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Structure-based prediction and biosynthesis of the major mammalian metabolite of the cardioactive prototype LASSBio-294

Emmanuel O. Carneiro [a], Carolina H. Andrade [a], Rodolpho C. Braga [a], Andréa C. B. Tôrres [b], Rosângela O. Alves [b], Luciano M. Lião [c], Carlos A. M. Fraga [d], Eliezer J. Barreiro [d], Valéria de Oliveira [a,*]

[a] Laboratório de Bioconversão, Faculdade de Farmácia, Universidade Federal de Goiás, 1ª Avenida esquina c/Praça Universitária, S/N. Caixa Postal 131, Setor Universitário, CEP: 74.605-220, Goiânia, GO, Brazil
[b] Escola de Veterinária, Universidade Federal de Goiás, Goiânia, GO, Brazil
[c] Laboratório de Ressonância Magnética Nuclear, Instituto de Química, Universidade Federal de Goiás, Goiânia, GO, Brazil
[d] Laboratório de Avaliação e Síntese de Substâncias Bioativas (LASSBio), Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

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ABSTRACT

A new bioactive compound of the *N*-acylhydrazone class, LASSBio-294, was shown to produce a cardioinotropic effect and vasodilation. In this study, we report the structure-based drug metabolism prediction, biosynthesis and identification of the major mammalian metabolite of LASSBio-294.

Drug discovery and development is a lengthy, complex and expensive process. Nowadays, a combination of modern technologies and innovative strategies is increasing the demand for new compounds that need to be screened in a wide range of biological assays. New chemical entities expected to advance into clinical trials should have an ideal balance of pharmacokinetic properties: absorption, distribution, metabolism, and excretion (ADME).[1] In this context, it is very important to know the pharmacokinetic profile of compounds under biological investigation. Drug metabolism is a major consideration for modifying drug clearance and also a primary source for drug metabolite-induced toxicity. With major cytochrome P450 structures identified and characterized recently, structure-based drug metabolism prediction becomes increasingly attractive.[2]

Conventional therapy to treat hypertension often involves relaxation of vascular smooth muscle. Decrease of blood pressure by vasodilators is normally associated with adverse effects because of their low vascular selectivity.[3] Several bioactive agents have been synthesized but none of them has had a specific action, free of side effects, reinforcing the importance of identifying new clinically safe useful vasodilator agents.[3] New bioactive compounds of the *N*-acylhydrazone class (NAH) were synthesized from safrole (**1**), a Brazilian natural product.[4] Replacing the phenyl ring attached to the imine moiety by the isosteric 2-thienyl ring resulted in the design of 3,4-methylenedioxybenzoyl-2-thienylhydrazone, named LASSBio-294 (**2**) (Fig. 1).[5] LASSBio-294 was described as a potent positive cardio inotropic agent due to an increase in the Ca^{2+} accumulation in the sarcoplasmic reticulum (SR).[6] In addition, LASSBio-294 also promoted vasodilation in aortic rings, mediated by the guanylate cyclase/cyclic guanylate monophosphate pathway.[6] The next step in the design process is the pharmacokinetics studies.

The use of microorganisms as models of mammalian metabolism was introduced in the early 1970s[7] and it is a very interesting tool in the production of mammalian metabolites that can be used as reference standards in animal models of drug metabolism studies. Compared with more traditional methods, there are clearly a number of practical advantages in using microbial transformation as a model for drug metabolism, such as its low cost, high yield and easiness to regulate.[7]

In this work we describe the structure-based metabolism prediction, biosynthesis and identification of the major mammalian metabolite of LASSBio-294.

The binding poses of LASSBio-294 in the active site of CYP2C9 were analyzed using the MOE-Dock software allowing receptor flexibility.[8] The analysis was restricted to CYP2C9 according to previous results, based upon Lewis decision tree approach.[9] The

* Corresponding author. Tel.: +55 62 3209 6449; fax: +55 62 3209 6037.
E-mail address: valeria@farmacia.ufg.br (V. de Oliveira).

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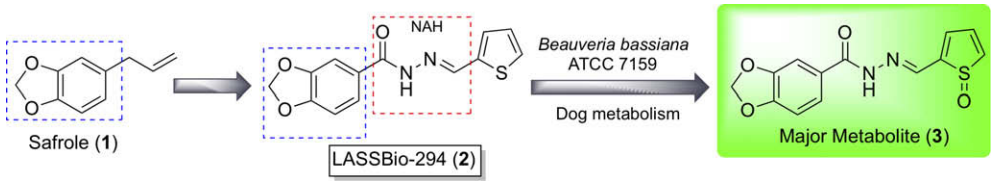

Figure 1. Structural design concept of LASSBio-294 and the biosynthesis of its major mammalian metabolite.

binding energy of the best pose obtained from docking was −4.81 kcal/mol. Figure 2 illustrates the energetically favored docking mode for LASSBio-294. The sulfur atom of the thiophene ring is in close proximity to the heme iron (~3.8 Å), which suggests that metabolism may occur by sulfoxidation.

The in vitro metabolism study of LASSBio-294 was conducted using the fungus *Beauveria bassiana* ATCC 7159, while in vivo metabolism evaluation was conducted in dog. The soil fungus *B. bassiana* ATCC 7159, was chosen as microbial model to investigate LASSBio-294 metabolism because it is one of the most frequently used and versatile whole-cell biocatalysts and is able to perform a wide range of reactions, including the cleavage of dioxolo ring.[10]

Erlenmeyer flasks containing 100 mL of liquid medium PDSM were inoculated with 0.5 mL of a spore suspension of *B. bassiana* ATCC 7159 obtained from seven days grown potato agar slants and glycerol 25%. The flasks were incubated with LASSBio-294 dissolved in a solution of dimethylformamide at a final concentration of 25 mg/100 mL. The flasks were kept in shaker under 200 rpm at 27 °C ± 2 °C for 96 h. Aliquots (1.0 mL) of the supernatant were taken every 24 h, up to 96 h and analyzed by HPLC. Control flasks consisted of culture broth without substrate to exclude components of cells walls fungi possibly detected by HPLC. The experiments without microorganisms were carried out to verify the stability of the substrate by addition of 1 mL of a solution of dimethylformamide. No oxidation products could be observed under these conditions. At the end of the process, the incubation medium was extracted with ethyl acetate to give oily crude which was purified by silica gel column chromatography using ethyl acetate/methanol (50:50 v/v) as eluent. Final purifications were achieved by recrystallization.

In a previous study, LASSBio-294 was tested for toxicological properties in mouse, rat, and dog models; and has very low toxic effects.[11] For the in vivo metabolism evaluation, capsules containing 7.5 mg of LASSBio-294 were given to a dog (1.0 mg/Kg), in a pilot study, in order to compare the metabolites obtained. Dog's serum and urine samples were collected before the administration and after 30 min, 90 min, 2 h, 4 h, and 8 h of administration and analyzed by HPLC.

According to HPLC analysis, *B. bassiana* ATCC 7159 produced one major metabolite at high concentrations in 24 h. Moreover, this metabolite was found identical to that observed in dog serum samples after 4 h of administration.

This result demonstrates the ability of this fungus to mimic mammalian metabolism of LASSBio-294. The metabolite produced by *B. bassiana* ATCC 7159 in 24 h of incubation is the same found in the dog serum, named as compound **3**. Compound **3** was obtained after recrystallization as white crystals, yield of 6.0%.

The structure of compound **3** was elucidated by ^{13}C and ^{1}H NMR analysis and it was consistent with expected structure.[12] The mass of **3** was confirmed by ESI-MS/MS as 291 for $[M+H]^+ + 16$ (O). Figure 3 shows the product-ion for the sulfoxide metabolite $[M+H]^+$, *m/z* 291, resulted in four major fragments at *m/z* 191.2,

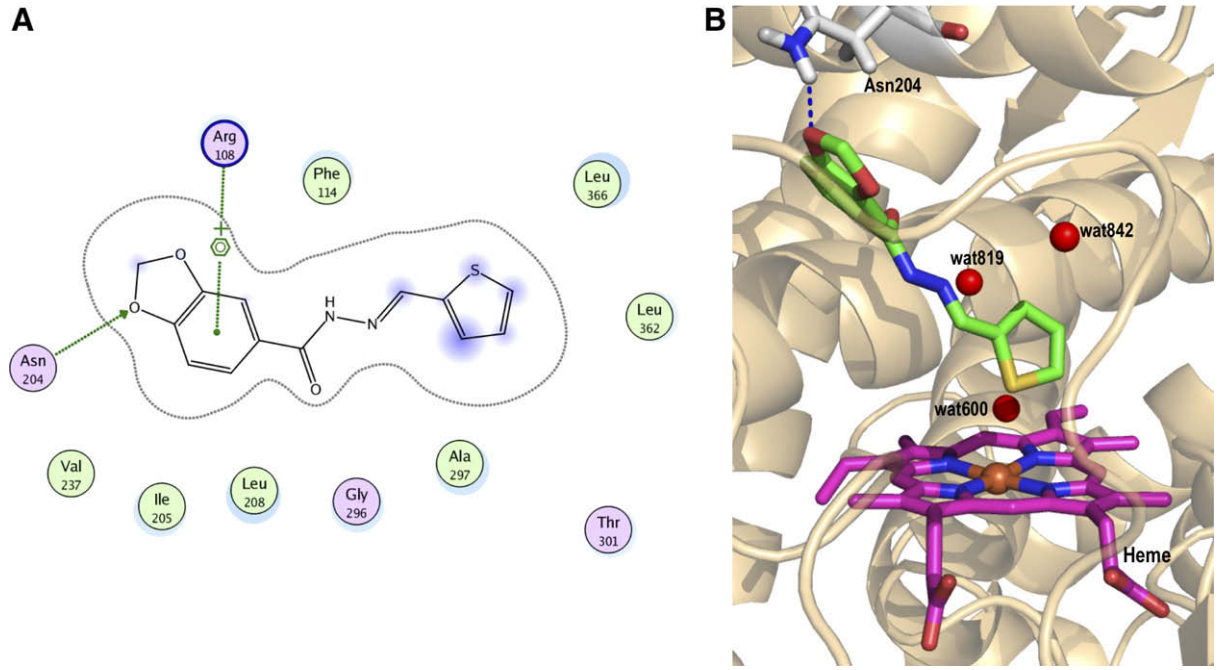

Figure 2. The binding pose of LASSBio-294 in the active site of CYP2C9 predicted by docking. (A) Schematic representation of active site residues and LASSBio-294 interactions. (B) 3D representation including the heme group (magenta), the active site water molecules (red spheres) and LASSBio-294 (green) from docking. The distance between LASSBio-294 and heme iron is ~3.8 Å. LASSBio-294 is shown in color-coded sticks: carbon = green, nitrogen = blue, oxygen = red, and sulfur = yellow.

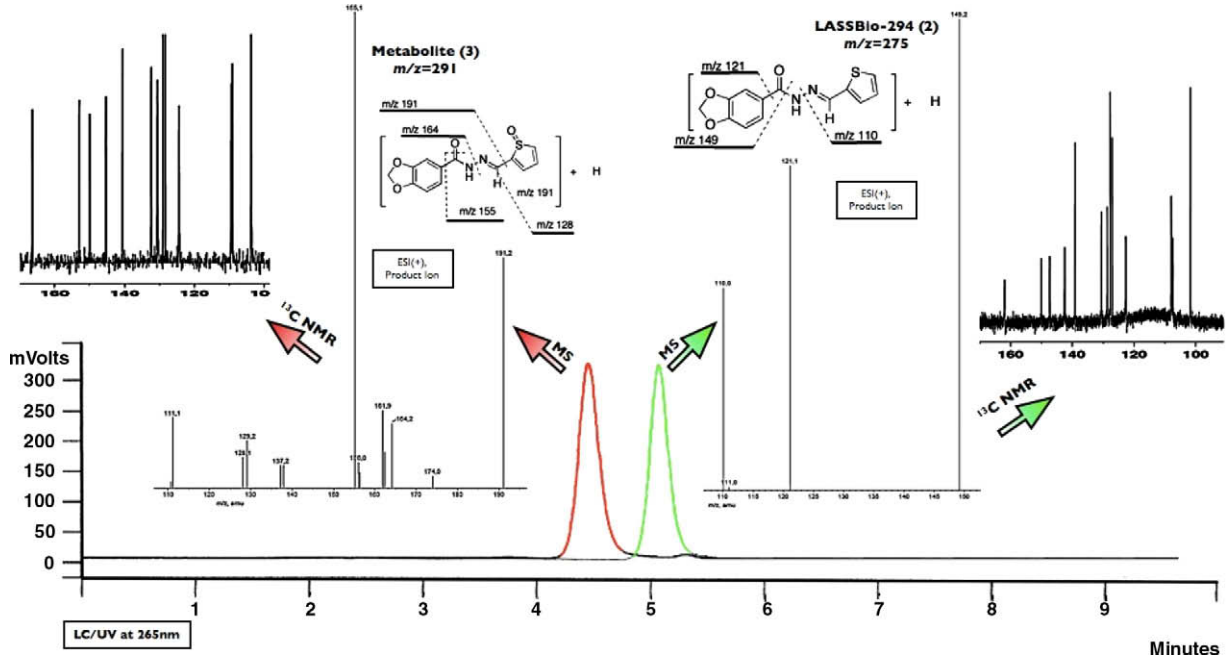

Figure 3. HPLC chromatograms, ^{13}C NMR and MS spectra of LASSBio-294 *m*/*z* 274 (A) and its sulfoxide metabolite (**3**) *m*/*z* 291 (B).

164.2, 155.1 and 128.1, with a shorter retention time than LASSBio-294. From these spectral data, **3** was characterized as sulfoxide metabolite of LASSBio-294.

The results reported here have showed that the sulfoxide metabolite was the major product produced by *B. bassiana* ATCC 7159, and also the major metabolite found in the dog serum. A number of known drugs are metabolized in mammals to its sulfoxides. The formation of sulfoxides (sulfoxidation) occurs in the metabolism of phenothiazine,[13] promazine,[13] chlorpromazine,[13] thioridazine,[13] albendazole,[14] and the proton pump inhibitors omeprazole, lansoprazole, pantoprazole and rabeprazole.[15] In some cases, the sulfoxide metabolite is more active than the parent drug, or has a different activity, reinforcing the importance of metabolism studies of a new chemical entity. All these findings demonstrate the relevance of this sulfoxide in mammalian metabolism.

As concluding remarks, the results from the CYP2C9 docking analysis were consistent with experimental data, as LASSBio-294 adopted an orientation in favor of sulfoxidation. The sulfoxide metabolite (**3**) of the cardioactive prototype LASSBio-294 was successfully biotransformated and characterized using the fungus *B. bassiana* ATCC 7159, which will be further evaluated for its pharmacological and toxicological profiles.

Acknowledgements

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8. *Molecular docking*: Docking studies were performed using Molecular Operating Environment (MOE) version 2008.10 (Chemical Computing Group Inc., Montreal, Canada). The crystal structure of LASSBio-294 was obtained from Cambridge Crystallographic Data Centre (CCDC code 707596). The MMFF94x force-field was used to calculate the partial charges. The X-ray crystal structure of CYP2C9 complexed with flurbiprofen at 2.0 Å resolution (PDB ID: 1R9O) was obtained from the Protein Data Bank. The enzyme was prepared for docking studies where: (i) ligand molecule was removed from the enzyme active site. (ii) Water molecules were removed, keeping just the important active site water molecules (wat600, wat819 and wat842). (iii) Hydrogen atoms were added to the structure with their standard geometry. (iv) MOE Alpha Site Finder was used for the active sites search in the enzyme structure and dummy atoms were created from the obtained alpha spheres. The MOE-Dock function was used for docking. Ligand placement was performed using alpha sphere triangle matching, with Affinity dG scoring. The top 30 poses were retained and refined using MMFF94x forcefield energy minimization with Generalized Born solvation model, allowing the receptor residues within 6 Å to relax around the mobile ligand. The receptor side chains were tethered with a force constant of 1.0 kcal/(mol Å^2). Energy minimization was stopped when the root-mean-squared gradient cutoff of 0.01 kcal/(mol Å) was reached. Final poses were ranked using the Affinity dG method to calculate the free energy of binding.
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12. *Experimental data for LASSBio-294*: ^1H NMR (500 MHz, MeOD): δ 11.56 (s, 1H), 8.62 (s, 1H), 7.64 (d, 1H), 7.49 (dd, 1H), 7.44–7.41 (m, 2H), 7.12 (dd, 1H), 7.03 (d, 1H), 6.18 (s, 2H). ^{13}C NMR (126 MHz): δ 101.2, 112.7, 115.4, 120.8 125.1, 125.8, 127.1, 127.4, 127.5, 144.2, 148.9, 152.2, 162.0. Compound **3** ^1H NMR (500 MHz): δ 11.56 (s, 1H), 8.50 (s, 1 H), 7.55 (d, 1H), 7.51 (dd, 1H), 7.42–7.38 (m, 2H), 7.11 (dd, 1H), 6.93 (d, 1H), 6.18 (s, 2H). ^{13}C NMR (126 MHz): δ 101.79, 104.49, 109.04, 124.13, 128.22, 128.62, 130.29, 132.12, 140.31, 145.01, 149.67, 152.68, 166.09. Elemental Anal. (%) for $C_{13}H_{10}N_2O_4S$: C, 53.79; H, 3.47; N, 9.65.
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