacologically active metabolites as potential resources for drug discovery and development. There are several advantages for screening drug candidates for active metabolites during drug discovery.[4] By creating a so-called prodrug it is possible for the drug to reach the target area in the body and gain the pharmacological effect when it undergoes a biotransformation process, an example is drug amitriptyline. Amitriptyline is demethylated in the liver to nortriptyline which is an active metabolite to give its pharmacological action.[5] Drug toxicity can arise from the biotransformation when a metabolite is electrophilic and react with *e.g.*, a protein. A classic example is paracetamol that can cause liver damage. Damage to the liver is due to a toxic metabolite (N-acetyl-p-benzoquinone imine (NAPQI)) produced by cytochrome P450 enzymes in the liver. The drug could also cause rare and possibly fatal, skin reactions; asthma and hearing loss. This damage is averted by conjugation of paracetamol' s reactive metabolite with glutathione but when the reserves of glutathione run low, serious liver damage can be the result.[6]

Microbial metabolism model evolved as one of the *in vitro* model to subjugate the flaws and disadvantages of other mammalian models. Microbial models can be used as a supplementary tool to imitate mammalian metabolism by minimizing the usage of animals and human subjects in the drug discovery and development process. Microorganisms such as bacteria, yeast and fungi can be used as *in vitro* models for successful prediction of mammalian drug metabolism with significant applications. They have shown their possible use *in vitro* models of drug metabolism. There are studies that have shown that fungi *Cunninghamella* species produce mammalian metabolites of different drugs such as meloxicam, naproxen, amitriptyline, omeprazole, clemastine and more.[7–10] These fungi produce metabolizing enzymes that can perform both phase I and phase II biotransformation reactions and their metabolism of numerous substances have been investigated. *C. elegans* has been proven to carry at least one gene coding for a CYP enzyme closely related to the CYP51 family.[11] *Cunninghamella* species are mainly soil fungi of the Mediterranean and subtropical zones; they are rarely isolated in less temperate regions. The experimental procedure of cultivating the fungi and using them in biotransformation is simple, requires a low input of work and has a low cost.[12] Different approaches can be identified for the development of microbial models for a given drug; the prediction and confirmation of metabolite production in mammals can be facilitated by prior generation of analytical standards of metabolites using the microbial model or a parallel microbial and mammalian metabolism may be conducted. In any approach, the preparative scale production of metabolites would facilitate their biological and toxicological evaluation, stereochemistry, and mechanism of formation.

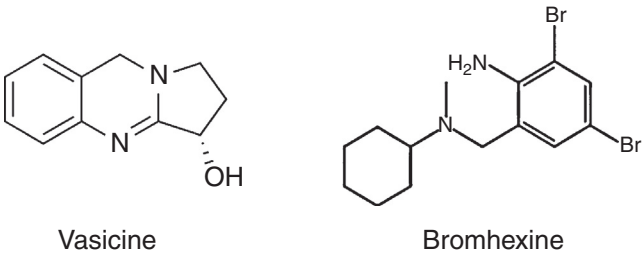

Fig. 1 – Bromhexine, a synthetic derivative of vasicine from *Adhatoda vasica*.

Bromhexine (2-amino-3, 5-dibromo-N-cyclohexyl-N-methylbenzylamine) is a synthetic derivative of vasicine, one of the active ingredients of the Asian plant *Adhatoda vasica* (Fig. 1). Bromhexine has proven its effectiveness in normalizing mucus in the respiratory tract so that a natural cough response can clear the airway. Introduced for the first time in 1963, as a secretolytic or mucolytic medicine by Boehringer Ingelheim, bromhexine became one of the most popular cough remedies. Today, it is still widely used as an over-the-counter drug. CYP3A4 enzymes are responsible for the drug's metabolism and in clinical use bromhexine is administered orally three times a day at dosage of 8 or 16 mg per dose.[13] Ambroxol is an active N-desmethyl metabolite of bromhexine. It is formed after deletion of a methyl group and introduction of a hydroxyl group in a para-trans position of cyclohexyl ring. It gains several important pharmacological properties like surfactant stimulatory, anti-inflammatory, anti-oxidant, and local anesthetic effect besides the muco-kinetic and muco-ciliary effects of the parent compound bromhexine.[12] Some drugs that inhibit CYP3A4 activity increases the plasma concentrations of the CYP3A4 substrate drug. Drugs, such as clarithromycin, itraconazole, and ketoconazole, are potent inhibitors of CYP3A4; these drugs may have markedly reduced CYP3A4 activity in patients.[14] The metabolism of bromhexine has been studied using pig hepatocyte cultures. They maintain both phase I and phase II biotransformation reactions and considered to be an established *in vitro* model. Phase I reactions, *i.e.* hydroxylation and demethylation occurs fast whereas hydroxylated/demethylated and aminal hydroxylated metabolites formation from multiple-step reactions takes longer time. Besides metabolites formed *in vivo*, three unknown components were also detected.[15]