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CYP1A2-mediated biotransformation of cardioactive 2-thienylidene-3,4-methylenedioxybenzoylhydrazine (LASSBio-294) by rat liver microsomes and human recombinant CYP enzymes

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ABSTRACT

We describe herein the metabolic fate of cardioactive 1,3-benzodioxolyl *N*-acylhydrazone prototype LASSBio-294 (**4**) and the structural identification of its major phase I metabolite from rat liver microsomal assays. Our results confirmed the hard-metabolic character of *N*-acylhydrazone (*NAH*) framework of LASSBio-294 (**4**). The development of a reproducible analytical methodology for the major metabolite by using HPLC–MS and the comparison with an authentic synthetic sample, allowed us to identify 2-thienylidene 3,4-dihydroxybenzoylhydrazine derivative (**7**), formed by oxidative scission of methylenedioxy bridge of LASSBio-294, as the main metabolite formed by action of CYP1A2 isoform. The identification of this isoform in the LASSBio-294 in the clearance of LASSBio-294 (**4**) oxidation was performed by the use of selective CYP inhibitors or human recombinant CYP enzymes.

1. Introduction

The metabolism is one of the most important factors that can affect an overall pharmacokinetics profile of xenobiotics, including drugs [1], contributing to the inadequate absorption, distribution, metabolism and excretion (ADME) properties of a drug candidate in the late stages of drug development. The monooxygenase cytochrome CYP450 enzymes are involved in a significant number of events associated with drug metabolism [2]. The characterization of the major metabolites or principal soft-metabolic points in the structure of a drug candidate, at an early phase of drug discovery process, is crucial to give information about several important structural properties as new pharmacologically inactive or toxic entities, poor bioavailability, efficacy and safety-related events [3].

N-Acylhydrazone (*NAH*) is a versatile moiety in medicinal chemistry [4]. Recently, Duarte and co-workers [5] have classified this spacer structural unit as a privileged structure, which is able to interact selectively with different target biomacromolecules [4]. Illustratively, the 6-nitro-3,4-methylenedioxyphenyl-*N*-acylhydrazone derivative (**1**), that presents 3,5-di-*tert*-butyl-4-hydroxyphenyl sub-unit, was able to act as TRPV1 (transient receptor potential vanilloid subfamily type 1) antagonist [6] whereas 6-methanesulfonamide-3,4-methylenedioxyphenyl-*N*-acylhydrazone derivative (**2**) was pointed out as a novel anti-inflammatory prototype, acting mainly as a non-selective cyclooxygenase (COX) inhibitor [7]. For instance, arylsulfonate–acylhydrazone derivative (**3**) represents a promising anti-platelet lead-candidate with significant antithrombotic activity *in vivo*, inhibiting the platelet aggregation induced by thrombin [8] (Fig. 1)

2-Thienylidene-3,4-methylenedioxybenzoylhydrazine (LASSBio-294; **4**) [9], was originally designed as phosphodiesterase (PDE) inhibitor candidate synthesized from natural safrole (Fig. 1), an

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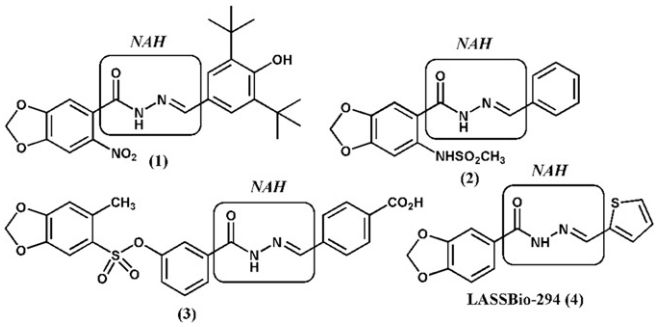


Fig. 1. Structures of different bioactive prototypes (**1**–**4**) possessing *NAH* moiety.

abundant Brazilian natural product, obtained from Sassafras oil in high yield by classic distillation (*Ocotea pretiosa* and *Piper hispidinervum*) [10,11]. This *N*-acylhydrazone (*NAH*) derivative was characterized as a novel possible alternative for treatment of cardiac failure, once it was able to promote an effective positive inotropic and vasodilatory activity [9] through a mechanism different from that displayed by cardiac glycosides and β-adrenergic agonists. LASSBio-294 (**4**) increased the spontaneous contraction of isolated hearts of Wistar rats in a dose-dependent manner (maximum effect at 25 μM) and the Ca^{2+} uptake into sarcoplasmic reticulum (SR) without changing the sensitivity of the contractile proteins to Ca^{2+} [12]. Moreover, this compound induced relaxation of isolated rat aorta with an IC_{50} of 74 μM [13] by increasing intracellular cyclic GMP levels. These pharmacological evidences suggest a novel mechanism of action, circumventing the toxic effects resulted from calcium homeostasis alteration [13]. More recently, LASSBio-294 (**4**) was also described to prevent myocardial infarction induced cardiac dysfunction through improving intracellular Ca^{++} regulation [14].

Taken together, the cardioinotropic and vasodilatory profiles of the *N*-acylhydrazone prototype (**4**) strongly indicated that it is a novel drug candidate, effective and safer to treat pathological conditions in which cardiac muscle fatigue is a debilitating disorder [15]. In this context, the development of a new therapeutic candidate at the preclinical stage involves the investigation of its biotransformation profile in order to access important information about its pharmacokinetic behavior [16]. Despite the structure-based prediction and mammalian biomimetic biosynthesis of thiophene sulfoxide (**5**) as the main metabolite of LASSBio-294 (**4**) produced by fungus *Beauveria bassiana* ATCC 7159 strains [17], the reactivity of this kind of compound as electrophile generally prevent its isolation [18–20] (Scheme 1).

The classical, usual and feasible *in vitro* tool to determinate the metabolic fate of a drug candidate consists in the exploitation of liver microsomal fractions obtained by differential centrifugation [21]. Additionally this procedure allows identifying which CYP isoforms are oxidizing the drug candidate by the use of selective CYP inhibitors [22]. In this context we performed this study in order to investigate the metabolic fate of this important cardioactive lead-compound, LASSBio-294 (**4**), and the structural identification and characterization of major metabolites evidenced from rat and human *in vitro* assays.

2. Results and discussion

2.1. Molecular docking studies of LASSBio-294 (4) on CYP1A2

Preliminary molecular docking studies with the principal human CYP isoforms indicated by the decision tree for evaluating P450 specificity developed by Lewis [23], was performed by using

Scheme 1.

GOLD 4.0.1 software [24]. The obtained results have pointed that CYP1A2 [25] possess important molecular recognition pattern to LASSBio-294 (**4**). The GoldScore fitness function [26] was used due the program performance to accurately predict the ligand binding mode. Five consecutive docking runs using the same input file were performed, in order to validate the experiments. The CYP1A2-LASSBio-294 complex with the higher fitness score among the top-scoring complexes was used for further visual inspection of the principal interactions. The binding position of LASSBio-294 (**4**) indicates that 1,3-benzodioxole ring is close to heme-iron atom (*ca.* 2.5 Å), indicating that this moiety is the principal soft-metabolism point of (**4**). The molecular recognition is reinforced by favorable hydrophobic π–π stacking interactions among three phenylalanine amino acids residues - Phe226, Phe256 and Phe260 - with thiophene ring of (**4**), as illustrated in Fig. 2.

2.2. Analytical procedures to LASSBio-294 (4)

The characterization and determination of (**4**) in the *in vitro* assays was validated regarding calibration curve (data not shown), limit of detection (LOD) and limit of quantification (LOQ). The

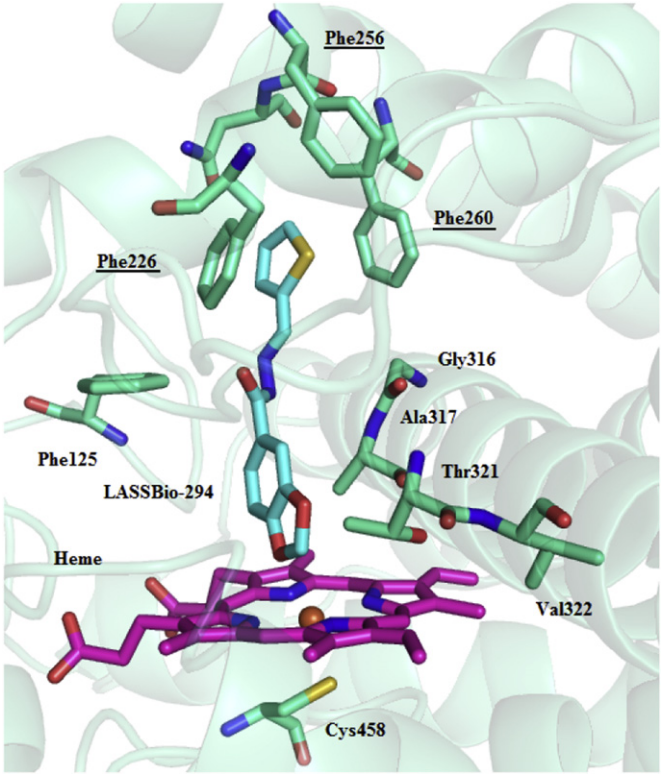
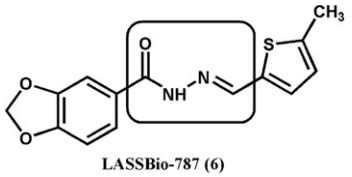


Fig. 2. Docking of LASSBio-294 in the active site of CYP1A2. The hydrogen atoms were removed for better visualization. 3D representation including the heme group (magenta), the active site and LASSBio-294 (**4**) is shown in color-coded sticks: carbon = cyan (ligand) or green (aminoacid residues), nitrogen = blue, oxygen = red and sulfur = yellow. The figure was generated using PyMOL [27]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).



LASSBio-787 (6)

Fig. 3. Structure of LASSBio-787 (**6**), used as internal standard in HPLC analysis.

lowest level at which the prototype (**4**) could be reliably detected was 0.25 μg/mL. For quantitative methods, the lowest measured level of (**4**) is often the lowest point of the calibration curve, *i.e.* 0.5 μg/mL. The biotransformation profile of LASSBio-294 (**4**) and its concentration after *in vitro* assay was determined by comparison with an internal standard (IS), *i.e. NAH* derivative LASSBio-787 (**6**) [28] due to its structural similarity and different retention time in comparison with our analyte (**4**) (Fig. 3).

2.3. HPLC analysis of the in vitro metabolic profile of LASSBio-294 (*4*)

The NADP-dependent metabolism of LASSBio-294 (**4**) was studied in rat liver microsomes after incubation at 37 °C for 1 h. Only one product (**7**) was identified on HPLC with shorter retention time (4.2 min) than the prototype (**4**) and the internal standard (**6**), which eluted at 9.4 and 13.5 min, respectively (representative chromatograms are shown in Fig. 4). The analysis of the blank sample indicated no presence of interfering substances at that retention time. The presence of LASSBio-294 (**4**) was confirmed by the comparison of the retention time, analysis of UV spectra and co-elution with the authentic standard.

2.4. LC-MS analysis of the in vitro metabolic profile of LASSBio-294 (*4*)

LASSBio-294 (**4**) was observed in rat liver microsomes incubation samples. The mass spectra showed the pseudo-molecular ion $[M + H]^+$ ion at *m*/*z* 275 and its MS spectra indicated a characteristic fragmentation pathway of *m*/*z*: 275–191–149 and retention time (Fig. 5) which was identical to the standard samples of LASSBio-294 (**4**). These data confirmed that the peak at 9.4 min was relative the prototype (**4**).

2.5. Characterization of the major metabolite (*7*)

The LC-ESI-MS method was used for characterization of the single metabolite detected after the rat liver microsomes incubation.

Mass spectra of (**7**) (Fig. 6) showed the pseudo-molecular ion $[M + H]^+$ shifts to *m*/*z* 263. The *m*/*z* 245 is indicative of the loss of water (18 Da) and suggested the presence of at least one hydroxyl group. On the other hand, the *m*/*z* 110 and *m*/*z* 127 derived respectively from fragmentation of the hydrazone ($RNH = NCH_2R$) and the amide groups, similarly to that described previously for the prototype LASSBio-294 (**4**).

Additionally, the McLafferty rearrangement in the *N*-acylhydrazone group of (**7**) produces the ions *m*/*z* 153 and 110, as described previously for (**4**) and other *NAH* analogues [29,30], whereas the fragmentation of the amide unit yielded the corresponding acylium ion (*m*/*z* 137) (Fig. 6), indicating that oxidative metabolic pathway occurs only at 1,3-benzodioxole ring of compound (**4**).

Based on these evidences, the unique metabolite of LASSBio-294 (**4**) obtained from *in vitro* metabolism employing rat liver

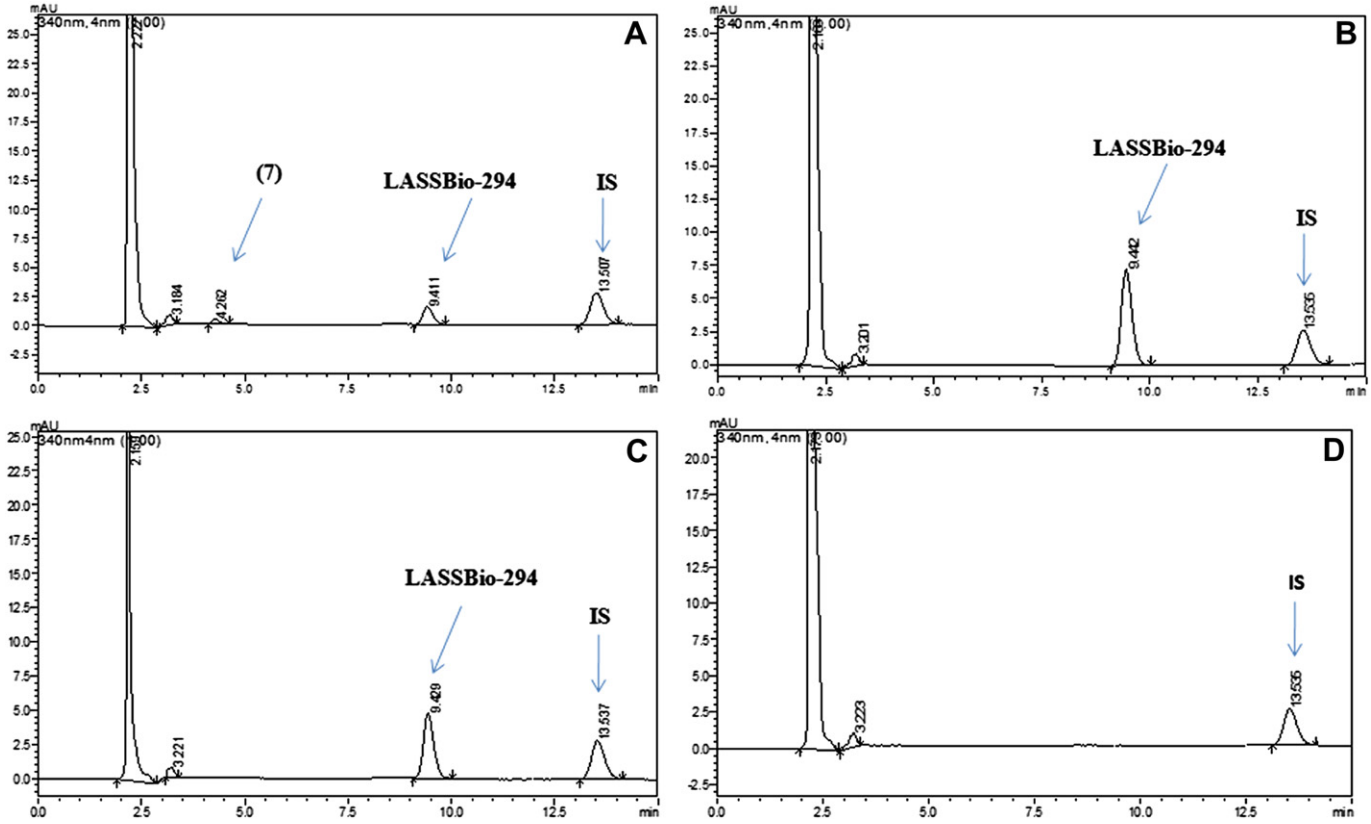


Fig. 4. Representative chromatograms of rat liver microsome incubations with LASSBio-294 (**4**). A) Incubation of (**4**) with 1 mg/mL microsomal proteins in the presence of NADPH-generating system; B) Incubation of (**4**) only with the NADPH-generating system; C) Incubation of (**4**) with 1 mg/mL microsomal proteins in the absence of NADPH-generating system; D) Blank experiment, *i.e.* 1 mg/mL microsomal proteins plus NADPH-generating system in the absence of (**4**). IS = internal standard LASSBio-787 (**6**).

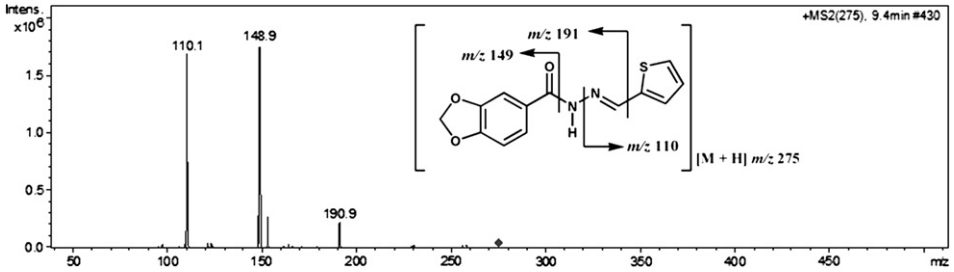


Fig. 5. Mass spectrum of LASSBio-294 (**4**) detected in rat liver microsome incubation.

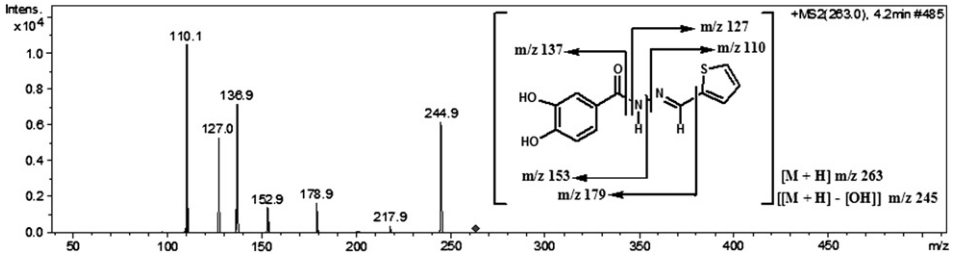


Fig. 6. Mass spectrum of metabolite (**7**) obtained from rat liver microsome incubation of *NAH* prototype (**4**).

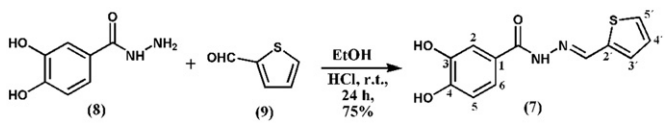
microsomes was identified as the cathecol-derivative (**7**), which was synthesized as illustrated in Scheme 2. Compound **7** could be synthesized in 75% yield through the acid-catalyzed condensation of 3,4-dihydroxybenzoylhydrazine (**8**) [31] with 2-thiophenecarbaldehyde (**9**). Analysis of the ^{1}H NMR spectrum of the reaction product after purification showed the presence of a broad signal referent to two hydrogen atoms from cathecol moiety at 9.40 ppm and only one signal referent to imine hydrogen at 8.62 ppm, which is indicative of the diastereoselective construction of the acylhydrazone group, as reported previously [28,30].

Furthermore, the same retention time of the co-elution of (**7**) with the synthetic authentic standard got further confidence to this peak assignment. Mass spectra analysis of synthetic samples of (**7**) is consistent with the fragmentation pattern described previously for the metabolite derived from LASSBio-294 (**4**) biotransformation.

The metabolism of related bioactive substances containing this kind of bis-oxygenated heterocyclic ring, as paroxetine [32], MBDB [33] and sanguinarine [34] also involves the oxidation of the 1,3-benzodioxolyl group, resulting in the ring-cleavage and *O*-demethylenation to produce the respective 3,4-dihydroxyphenyl metabolite. The higher polarity of metabolic oxidation product (**7**) is in agreement with its shorter HPLC retention time (4.2 min) in comparison with the parent prototype (**4**) (9.4 min), as displayed in the Fig. 4A.

2.6. Characterization of in vitro metabolic profile of LASSBio-294 (*4*)

Few reports on the oxidative metabolism of the *NAH* derivatives have been described [35,36]. On the other hand, the present study indicates that when the *NAH* derivative LASSBio-294 (10 μM) was incubated with rat liver microsomes (1 mg/mL) it underwent oxidative metabolism with the formation of only one metabolite (**7**). The appearance of (**7**) was NADP-dependent once no products were observed when this co-factor was omitted from the incubation medium (Fig. 4C), suggesting that it was biosynthesized by catalytic activity of CYP450 enzymes. The $t_{1/2}$ for LASSBio-294 (**4**) consuming was 22.8 min and the $t_{1/2}$ for the metabolite (**7**) formation was 13.8 min after incubation with rat liver microsomes until 120 min.



Scheme 2.

The most probably isoform of CYP involved in metabolic transformation could be appointed by use of some selective and non-selective inhibitors. The effect of these CYP inhibitors, CYP1A2 (furafylline) [37], CYP2C9 (sulfaphenazole) [34], CYP2D6 (quinidine) [38], and non-selective (ketoconazole) [39], on the production of (**7**) after incubation of 10 μM of (**4**) with 1.0 mg/mL of rat liver microsomes was compared with the control assay, where the relative concentration of metabolite was 1.07 μM after 60 min (Table 1). The concentration of metabolite (**7**) was reduced to 11%, 29%, 82% and 85% of original biotransformation when furafylline, ketoconazole, sulfaphenazole and quinidine were used, respectively, corresponding to a decrease in 89%, 71%, 18% and 15% of

Table 1
Effect of various inhibitors of CYP enzymes on the formation of metabolite (**7**) by rat liver microsomes.

CYP inhibitor[a]	Concentration of metabolite (**7**)[b] (μM)	Inhibition of the formation of (**7**)[c,d] (%)
Control	1.07	0
Furafylline	0.12	89
Ketoconazole	0.31	71
Sulfaphenazole	0.85	18
Quinidine	0.91	15

[a] The employed concentration of the inhibitors are described in Experimental section.
[b] Determined from HPLC–MS analysis using LASSBio-787 (**6**) as internal standard.
[c] The results are the mean of three independent experiments.
[d] These results were obtained after comparison with the control assay employing 10 μM of LASSBio-294 (**4**), 1 mg/mL of rat liver microsomal proteins for 60 min of incubation, in the absence of CYP inhibitors.

Table 2
Metabolic stability of LASBio-294 (**4**) by human recombinant of CYP enzymes.

Human recombinant CYP isoform[a]	Recovered amount of LASSBio-294[b,c] (%)
CYP1A2	67
CYP2B6	98
CYP2C8	99
CYP2C9	81
CYP2C19	96
CYP2D6	95
CYP3A4	92
CYP3A5	100

[a] The employed concentration of the human recombinant CYP enzymes are described in experimental section.
[b] The results are expressed as the mean of two independent experiments.
[c] The concentration of LASSBio-294 (**4**) used was 1 μM during 60 min of incubation.

the metabolism. Its results suggested once again that the formation of (**7**) is CYP-dependent and more specifically CYP1A2 seems to be the major isoform involved in the LASSBio-294 (**4**) metabolism in rats. Moreover, experiments using ketoconazole, a CYP-non-selective inhibitor prevented the bioformation metabolite (**7**) by 71% [39].

The metabolism of LASSBio-294 was reduced to 11%, 29%, 81% and 85% of original biotransformation step when furafylline, ketoconazole, sulfaphenazole and quinidine were used, respectively. Its results suggested once again that the formation of (**7**) is CYP-dependent and more specifically CYP1A2 seems to be the major isoform involved in the LASSBio-294 (**4**) metabolism in rats Moreover, experiments using ketoconazole, a CYP-non-selective inhibitor prevented the bioformation metabolite (**7**) by 71% [39].

Moreover, the *in vitro* metabolic stability of LASSBio-294 (**4**) was performed employing human recombinant CYP enzymes [40]. CYP1A2 was responsible for the highest consumption of the prototype with 33% of biotransformation (67% of recovered LASSBio-294), suggesting that this CYP isoform also exerts a major contribution for the oxidative metabolism of the LASSBio-294 (**4**) in humans, followed by a less pronounced clearance mediated by CYP2C9 (81% of recovered LASSBio-294) (Table 2).

The formation of metabolite (**7**) and the main CYP isoform involved in biotransformation of LASSBio-294 (**4**), could be adequately predicted by using the Lewis' decision tree [23] coupled to molecular docking studies, which indicated the vulnerability of the methylene unit at 1,3-benzodioxole ring of LASSBio-294 (**4**) to the oxidation step promoted by CYP1A2, over other possible metabolism sites.

3. Conclusions

A pattern of biotransformation for the *NAH* compound LASSBio-294 (**4**), a cardioactive drug candidate, was determined by a useful assay employing rat liver microsomes. The development of this *in vitro* methodology allowed the investigation of the metabolic behavior of *NAH* framework, which demonstrated to be a hard-metabolophoric unit when exposed to microsomal enzymes. Our results pointed to the formation as only major metabolite compound **7**, which was structural characterized by LC–MS analysis, and by comparison with a fully characterized authentic synthetic standard. The use of selective CYP inhibitors characterized CYP1A2 as the main isoform involved in the oxidative metabolism of LASSBio-294 by rat liver microsomes and a similar behavior was revealed when human recombinant CYP enzymes were exploited, indicating that *NAH* moiety has a hard-metabolic character in this cardioactive drug candidate (**4**).

4. Experimental part

4.1. Chemistry

Melting points were determined with a Quimis 340 apparatus and are uncorrected. ^{1}H NMR spectra were obtained in a Bruker AC 200 spectrometer at 200 MHz, using deuterated dimethylsulfoxide as solvent. Splitting patterns are as follows: s, singlet; d, doublet; dd, double doublet; br, broad. ^{13}C NMR spectra were obtained using the same spectrometer described above at 50 MHz. Infrared (IR) spectra were obtained with a Bomem FTLA spectrophotometer by using potassium bromide plates. Microanalysis data were obtained using a Thermo Finnigan EA 1112 Series Flash Elemental Analyzer, using a Perkin–Elmer AD-4 balance. The progress of all reactions was monitored by TLC performed on 2.5 × 7.5 cm aluminium sheets precoated with silica gel 60 (HF-254, E. Merck) and thickness of 0.25 mm. The developed chromatograms were viewed under ultraviolet light at 254 nm. The target *N*-acylhydrazone derivative LASSBio-294 (**4**) and the internal standard LASSBio-787 (**6**), were respectively prepared in *ca.* 70% and 89% yield, exploiting at the key-step the condensation of acylhydrazine intermediate with corresponding 2-thiophenecarbaldehyde, as described previously [9,28]. Nicotinamide adenine dinucleotide phosphate (NADP), D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase, furafylline, ketoconazole, sulfaphenazole, quinidine, HPLC-grade acetonitrile, methanol, dimethylsulfoxide, 2-thiophenecarbaldehyde (**9**) and the salts for buffers were purchased from Sigma–Aldrich (St Louis, MO, USA). The 3,4-dihydroxybenzoylhydrazine (**8**) was purchased from Alfa Aesar (Ward Hill, EUA). Water was purified and filtered using a Milli-Q system (Millipore, St Quentin-en-Yvelines, France).

4.2. Synthesis of 2-thienylidene 3,4-dihydroxybenzoylhydrazine (7)

To a suspension of 3,4-dihydroxybenzoylhydrazine (0.83 mmol) (**8**) in absolute EtOH containing three drops of hydrochloric acid was added 2-thiophenecarbaldehyde (0.87 mmol) (**9**). The reaction mixture was stirred at room temperature for 24 h when the end of reaction was observed by TLC [hexane/ethyl acetate (30:70)]. Next, the solvent was partially concentrated at reduced pressure and the resulting mixture was poured into cold water (*ca.* 10 mL). The obtained precipitate was collected by filtration, under reduced pressure and recrystallized from ethanol/water mixture to give the desired cathecol-derivative (**7**) in 75% yield, as a pale yellow solid, mp 209–210 °C ^{1}H NMR (200 MHz): δ 11.55 (s, 1H, –CONH–), 9.40 (br, 2H, –OH), 8.62 (s, 1H, –N=CH–), 7.62 (d, 1H, thiophene-H$_{5'}$, J = 5.0 Hz), 7.41 (d, 1H, thiophene-H$_{3'}$, J = 2.8 Hz), 7.35 (d, 1H, Ar–H$_2$, Jax = 1.9 Hz), 7.30 (dd, 1H, Ar–H$_6$, Jax = 8.2 Hz, Jbx = 2.0 Hz), 7.12 (dd, 1H, thiophene-H$_{4'}$, Jax = 4.9 Hz, Jbx = 3.6 Hz), 6.82 (d, 1H, Ar–H$_5$, J = 8.2 Hz); ^{13}C NMR (50 MHz): δ 162.9 (C=O), 148.9 (Ar–C$_4$), 144.9 (Ar–C$_3$), 141.8 (thiophene-C$_{2'}$), 139.3 (N=CH), 130.3 (thiophene-C$_{3'}$), 128.4 (thiophene-C$_{5'}$), 127.7 (thiophene-C$_{4'}$), 124.2 (Ar–C$_1$), 119.5 (Ar–C$_6$), 115.3 (Ar–C$_2$), 114.9 (Ar–C$_5$); IR (KBr, cm^{-1}): 3402 (O–H), 3209 (N–H), 1592 (C=O), 1556 (C=N), 1295 and 1227 (C–O). Anal. Calcd for $C_{12}H_{10}N_2O_3S$: C 55.95, H 3.84, N 10.68; Found: C 55.14, H 3.83, N 10.67.

4.3. Docking studies

Automated docking studies were performed with GOLD software version 4.0.1 (license G/414/2006) [24]. The X-ray crystal structure of CYP1A2 complexed with α-naphthoflavone at 1.95 Å resolution (PDB code 2HI4) was obtained by Protein Data Bank [25]. The ligand was extract from the crystal structure and

protein was treated as determined by *default* conditions of GOLD software. Compound structures (LASSBio-294 and α-naphthoflavone) were built manually with Spartan for Linux v. O8 software (Wavefunction Inc.). Geometry optimization was performed using Density Functional Model (hybrid functional B3LYP, base set 6-31G*) [41]. The GoldScore fitness function [26] in the Gold docking software was used to score the docked binding mode of the ligand. The active site was defined as the atoms within 10 Å of heme iron. The same recognition of the coordination geometry was observed when the metal ion was assigned as *default* or *octahedral* form. Five consecutive dockings runs using the same input file were performed and the best-ranking fitness value was collected and the results represent the mean of these values.

4.4. Biological

4.4.1. Preparation of microsomes from rat liver

Livers were obtained from male Wistar rats (250–300 g). The animals were allowed food and water *ad libitum* and the preparation of liver microsomes were conducted using the method of Cabrera et al. [42].

Protein concentration of the microsomal fraction was determined by the bicinchoninic acid assay [43] using the commercial protein determination kit obtained from Sigma–Aldrich (St Louis, MO, USA), following the procedure suggested by the manufacturer.

4.4.2. Rat liver microsomes incubation studies

The incubation was conducted at 37 °C for 60 min, in 24-well plates open to air. The experiments contains $MgCl_2$ (1.3 mM), $NADP^+$ (0.4 mM), glucose-6-phosphate (3.5 mM), 0.5 U/mL glucose-6-phosphate dehydrogenase in a phosphate buffer (0.1 M, pH 7.4) containing EDTA (1.5 mM) and the LASSBio-294 (10 μM, added from stock solutions of 4 mM in DMSO) with 1 mL of final volume. After the pre-warming of the mixture at 37 °C, the microsomal proteins were added to give a final protein concentration of 1 mg/mL. At the end of the incubation time the reaction was stopped by the addition of 400 μL of the extraction solvent, *i.e.* MeOH. The experiments were performed in duplicate. Three control incubations were realized: (a) without NADPH-generating system, (b) without the microsomal proteins and (c) blank experiment (without LASSBio-294, **4**).

4.4.3. Inhibition of LASSBio-294 (**4**) metabolism in rat liver microsomes

The identification of the most probably enzymatic pathway involved in the *in vitro* oxidative metabolism of LASSBio-294 (**4**) was carried out with rat liver microsomes in the absence of NADPH-generating system (positive control), in the presence of NADPH-generating system and with or without LASSBio-294 (**4**). The following CYP inhibitors, CYP1A2-specific inhibitor furafylline (50 μM) [37], CYP2C9-specific inhibitor sulfaphenazole (10 μM) [34], CYP2D6-specific inhibitor quinidine (10 μM) [38], non-selective CYP inhibitor ketoconazole (10 μM) [39] were pre-incubated during 10 min at 37 °C with rat liver microsomes in the presence of the NADPH-generating system. The effect of these inhibitors on the metabolic clearance of LASSBio-294 (**4**) was evaluated by comparison of the metabolite (**7**) concentrations with the control values obtained without the use of inhibitors and expressed as percentages of inhibition.

4.4.4. Metabolic stability of LASSBio-294 (**4**) with recombinant human CYP isoform

The basic incubation medium contained (40 pmol/mL of CYP1A2, 50 pmol/mL of CYP2C8 and 10 pmol/mL of CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A5) human recombinant and 1 μM of (**4**) were incubated at 37 °C in a shaking water bath for 0 and 60 min. At the end of incubation at each time, an equal volume of an organic mixture (acetonitrile/methanol, 50/50, v/v) was added to the incubation mixture followed by centrifugation at 10,000 rpm for 1 min and the supernatants were injected onto an HPLC. Peaks area corresponding to the analyte was determined by HPLC–MS/MS. The ratio of precursor compound remaining at the end of the incubation relative to the amount remaining at time zero, expressed as percent, is reported as metabolic stability [40].

4.4.5. Sample preparation

At the end of rat liver microsomes incubation was added 0.4 mL of ice-cold methanol and the mixture was kept at 4 °C for protein precipitation. The mixture was centrifuged at 13,000 rpm for 10 min. 1 mL of the supernatant fraction was separated and 0.3 mL of CH_3CN was added, vortex mixed and centrifuged at 13,000 rpm for 5 min. 1 mL of the supernatant fraction was filtered through RC regenerated cellulose filters 0.45 μm pore size (Agilent, Germany) and then analyzed.

4.4.6. HPLC/UV analysis

The organic fraction was analyzed with the Shimadzu Prominence HPLC system (Shimadzu, Tokio, Japan) consisting of a vacuum degasser (DGU-$20A_5$), a binary pump (LC-20AD), a autosampler (SIL-20A), UV/VIS Photodiode Array Detector (SPD-M20A) and fitted with a guard column (CLC G-ODS) and a Shimadzu (CLC-ODS, M) column (250 mm × 4.6 mm i.d.) running at room temperature. Isocratic elution was performed with acetonitrile–water (40:60 v/v), at a flow rate set at 1 mL/min. The detection was carried out at 340 nm wavelength.

4.4.7. LC–MS analysis

Qualitative analysis of LASSBio-294 (**4**) and it metabolite (**7**) was detected with HPLC-ESI-MS employing Shimadzu Prominence HPLC system (Shimadzu, Tokio, Japan) and the mass spectrometry HCT Ultra, Brucker Daltonics (Bruker Corporation, Germany). The HPLC consisted in a binary pump (LC-20AD), a vacuum solvent degasser (DGU-$20A_3$), a autosampler (SIL-20AC HT), UV/VIS Photodiode Array Detector (SPD-M20A), fitted with a guard column (CLC G-ODS) and a Shimadzu (CLC-ODS, M) column (250 mm × 4.6 mm i.d.) running at 23 °C (CTO-20A). Isocratic elution was performed with acetonitrile–water (40:60 v/v), at a flow rate set at 1 mL/min. The LASSBio-294 (**4**) and it metabolite (**7**) were detected with electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS) performed with ESI in positive mode. The capillary voltage was 4.0 mV. The collision energy was set at 30 eV using helium as collision gas. Nitrogen nebulizer gas flow was 4.0 L/min, temperature was 250 °C and pressure of 15 psi. The LC-ESI-Ms chromatograms were obtained by scanning over m/z 50–3000 range.

To get the fragmentation pathways, the LASSBio-294 (**4**) and metabolite (**7**) were dissolved in acetonitrile and then introduced to spectrometer by a syringe pump.

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