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Article

CYP450 Metabolism of a Semisynthetic Naphthoquinone, an Anticancer Drug Candidate, by Human Liver Microsomes

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CNFD (6b,7-dihydro-5*H*-cyclopenta[*b*]naphtho[2,1-*d*]furan-5,6(9aH)-dione) is a semisynthetic naphthoquinone derived from lawsone that has cytotoxic action in different tumor lines and anticancer activity *in vivo*. Therefore, this molecule is a relevant candidate for drug development, but there is still no information on its human metabolism and systemic elimination. This study aimed to investigate the *in vitro* metabolism of this naphthoquinone by human liver microsomes. Initially, in order to determine the *in vitro* enzymatic kinetic parameters, a high performance liquid chromatography (HPLC) method to quantify the CNFD was developed and validated. In addition, the enzymatic kinetic data, the predicted pharmacokinetic *in vivo* parameters and the phenotyping study were presented. The main metabolism sites and metabolites have been suggested *in silico*. The developed HPLC method was linear, reproducible, selective, accurate, and stable. The enzymatic kinetic parameters revealed a sigmoidal profile. *In vitro* to *in vivo* extrapolation hepatic metabolic clearance was 10.39 mL min^{-1} kg^{-1} protein and the liver extraction rate was 51%. The clearance *in vivo* associated with a hepatic extraction ratio indicates that the hepatic metabolism is the main route of elimination. Although all cytochrome P450 enzymes evaluated metabolized CNFD, CYP2C9 and CYP3A4 showed higher metabolic capacity. For the first time, metabolism studies of CNFD were demonstrated.

Keywords: lawsone, drug development, biotransformation, pharmacokinetics, preclinical drug evaluation

Introduction

Cancer is one of the main public health problems worldwide. It is the first or second leading cause of premature death in 134 countries.[1,2] Generally, the chemotherapy treatment currently available for cancer is still inefficient, with high toxicity and low selectivity. Thus, the search for new, more effective and selective, and low toxic antineoplastic drugs is necessary.[3,4] In this context, plant-derived molecules, such as naphthoquinones, are promising candidates for new anticancer agents.[5]

Naphthoquinones are secondary metabolites of plants that have considerable biological activities, such

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as antimicrobial, antifungal, antiviral, antimalarial, and antitumor activity.[5-8] Among natural naphthoquinones, lawsone (2-hydroxy-1,4-naphthoquinone), found in the leaves of *Lawsonia inermis* L. (Lythraceae), a plant known as henna, is widely used as natural dye for skin and hair.[5,9] Lawsone derivatives are attractive for the development of new drugs due to the broad spectrum of their biological activities.[1] Several pharmacological properties of them have already been reported, such as antibacterial,[10] antifungal[11] and antitumor.[12]

The CNFD (6b,7-dihydro-5*H*-cyclopenta[*b*] naphtho[2,1-*d*]furan-5,6(9aH)-dione) (Figure 1) is a naphthoquinone synthesized from lawsone with remarkable antifungal activity.[13] In addition, this derivative has also demonstrated potential as an antineoplastic agent in different cell lines, especially in human breast adenocarcinoma cells MCF-7 with an half-maximal inhibitory concentration (IC_{50}) of less than 1 μmol L^{-1}. CNFD has the ability to induce cell apoptosis. In an *in vivo* study using a murine model of melanoma (B16F10), CNFD led to tumor regression in animals (inhibition of 46, 50, and 52% at doses of 10, 30 and 60 mg kg^{-1} day^{-1}, respectively), becoming a candidate for an antineoplastic drug.[14] A patent, related to the antineoplastic properties of CNFD, was already deposited under the registration number BR10201700717.[15]

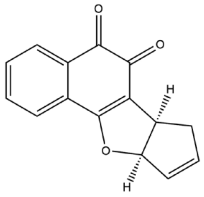

Figure 1. Chemical structure of CNFD (6b,7-dihidro-5*H*-ciclopenta[*b*] nafto[2,1-*d*]furano-5,6(9aH)-dione).

During the development of new drugs, drug candidates must undergo pre-clinical studies to obtain the greatest possible knowledge about the pharmacological, pharmacokinetic properties, and toxicity.[16] In this context, the contribution of cytochrome P450 (CYP450) enzymes to the metabolism of this new candidate is fundamental, since these enzymes are responsible for the metabolism of most commercialized drugs.[17] *In vitro* and *in silico* studies during drug discovery can be useful to predict issues related to drug safety, such as pharmacokinetic parameters and drug-drug interactions.[18,19]

Anti-tumor drugs have low specificity, that is, they do not only affect cancer cells, but also affect normal cells. The metabolites formed by the metabolism of these drugs can induce several adverse effects, including other serious problems and even the patient's death.[20]

Despite the antitumor activity of CNFD, its metabolic pathway within the human body has not been reported. Thus, this study aimed to evaluate the *in vitro* metabolism of CNFD by CYP450 enzymes, including elucidation of the enzymatic kinetic profile, identification of the main CYP450 isoforms responsible for its metabolism, and prediction of *in vivo* pharmacokinetic parameters. In addition, predict the CNFD metabolism sites by CYP enzymes and the possible metabolites formed *in silico.*

Experimental

Reagents and solvents

The lawsone derivative, CNFD (6b,7-dihydro-5*H*-cyclopenta[*b*]naphtho[2,1-*d*]furan-5,6(9aH)-dione), was kindly provided by Prof Dr Vitor Francisco Ferreira.[13] The standard stock solution of CNFD was prepared at 4000 μmol L^{-1} in methanol. Phenacetin (≥ 98.0%), which was used as internal standard (IS), was acquired from Sigma-Aldrich (St. Louis, USA). The standard stock solution of phenacetin was prepared at 200 μmol L^{-1} in methanol. Human plasma was obtained from Sigma-Aldrich (St. Louis, USA), and human liver microsomes (HLMs) (150-donor pool) and recombinant CYP450 (rCYP450) isoforms (Supersomes®) were purchased from Corning Life Science (Phoenix, USA). Ultrapure water (18.2 MΩ cm) was obtained from a Milli-Q Direct-Q3 UV (Millipore, Bedford, USA). HPLC (high-performance liquid chromatography) grade solvents methanol and ethyl acetate were obtained from Panreac (Castellar Del Vallès, Barcelona, Spain). The glucose-6-phosphate sodium salt, glucose-6-phosphate dehydrogenase, and β-nicotinamide adenine dinucleotide phosphate hydrate ($NADP^+$) components of the NADPH (β-nicotinamide adenine dinucleotide phosphate) cofactor system were acquired from Sigma-Aldrich (St. Louis, USA). The solutions were prepared in a tris-KCl buffer (tris(hydroxymethyl) aminomethane 0.05 mol L^{-1} and KCl 0.15 mol L^{-1}, pH 7.4) at the following concentrations: glucose-6-phosphate (50 mmol L^{-1}), and glucose-6-phosphate dehydrogenase (8.0 U mL^{-1}), and $NADP^+$ (2.5 mmol L^{-1}) and stored at −20 °C. Other analytical grade reagents used were tris(hydroxymethyl) aminomethane (JT Baker, Phillipsburg, USA), Cremophor® (Sigma-Aldrich, St. Louis, USA), potassium chloride (Mallinckrodt Chemicals, Phillipsburg, USA), sodium phosphate monobasic, and sodium phosphate dibasic (Synth, Diadema, Brazil).

Microsomal incubation conditions

The microsomal incubation medium consisted of the substrate (CNFD), NADPH cofactor system, HLMs or rCYP450, and phosphate buffer (0.1 mol L^{-1}, pH 7.4) with 0.1% (m/v) of Cremophor®, in a final volume of 200 µL. The samples were pre-incubated for 5 min at 37 °C, in a water bath. The metabolism was initiated by the addition of the NADPH cofactor system. After the incubation time, the reaction was stopped with the addition of 1 mL of ethyl acetate and 50 µL of internal standard (IS). Next, the samples were shaken for 15 min at 1500 rpm in a Vibrax VXR® agitator (IKA, Staufen, Germany) and centrifuged at 1800 × g for 15 min at 4 °C in a HIMAC CF15D2 centrifuge (Hitachi, Tokyo, Japan). Then, the organic phase was collected and evaporated in a Concentrator Plus speed vacuum (Eppendorf, Hamburg, Germany). The final residue was reconstituted in the mobile phase and analyzed by HPLC.

HPLC

A Shimadzu HPLC system (Kyoto, Japan), which comprised a DGU-20A5 online degasser, a LC-20AT solvent pump unit, a SIL-10AF automatic injector, a CTO-20A column oven, an SPD-M20A (190-800 nm) diode array detector, and a CBM-20A system controller, was employed. The separation was carried out using an Ascentis Express C18 (3.0 mm × 4.6 mm, 2.7 µm) guard column and an Ascentis Express Fused Core C18 (100 mm × 4.6 mm, 2.7 µm) analytical column (Supelco, Bellefonte, USA). The mobile phase consisted of methanol:water (55:45, v/v) at flow rate of 0.8 mL min^{-1}. The injection volume was 30 µL and the temperature of analysis was 30 °C. The detection was performed at 260 and 247 nm for CNFD and IS, respectively. Data were collected using the LC solution software 1.25 SP1 (Shimadzu, Kyoto, Japan).

Method validation

The analytical method was validated according to the ANVISA guidelines on bioanalytical method validation.[21] The evaluated parameters were linearity, limit of quantification, carryover, selectivity, accuracy, precision, and stability.

Linearity was assessed for the following concentrations of CNFD: 0.3 (lower limit of quantification (LLOQ)), 2.56 (low quality control (LQC)), 10.07 (quality control (QC)), 20.14 (medium quality control (MQC)), 80.59 (high quality control (HQC)) and 100.73 µmol L^{-1} (upper limit of quantification (ULOQ)). The calibration curves were constructed plotting the normalized area *versus* concentration of CNFD. The analytical curve was weighted (weighting factor $1/X^2$) and the determination coefficient (r^2) and linear regression equation were calculated. In addition, the linearity was assessed using an analysis of variance (ANOVA) lack-of-fit test, calculating the values of F and p with Minitab 16 Statistical Software[22] (State College, USA). The LLOQ was determined by analyzing the lowest concentration of the CNFD with relative standard deviation (RSD) and relative error (RE) lower than 20%. Carryover was evaluated assessing the presence of interferences in the CNFD and IS retention time. Selectivity was evaluated analyzing a blank sample of the microsomal medium (without CNFD and IS) to assess the presence of matrix interferents. Intraday (n = 5) and interday (n = 5) accuracy and precision were evaluated for the LLOQ, LQC, MQC, HQC and ULOQ levels. The results were acceptable if RSD and RE were lower than 20% for the LLOQ and lower than 15% for the other concentrations levels. Stability was evaluated at incubation conditions (37 °C for 60 min) and auto-injector for 24 h, for the LQC and ULOQ samples.

Enzymatic kinetic

The enzymatic kinetic of the metabolism of CNFD by CYP450 enzymes (n = 4) was determined at initial velocity conditions (microsomal protein content of 0.20 mg mL^{-1} and incubation time of 60 min) for the following concentration range of CNFD: 0.3-80 µmol L^{-1}. After metabolism, the samples were analyzed and quantified by HPLC, using a calibration curve (n = 3), prepared on the same day of the study. The rate of enzymatic reaction, for each concentration of CNFD, was determined by the metabolized concentration of CNFD by the protein concentration and incubation time. The obtained results were plotted on a graph of rate of enzymatic reaction *versus* concentration of CNFD and analyzed by non-linear regression in enzymatic models, using the GraphPad Prism 6 software[23] (San Diego, USA). The enzymatic kinetic parameters of the metabolism reaction were obtained and the *in vitro* intrinsic clearance ($CL_{INT,\ in\ vitro}$) was calculated using equation 1.[24] Then, the $CL_{INT,\ in\ vitro}$ was extrapolated to the *in vivo* intrinsic liver clearance ($CL_{INT,\ in\ vivo}$), according to equation 2.[25]

$$CL_{INT,\ in\ vitro} = \frac{V_{MAX}}{S_{50}} \times \frac{(h-1)}{h\,(h-1)^{1/h}} \quad (1)$$

where $CL_{INT,\ in\ vitro}$: *in vitro* intrinsic clearance; V_{MAX}: maximum velocity of the enzymatic reaction; S_{50}: concentration of the substrate where V corresponds to half of V_{MAX}; h: Hill coefficient.

$$CL_{INT,\ in\ vivo} = CL_{INT,\ in\ vitro} \times \frac{40\ mg\ microsomal\ protein}{g\ liver} \times \frac{21.4\ g\ liver}{kg\ body} \quad (2)$$

where, $CL_{INT,\ in\ vivo}$: *in vivo* intrinsic clearance and $CL_{INT,\ in\ vitro}$: *in vitro* intrinsic clearance.

Prediction of *in vivo* pharmacokinetic parameters

For the prediction of the *in vivo* pharmacokinetic parameters, the percentage of binding of CNFD to plasma proteins and microsomal proteins were determined using the substrate concentration below the S_{50} value determined in enzymatic kinetic. The microsomal medium was prepared with the substrate, HLMs (0.2 mg mL^{-1}) and phosphate buffer (0.1 mol L^{-1}, pH 7.4) with 0.1% (m/v) of Cremophor®. The plasma medium was made of substrate and human plasma (protein concentration 42 mg mL^{-1}). Control samples (in the absence of plasma or microsomal proteins) were prepared with the substrate and phosphate buffer (0.1 mol L^{-1}, pH 7.4) with 0.1% (m/v) of Cremophor®. All samples were incubated at 37 °C for 60 min. After incubation, the samples were subjected to ultracentrifugation at 150060 × g and 4 °C for 3 h, in a Beckman Optima XL-100K (Brea, CA, USA). The supernatant was collected, subjected to the sample preparation, and analyzed using HPLC. The unbonded fraction of the substrate (f_u) was determined according to equation 3.[26]

$$f_u = \frac{C_A}{C_C} \quad (3)$$

where f_u: substrate unbonded fraction, C_A: sample concentration and C_C: control concentration.

Using the obtained results, unbonded fraction of the substrate in microsome and plasma, $CL_{INT,\ in\ vivo}$ and the hepatic blood flow (Q) 20 mL min^{-1} kg^{-1},[27] the *in vivo* pharmacokinetic parameters hepatic clearance (CL_H) and hepatic extraction rate (E_H) were calculated using equations 4 and 5, respectively.[25]

$$CL_H = \frac{Q \frac{f_{u,\,p}}{f_{u,m}} CL_{INT,\ in\ vivo}}{Q + \left(\frac{f_{u,p}}{f_{u,m}} CL_{INT,\ in\ vivo} \right)} \quad (4)$$

where CL_H: hepatic clearance, Q: hepatic blood flow, $f_{u,p}$: unbonded fraction of the substrate in plasma, $f_{u,m}$: unbonded fraction of the substrate in microsomal medium and $CL_{INT,\ in\ vivo}$: *in vivo* intrinsic clearance.

$$E_H = \frac{CL_H}{Q} \quad (5)$$

where E_H: hepatic extraction rate, CL_H: hepatic clearance, Q: hepatic blood flow.

CYP450 phenotyping

The determination of the main human CYP450 isoforms responsible for CNFD metabolism was performed using rCYP450. The substrate was incubated with a rCYP450 isoform (50 pmol mL^{-1}) (rCYP1A2, rCYP2B6, rCYP2C8, rCYP2C9, rCYP2C19, rCYP2D6, rCYP2E1, rCYP3A4 or rCYP3A5). Controls containing insect cells instead of the rCYP450 isoform were prepared. The mixture was incubated at 37 °C for 60 min and the reaction was stopped by the addition of ethyl acetate. Sample preparation and HPLC analysis were performed as previously described. The samples were quantified using analytical curves prepared on the same day and the reaction rates were determined. To obtain the normalized rate (TN) (equation 6), the abundance of each CYP450 isoform in human liver microsomes was considered. The total normalized rate (TNR) for each isoform was obtained by the ratio of TN to the sum of the TN of all isoforms of CYP450 (equation 7).[28]

$$TN = v_{rCY450} \times abundance_{(CYP450)} \quad (6)$$

where, TN: normalized rate of each isoform of CYP450 and $v_{rCYP450}$: rate of enzymatic reaction for each isoform of CYP450.

$$TNR\,(\%) = \frac{TN \times 100}{\sum TN} \quad (7)$$

where, TNR: percentage of the total normalized rate and TN: normalized rate.

Prediction of metabolism using SMARTCyp software

The prediction of CNFD metabolism sites by the enzymes CYP3A4, CYP2D6 and CYP2C9 was performed using the SMARTCyp online server.[29] According to this database, the lower the activation energies, the more likely a site is to be metabolized.[30]

CNFD was submitted to the online server MetaTox,[31] through a two-dimensional drawing in a database to predict the possible sites of metabolism, from phase I reactions (oxidation, reduction and hydroxylation) and phase 2 (conjugation).[32]

Results

Method validation

Chromatogram of the CNFD analysis can be seen in Figure 2. The calibration curve developed showed adequate linearity for studies evaluating the metabolism of CNFD in microsomal medium. The lack-of-fit test showed that the analytical curve obeys a linear model. The lower limit of quantification of CNFD in microsomal medium was 0.3 μmol L^{-1}. The method was selective and carry over was not observed. The intraday and interday precision and accuracy were lower than 7% for all concentrations. Stability results guaranteed the stability of CNFD in incubation conditions and auto-injector, since both the RE and RSD were lower than 8% (Table 1).

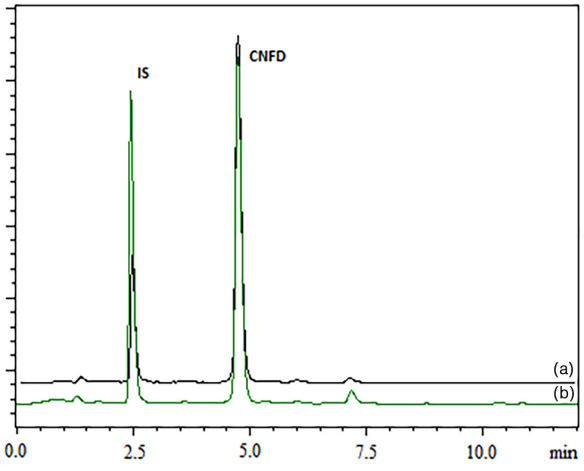

Figure 2. Representative chromatogram of the CNFD analysis. Chromatographic conditions (HPLC-DAD): Ascentis Express C18 (3.0 mm × 4.6 mm, 2.7 μm) guard column and an Ascentis Express Fused Core C18 (100 mm × 4.6 mm, 2.7 μm) analytical column, mobile phase methanol:water (55:45, v/v), flow rate of 0.8 mL min^{-1}, injection volume was 30 μL and the temperature was 30 °C. The detection was performed at 260 (a) for CNFD and 247 nm (b) for IS.

Enzymatic kinetic

The enzymatic kinetic of CNFD metabolism by HLMs demonstrated a sigmoidal kinetic profile (Figure 3). The enzymatic parameters were V_{MAX} = 8.6 ± 1.7 nmol mg^{-1} protein min^{-1}, S_{50} = 137.1 ± 36.4 μmol L^{-1} and a Hill coefficient of 1.23 ± 0.05. The Eadie-Hofstee plot resulted in a convex curve.

Prediction of *in vivo* pharmacokinetic parameters

The binding of CNFD to microsomal and plasma proteins was 4 and 37%, respectively. The predicted parameters *in vitro* intrinsic clearance, *in vivo* intrinsic clearance, hepatic clearance and hepatic extraction ratio were expressed in Table 2.

Table 1. Confidence limits obtained for the analysis the CNFD method in microsomal media

Linearity (n = 5)		
Linear equation / (μmol L^{-1})	$y = 0.1027x + 0.0027$	
Range / (μmol L^{-1})	0.3-100.73	
Determination coefficient (r^2)	0.9994	
Lack of fit	$F = 1.73$; $p = 0.208$	
Limit of quantification (n = 5)		
Concentration / (μmol L^{-1})	0.3	
Precision RSD / %	7	
Accuracy RE / %	7	
Stability (n = 3)		
Incubation 37 °C for 60 min		
Concentration / (μmol L^{-1})	2.56	
Precision RSD / %	0	
Accuracy RE / %	–8	
Concentration / (μmol L^{-1})	80.59	
Precision RSD / %	2	
Accuracy RE / %	1	
Incubation auto-injector for 24 h		
Concentration / (μmol L^{-1})	2.56	
Precision RSD / %	1	
Accuracy RE / %	–3	
Concentration / (μmol L^{-1})	80.59	
Precision RSD / %	1	
Accuracy RE / %	0	
	Precision RSD (n = 5) / %	
	Intraday	Interday
0.3 μmol L^{-1}	7	6
2.56 μmol L^{-1}	3	2
20.14 μmol L^{-1}	3	3
80.59 μmol L^{-1}	5	3
100.73 μmol L^{-1}	7	5
	Accuracy RE (n = 5) / %	
	Intraday	Interday
0.3 μmol L^{-1}	7	3
2.56 μmol L^{-1}	0	0
20.14 μmol L^{-1}	4	4
80.59 μmol L^{-1}	6	6
100.73 μmol L^{-1}	0	–5

RSD: relative standard deviation; RE: relative error.

CYP450 phenotyping

rCYP450 were used to determine the role of each CYP450 isoform involved in the metabolism of CNFD. As show in Figure 4, all the evaluated rCYP450 isoforms contributed to CNFD metabolism.

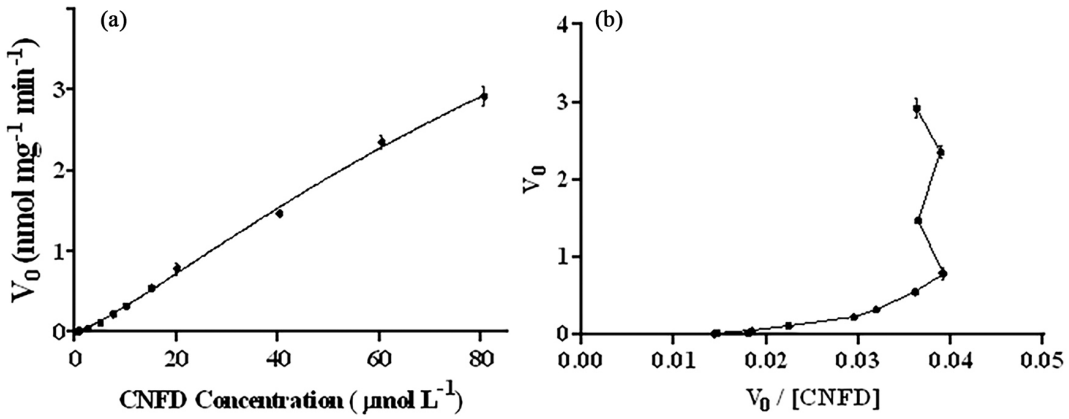

Figure 3. (a) *In vitro* kinetic profile (sigmoidal plot) of CNFD catalyzed by CYP enzymes. (b) Eadie-Hofstee graph.

Table 2. Determination of the free fraction not bound to microsomal and plasma proteins and *in vivo* pharmacokinetic parameters

$f_{u,m}$ / %	$f_{u,p}$ / %	$CL_{INT,\ in\ vitro}$ / (μL mg^{-1} min^{-1})	$CL_{INT,\ in\ vivo}$ / (mL min^{-1} kg^{-1})	CL_H / (mL min^{-1} kg^{-1})	E_H / %
96	63	38.5	32.98	10.39	51

$f_{u,m}$: unbonded fraction of the substrate in microsomal medium; $f_{u,p}$: unbonded fraction of the substrate in plasma medium; $CL_{INT,\ in\ vitro}$: *in vitro* intrinsic clearance; $CL_{INT,\ in\ vivo}$: *in vivo* intrinsic clearance; CL_H: hepatic clearance; E_H: hepatic extraction rate.

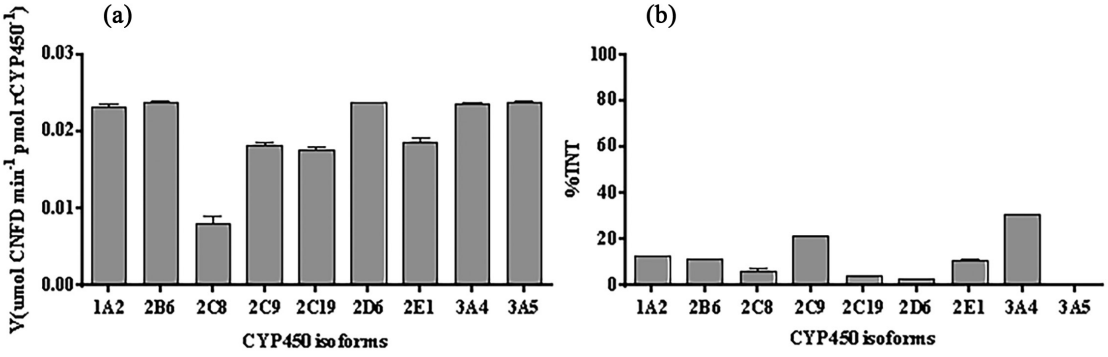

Figure 4. Determination of the CYP isoform involving CNFD metabolism using recombinant enzymes. (a) Velocity of the enzymatic reaction. (b) Total normalized rate.

Prediction of metabolism using SMARTCyp and Metatox software

The prediction of CNFD metabolism sites by CYP enzymes was evaluated by the SMARTCyp server. The platform classified the relevance of color metabolism and the main site of metabolism by CYP3A4 and CYP2D6 was carbon 8. For CYP2C9, the detected metabolism site was carbon 12. The results are available in Table 3 and Figure 5.

The metabolic chemical reactions predicted by the CYP450 enzyme are phase I reactions, where a greater prediction was observed for hydrogenation, epoxidation and oxidation reactions. Among the predicted metabolites, the M1 metabolite obtained by the hydrogenation reaction, was the one with the highest probability of occurrence. The results are available in Figure 6.

Table 3. Prediction of CNFD metabolism using the online platform (values were computed by SMARTCyp server)

Ranking (CYP3A4)	Atom	Score / (KJ mol^{-1})	Energy / (KJ mol^{-1})
1 (red)	C8	39.8	46.2
2 (orange)	C9	55.7	62.2
3 (yellow)	C12	56.2	65.6
Ranking (CYP2D6)			
1 (red)	C8	59.2	46.2
2 (orange)	C12	64.2	65.6
3 (yellow)	C11	71.1	65.6
Ranking (CYP2C9)			
1 (red)	C12	81.9	65.6
2 (orange)	C8	87.1	46.2
3 (yellow)	C11	88	65.6

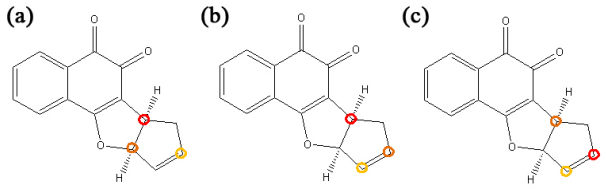

Figure 5. Probable sites of drug metabolism prompted by CYP3A4 (a) CYP2D6 (b) CYP2C9 (c), according to the online platform. The color code can be found in Table 3.

Discussion

The development of a new drug undergoes pre-clinical studies, including the evaluation of the contribution of CYP450 enzymes in the metabolism of this new drug candidate.[33,34] Through these studies, it is possible to predict the enzymes involved in metabolism and drug interactions.[35] Since the CNFD is still in the early stages of the drug development process, the present study reports for the first-time evidence of its metabolism by CYP450 enzymes.

The enzymatic kinetics obtained showed a sigmoidal profile, with positive cooperativity for having exhibited h above 1 (one) (h = 1.23) and the Eadie-Hofstee plot resulted in a convex curve.[36] This result shows that the binding of the substrate with the enzyme occurs in more than one active site.[37] In addition, the velocity of the enzymatic reaction may be the sum of the catalysis of several CYP enzymes, as the phenotyping study showed the contribution of all isoforms to the metabolism of CNFD, except for CYP3A5. The kinetic parameters obtained were used to determine the *in vitro* intrinsic clearance.

From the data obtained in enzymatic kinetics, it was possible to extrapolate these results *in vitro* to *in vivo*, predicting the pharmacokinetic parameters. Liver clearance is extremely important during the development of a new drug, as it is related to drug exposure and half-life.[19] The rate of hepatic extraction (E_H = 51%) indicates that part of the drug undergoes first-pass metabolism.[38] In addition, liver clearance (CL_H = 10.39 mL min^{-1} kg^{-1}) indicates that CNFD is rapidly metabolized by the liver. CNFD was weakly bound to microsomal proteins, thereby minimizing non-specific binding. In addition, only 37% of the CNFD was bound to plasma proteins. In drug development, the low binding of a molecule with plasma proteins is desirable since the risk of competitive interactions is reduced.

As previously mentioned, the phenotyping study suggests the mediation of the evaluated CYP isoforms, mainly CYP3A4 and CYP2C9. The metabolism by multiple enzymes is also important for an ideal drug.[35] If any enzymatic pathway is impaired, other enzymes can carry out the metabolism of the drug, ensuring the elimination from the organism. Furthermore, the metabolism performed by different CYP enzymes is a positive result as more than one pathway may provide drug conversion, which minimizes the risk of interactions.

CNFD is a naphthoquinone derivative and naphthoquinones are known to have inhibitory potential in some human isoforms of CYP450, in addition to which cytochrome P450 and P450 reductase enzymes can promote reductive activation of quinones by one or two electron reductions, providing the production of unstable semiquinone.[39,40]

In silico approaches have been increasingly used to predict the metabolism of new drug candidates, allowing for cost and time savings.[18] One recent example in the literature was the analysis with the three different SMARTCyp algorithms in the study of the CYP-mediated metabolism

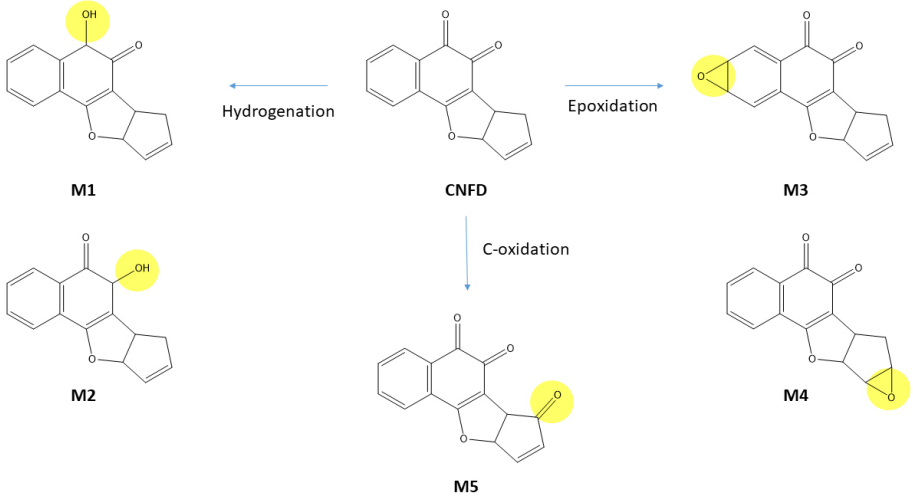

Figure 6. Prediction of CNFD metabolites and their respective chemical reactions. Hydrogenation: M1 (93.93%) and M2 (88.42%); epoxidation: M3 (84.82%) and M4 (71.09%); C-oxidation: M5 (67.39%).

of DA-Phen, a new dopaminergic agent.[41] The prediction of the metabolism sites by the SMARTCyp platform for CYP3A4, CYP2D6 and CYP2C9 occurred through the use of algorithms that use the activation energy of cytochrome P450 for reaction with a molecule.[30,34] In this study, the main metabolism sites have been suggested, providing important information for the identification of metabolites. Although the predictive approaches developed cannot completely replace standard protocols, this strategy can provide an earlier decision-making process until the necessary data is available.

The bioanalytical method developed and validated is simple and has been successfully used for its intended purpose. It was not possible to detect CNFD metabolites, which requires the use of more sensitive and selective assays employing liquid chromatography mass spectrometry (LC-MS) in order to determine, isolate and elucidate these molecules. However, the chromatographic method is fully compatible with mass spectrometry, being a starting point for future studies. Since CNFD is a drug candidate, assessing the toxicity of these metabolites is of great importance. In addition, the enzyme inhibition study may indicate the likely drug interactions that may occur.

Conclusions

In summary, the metabolism of the CNFD drug candidate was characterized for the first time. The data established in the kinetic study were used to predict important pharmacokinetic parameters, determining liver clearance. CYP450 is involved in CNFD metabolism, with all enzymes showing important roles, especially CYP3A4 and CYP2C9. Metabolism sites were predicted. These results may be useful for future *in vitro* studies, as well as for clinical studies.

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Author Contributions

Anderson R. M. Oliveira and Igor R. S. Magalhães were responsible for conceptualization; Edna M. A. Costa, Tallita M. Machado, Jade L. M. Bucci, Daniel B. Carrão for methodology and formal analysis; Anderson R. M. Oliveira and Igor R. S. Magalhães for funding acquisition; Vitor F. Ferreira, Émerson S. Lima for resources; Marne C. Vasconcellos, Anderson R. M. Oliveira and Igor R. S. Magalhães for writing original draft and writing-review and editing; Igor R. S. Magalhães for investigation, supervision and project administration.

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