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Research Paper

Synthesis and preclinical pharmacokinetic study of DHA-10, a novel potential antifungal pogostone analogue

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Keywords
pogostone analogue; synthesis; pharmacokinetics; tissue distribution; excretion

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Abstract

Objectives The emergence of fungal disease calls for the urgency of development of novel drug. In this study, we developed a novel pogostone analogue, DHA-10 and investigated its preclinical pharmacokinetics, tissue distribution, excretion and protein binding rate in rats.

Methods DHA-10 was synthesized with dehydroacetic acid (DHA) as the starting material and the structure confirmed by NMR and HRMS. The LC-MS/MS was applied to quantitative analysis of DHA-10 concentrations in the biological samples.

Key finding DHA-10 was eliminated rapidly in rat plasma with half-lives of 3.39 ± 0.5, 3.24 ± 0.32 and 3.80 ± 0.40 h after single oral doses of 70, 140 and 280 mg/kg, respectively, and showed linear pharmacokinetic within the examined dosage range. The oral bioavailability of DHA-10 was 69.09 ± 3.9%. DHA-10 distributed widely in tissues with highest tissue concentration was found in small intestine at 2.5 h postdose, followed by the stomach, liver and uterus. Approximately, 1.50 ± 0.26% and 9.12 ± 2.53% of parent drug was excreted via the urine and faeces within 48 h, respectively; 1.45 ± 0.12% was excreted into the bile up to 36 h after a single oral administration of 140 mg/kg. Binding rate of DHA-10 with plasma protein was about 78.80 ± 1.75% in a concentration-independent manner.

Conclusions DHA-10 was successfully synthesized and characterized. The preclinical pharmacokinetics study in rats supported the further development of this new antifungal candidate compound.

Introduction

The fungi have emerged as major causes of human disease, ranging from superficial infections of the mucosal surfaces or skin to systemic infections.[1,2] The invasive infections (IFIs) are an increasingly important cause of morbidity and mortality in immunocompromised patients, especially cancer patients with chemotherapy-induced neutropenia, transplant recipients receiving immunosuppressive therapy and human immunodeficiency virus (HIV)-infected patients.[3–7]

Candida species are the most common opportunistic fungal pathogens in humans, with *Candida albicans* being the most prevalent microbial population in mucosal and systemic infections.[8,9] So far, only a few classes of antifungal drugs (polyenes, azoles, echinocandins, allylamines and DNA analogues etc.) are available for candidiasis treatment[10] and the application of these drugs are greatly limited by their toxicities, side effects, drug interactions, drug delivery routes and the emergences of drug resistance.[11] Multiple adverse factors have stimulated research directed towards the discovery of novel chemical entities with alternative mode of action to treat fungal infections and prevent drug resistance. Pogostone (PO, $C_{12}H_{16}O_4$) has been investigated for its significant antimicrobial activity against a large number of clinically relevant fungal and (myco) bacterial strains, and showed anti-Candida activity both *in vitro* and *in vivo*.[12,13] Unlike major antifungal drugs in current clinical use (azole, polyenes, echinocandins bacteria),[10] PO belongs to a different chemical structure

family of pyranones. But the oil–water partition coefficient (Log *P*) of pogostone was not ideal and affected its biological transport in the body. Therefore, a novel pogostone analogue was synthesized which retained the biological actions 2-pyrone ring of the pogostone parent molecule with improved water solubility and better pharmacological properties by modifying the structure of green food grade antibacterial agent (DHA). The pharmacodynamic study of the cooperative group indicated that DHA-10 [3-(2-diethylamino acetyl-4-hydroxy-6-2H-pyran-2-one hydrochloride] has good effects on *Candida albicans*, *Candida glabra*, *Candida tropicalis* and *Candida famala*.

With the increasing significance of DHA-10 as a potential antimycotic agent, it is important to investigate the *in-vivo* process of DHA-10. An HPLC-MS/MS method was validated and applied to the study of the pharmacokinetics, distribution, excretion and plasma protein binding rate of DHA-10 in rats. To date, this is the first report on the synthesis and pharmacokinetics of novel pogostone analogues (DHA-10). The results could be used for the analysis of efficacy, and toxicity, more importantly guidance for lead optimization thus played a critical role in the development of potential pyrone antifungal drugs.

Materials and Methods

Chemicals and reagents

DHA-10 [3-(2-diethylamino acetyl-4-hydroxy-6-2H-pyran-2-one hydrochloride] was synthesized, purified and characterized in the Pharmaceutical Sciences College of Nanjing Tech University, (Nanjing, China). HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Formic acid of LC-MS grade was obtained from Sigma (St. Louis, MO, USA). Water was purified by employing a Milli-Q water purification system (Millipore, Billerica, USA). All other reagents were at analytical grade.

Synthesis and characterization of DHA-10

The synthesis route used to synthesize the pogostone analogues (DHA-10) is outlined in Figure 1. Firstly, a mixture of dehydroacetic acid (1.681 g, 10 mmol), 5, 5-Dibromobarbituric acid (2.859 g, 10 mmol), *p*-toluenesulfonic acid (0.172 g, 1 mmol) and $AlCl_3$ (0.067 g, 0.5 mmol) in CH_3CN (20 ml) was stirred under reflux for 15 h. After the reaction, the temperature was cooled down and solvent removed under vacuum. The residue was then dissolved in dichloromethane and washed with H_2O. The organic layer was dried over anhydrous sodium sulphate and solvent removed under vacuum to obtain the product. Secondly, the bromide intermediate (1.225 g, 5 mmol) was dissolved in CH_3CN (40 ml), stirring at room temperature for 0.5 h. Diethylamine (0.37 g, 5 mmol) was then added dropwise to the liquid followed by potassium carbonate (0.82 g, 6 mmol), stirring at room temperature for 5 h. After the reaction, the resultant mixture was filtered and solvent was removed under vacuum. The residue was then dissolved in ethyl acetate and washed with saturated aqueous NaCl. The organic layer was dried over anhydrous sodium sulphate and solvent removed under vacuum. The crude product was purified by flash chromatography on silica gel, gradient elution with ethyl acetate in petroleum ether to obtain the yellow product. The product obtained was then dissolved in CH_2Cl_2, stirring at −10 °C and hydrogen chloride solution was added. After the reaction, the desired product was recrystallized by ethyl acetate. Due to the poor solubility of DHA-10 in organic solvents, it is hard to characterize DHA-10 by nuclear magnetic resonance. Therefore, the free base form of DHA-10 was characterized with nuclear magnetic resonance shown in Appendix S2.

Animals

The Sprague-Dawley rats (male and female, weight 200 ± 20 g) were obtained from the Animal Center of Nanjing Medical University (Nanjing, China; certificate no. SCXK2009-001), were used in this study. All rats were acclimatized for one week under controlled environmental conditions (temperature was 23–25 °C; humidity was 50% with a 12 h-dark–light cycle). Before administration, rats were fasted for 12 h with free access to water. The experimental protocols were approved by the Animal Ethics Committee of Nanjing Tech University.

Experimental design

Pharmacokinetic study

Twenty-four Sprague-Dawley rats (12 males and 12 females) were divided into four groups randomly (six rats/per group, three males and three females). DHA-10 was dissolved in normal saline. The first three groups of rats received a single oral (i.g.) administration of DHA-10 at 70, 140 and 280 mg/kg, respectively, and blood samples (200 μl) were collected into heparinized tubes at predose, 0.25, 0.75, 1.5, 2.0, 2.5, 2.75, 3.0, 3.5, 4, 6, 8, 12 and 24 h postdose. The fourth group rats were given intravenous (i.v.) administration via lateral tail vein at the dose of 140 mg/kg and blood samples (200 μl) were collected into heparinized tubes at predose, 0.033, 0.083, 0.167, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8,12 and 24 h postdose. All samples were centrifuged at 5315 × g for 10 min to obtain the plasma that were separated and kept at −80 °C until analysis.

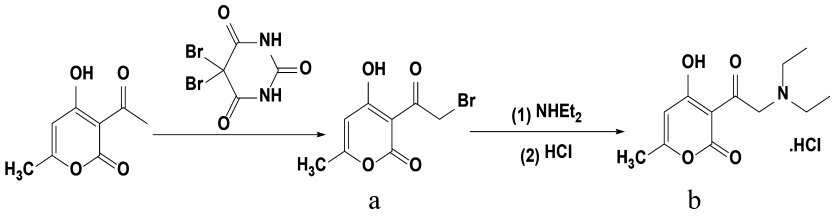

Figure 1 The synthesis route of DHA-10, where a and b are the first and second step reaction products.

Tissue distribution study

Eighteen rats were divided into three groups (n = 6 per group, three males and three females) randomly. All rats were oral administered single dose of 140 mg/kg DHA-10. At 0.5, 2.5 and 12.0 h postdose, one group rats were sacrificed, samples of heart, liver, spleen, lung, kidney, stomach, intestine, brain, gonadal (testis, uterus and ovary) were harvested, rinsed in normal saline and blotted dry with filter paper. Tissue homogenized was obtained by homogenization in normal saline (1 ml/0.2 g tissue) and stored at −80 °C until analysis.

Excretion study

After single-dose oral administration of DHA-10 at 140 mg/kg, six rats (half male and half female) were individually placed in stainless-steel metabolic cages with ad libitum access to food and water, allowing separate collection of urine and faeces. Urine samples were collected at intervals of 0–2, 2–4, 4–8, 8–12, 12–24, 24–36 and 36–48 h postdose. Faeces samples were collected at 0–12, 12–24, 24–36 and 36–48 h. The volume of urine for each collection period was recorded, respectively. The faeces were individually dried at 50 °C dry oven for 24 h, weighed and recorded, and then homogenized in normal saline to prepare the suspension (200 mg/ml; w/v) before storing at −80 °C.

To study the bile excretion, another six rats (half male and half female) were anesthetized by subcutaneous injection of 2% pentobarbital sodium solution (w/v). A cannula was inserted into the bile duct of each animal. Blank bile was collected before DHA-10 was administered. After oral administration of DHA-10 at the dose of 140 mg/kg, bile samples were collected at 0–2, 2–4, 4–6, 6–8, 8–10, 10–12, 12–24 and 24–36 h postdose and volume recorded before stored at −80 °C.

Plasma protein binding study

Ultrafiltration method was applied to the determination of plasma protein binding (PPB) rate. 25 μl DHA-10 working solution was added to 475 μl fresh rat plasma to obtain a final concentration of 6, 48 and 120 μg/ml (n = 3). The mixture of DHA-10 and plasma was incubated at 37 °C for 2 h to allow equilibration. Then a 100-μl aliquot was removed to the centrifugal filter unit for the total concentration analysis in each sample, including free and bound DHA-10 concentrations. The remaining volume using filters to ultrafiltration. Centrifugation was carried out at 12 000g for 45 min. 400 μl phosphoric acid buffer solution (PBS, PH 7.4) of DHA-10 at a concentration of 6, 48 and 120 μg/ml (n = 3) was processed with the same plasma ultrafiltration method to evaluate the adsorption of drug and membrane. Using the instrument LC-MS/MS for the determination of total drug concentration (A) and the free drug concentration (B), then according to the formula $F = (A - B)/A$ calculated plasma protein binding rate.

Sample processing

In this study, different samples were extracted by different methods. In detail, an aliquot of 100 μl of biological samples (plasma, urine, bile) were spiked with 10 μl IS (2 μg/ml) solution. Then, 400 μl ethyl acetate for plasma or 400 μl methanol for urine and bile was added and vortexed for 5 min immediately. The mixture solutions were cold centrifuged at 10600 × g for 10 min maintained at 4 °C temperature. Subsequently, 300 μl of supernatant layer was transferred into a new 1.5-ml Eppendorf tube (EP) and evaporated to dryness in vacuum concentrator. Residues were reconstituted in 100 μl mobile phase solvents (acetonitrile: water, 55 : 45, v/v) by vortexing for 5 min and centrifuged. 80 μl supernatant was transferred to a fresh vial and 5 μl was injected into LC-MS/MS analysis system for analysis.

Each weighed tissue sample was homogenized in normal saline (1 ml/0.2 g tissue, w/v). 100 μl of tissue homogenate was spiked with 10 μl of IS solution (2 μg/ml) and 400 μl of ethyl acetate solution, and then vortex-mixed for 5 min. Subsequent steps were in accordance with above-mentioned plasma sample preparation.

Faeces were dried at 50 °C dry oven for 24 h, individually weighed and recorded, and then homogenized in normal saline to prepare the suspension (200 mg/ml, w/v).

Subsequent steps were in accordance with above-mentioned plasma sample preparation.

Analytical methodology

DHA-10 concentrations in the plasma, tissue, faeces, urine and bile were quantified by LC-MS/MS and the analytical method was shown in Appendix S1. The separation was achieved on a Kromasil 100-5C18 (150 mm × 2.1 mm, 5 μm) column with MS detection operated in positive MRM mode at *m/z* 240.0 → 222.0 and *m/z* 199.0 → 125.2 for DHA-10 and IS, respectively. The assay was linear over the concentration range ($r^2 > 0.995$) with the LLOQ of 0.010 μg/ml. The intra- and interday precisions (R.S.D.) were ≤11.67%, whereas accuracy ranged from −13.96% to 14.66%. The extraction recovery, stability and matrix effect were within the acceptable limits.[14]

Data analysis

SPSS 19.0 (SPSS Inc., Chicago, USA) and Origin Pro 8.6 (Origin Lab Co., Northampton, USA) software were used for various calculations. The pharmacokinetic parameters and the concentration-time curves were analysed through a non-compartmental method by DAS 2.0 Software (Mathematical Pharmacology Professional Committee of China, Shanghai, China). Levels of statistical significance were assessed using the Student's *t*-test. $P < 0.05$ was considered as statistical significance. All the data were expressed as the mean ± SD.

Results

Synthesis and characterization

DHA-10 was synthesized and its spectral data given below:

1 White solid; m.p. = 118–119 °C. ^{1}HNMR ($CDCl_3$, 400 MHz) δ = 15.40(s, 1H), 5.95(s, 1H), 4.64(s, 2H), 2.25(s, 3H); HRMS (ESI) *m/z* calcd for $C_8H_7O_4Br$ $[M + H]^+$246.9528, found 246.9486.

2 ^{1}HNMR ($CDCl_3$, 400 MHz) δ = 12.05(s, 1H), 6.43(s, 1H), 4.70(s, 2H), 3.57(q, 2H, *J* = 7.1 Hz), 3.49(q, 2H, *J* = 7.2 Hz), 2.52(s, 3H), 1.34(t, 3H), 1.28(t, 3H, *J* = 7.2 Hz); HRMS (ESI) *m/z* calcd for $C_{12}H_{17}NO_4$ $[M + H]^+$240.123, found 240.1244.

Plasma pharmacokinetic study

Mean plasma concentration-time profiles and log of plasma concentration-time curves of DHA-10 after single oral and intravenous administration were shown in Figure 2. The major pharmacokinetic parameters were calculated by a noncompartmental model based on the statistical moment and listed in Table 1. The terminal elimination half-life ($T_{1/2}$) of three oral dose groups was 3.39 ± 0.58, 3.24 ± 0.32 and 3.80 ± 0.40, respectively, and the mean residence time (MRT) ranging from 5.66 to 6.96 h. Total plasma clearance CL (0.37 ± 0.045, 0.32 ± 0.019 and 0.34 ± 0.048 l/h per kg) was not different across the investigated dosage range, respectively. The mean plasma C_{max} and $AUC_{0-\infty}$ values of the three doses indicated an apparent dose-proportionality and correlation coefficient (*r*) is 0.995 and 0.998, respectively. As for the other parameters including T_{max}, MRT, CL, Vd and $T_{1/2}$, there were no significant differences in the three dosages ($P > 0.05$). All these results supported the linear plasma pharmacokinetics of DHA-10 among the investigated dose range in rats (70–280 mg/kg). The oral bioavailability (*F*) of DHA-10 at the dose of 140 mg/kg was estimated to be 69.09 ± 3.9%.

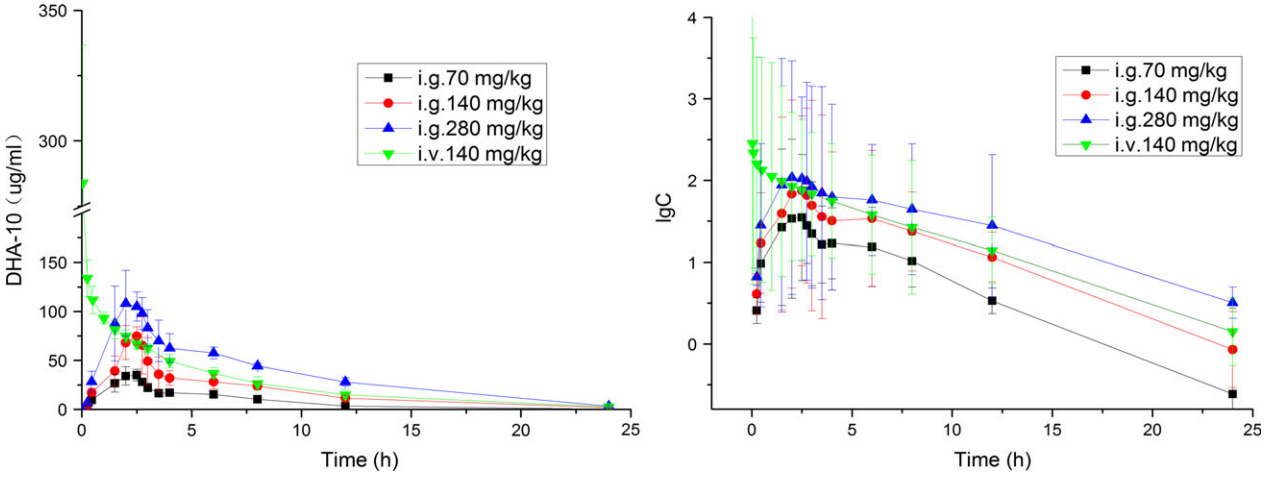

Figure 2 Mean plasma concentration-time curves of DHA-10 and log of plasma concentration-time curves after a single oral administration at a dose of 70 140 280 mg/kg ($n = 6$).

Table 1 Pharmacokinetic parameters of DHA-10 after a single-dose administration in rats (n = 6)

Parameter	Oral			Intravenous
	70 mg/kg	140 mg/kg	280 mg/kg	140 mg/kg
C_{max} (μg/l)	40.53 ± 4.88	79.79 ± 1.77	134.72 ± 8.30	283.91 ± 52.78
T_{max} (h)	2.25 ± 0.27	2.42 ± 0.38	2.21 ± 0.33	–
$T_{1/2z}$ (h)	3.39 ± 0.58	3.24 ± 0.324	3.80 ± 0.40	3.63 ± 0.15
AUC_{0-t} (μg/l × h)	192.43 ± 24.53	436.214 ± 24.63	820.89 ± 112.48	631.40 ± 90.23
$AUC_{0-\infty}$ (μg/l × h)	194.32 ± 25.77	440.357 ± 24.96	834.45 ± 118.34	637.40 ± 92.14
$CL_{z/F}$ (l/h per kg)	0.37 ± 0.045	0.32 ± 0.019	0.34 ± 0.048	0.223 ± 0.031
$V_{z/F}$ (l/kg)	1.776 ± 0.32	1.49 ± 0.17	1.86 ± 0.204	1.17 ± 0.17
MRT_{0-t} (h)	5.44 ± 0.30	6.34 ± 0.32	6.59 ± 0.27	4.72 ± 0.44
$MRT_{0-\infty}$ (h)	5.658 ± 0.40	6.55 ± 0.42	6.96 ± 0.27	4.94 ± 0.48

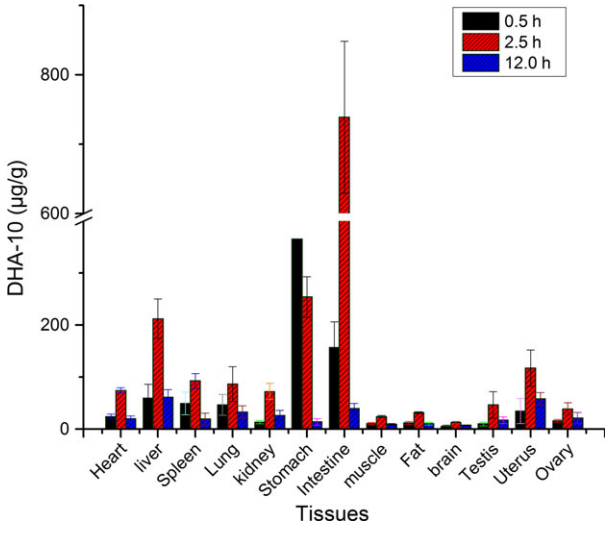

Figure 3 The distribution of DHA-10 in rats after oral administration of 140 mg/kg at 0.5, 2.5 and 12.0 h.

Tissue distribution study

The concentration of DHA-10 (Figure 3) was detected in heart, liver, spleen, lung, kidney, stomach, small intestine, brain, fat, muscle and gonadal (testis, uterus and ovary) samples. The results indicated that after oral administration, the DHA-10 underwent a wide distribution in rat tissues within the time course examined. The highest tissue concentration was found in small intestine at 2.5 h, followed by the stomach, liver and uterus with very little DHA-10 found in brain. DHA-10 concentration decreased obviously at 12 h postdose.

Excretion study

Excretion of DHA-10 in rat urine, faeces and bile was presented in Figure 4 and Table 2. The result indicated that after a single oral administration, 1.50 ± 0.26% of DHA-10 was excreted as original form in urine within 48 h and 9.12 ± 2.53% of DHA-10 was excreted as unchanged form

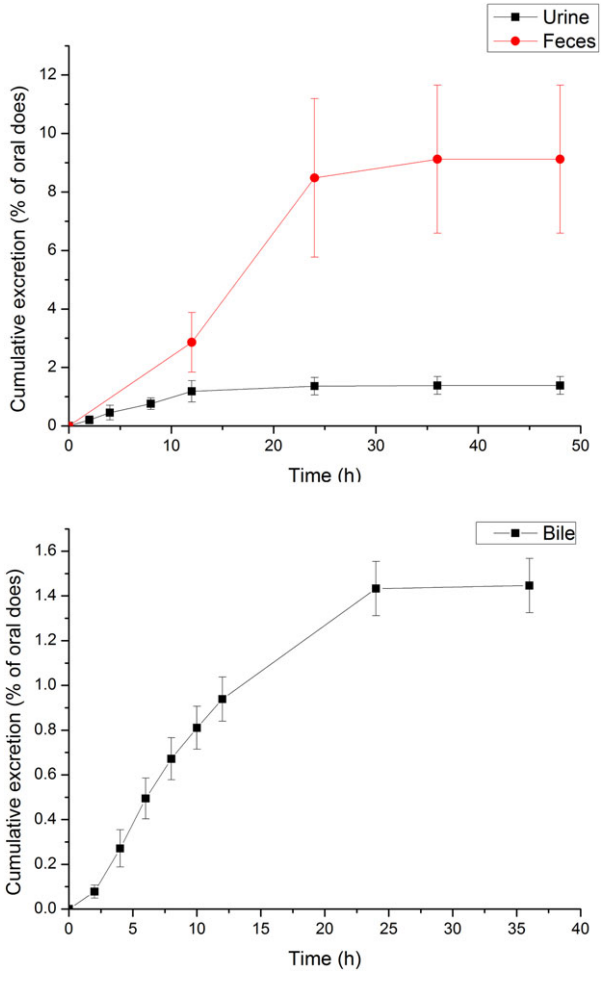

Figure 4 Urinary, faecal and biliary cumulative excretion of DHA-10 in rats after single-dose administration of 140 mg/kg.

in faeces within 48 h period. The amount of DHA-10 in faeces reached to 8.48 ± 2.71% of the dose within 24 h, and then increased slowly over time. Biliary excretion within 36 h was about 1.45 ± 0.12%.

Plasma protein binding study

The plasma protein binding rates of DHA-10 were measured to be 77.26 ± 1.72, 80.04 ± 0.92, 79.11 ± 0.61% for three different concentrations, respectively. The average plasma protein binding rate was 78.80 ± 1.75%. There was no significant differences at three different levels of DHA-10 in rats ($P > 0.05$), indicating that plasma protein binding to DHA-10 had no concentration dependence.

Discussion

Pogostone is one of the secondary metabolites from Pogostemon cablin (Blanco) Benth (Lamiaceae), serving as the effective component of the antimicrobial activity. Previous report showed that PO may develop as a serious novel antifungal compounds with proper structure modification.[15] We have synthesized a series of PO analogues and evaluated their antimicrobial activities, of which DHA-10 showed the most antifungal potential. Here, the synthesis and pharmacokinetic properties of DHA-10 were investigated for the first time.

The preclinical pharmacokinetic study including the plasma profiles, tissue distribution, excretion and plasma protein binding were designed and carried out based on the LC-MS/MS. method which proved to be sensitive, rapid and reproductive and suitable to the pharmacokinetic study in rats.

Pharmacokinetics of DHA-10 showed linear kinetic character within the dose range studied. $T_{1/2}$ of DHA-10 was 3.15–3.83 h, which was longer than that of PO, 53.31–51.96 min,[13] suggesting that DHA-10 eliminated more slowly. The highest tissue concentration was found in small intestine and the second in liver, which implied that the small intestine might be the main absorption site of the drug and liver played an important role in the biotransformation of DHA-10. High concentrations of DHA-10 were also found in uterus and ovary, which indicated that these two tissues could be the therapeutic targets. This discovery was obviously meaningful considering that gynecological diseases caused by fungal infections were common in women. DHA-10 was detectable in testis, suggesting that DHA-10 could get through the blood-testis barrier and have effects on testis infected by fungus. The amount of DHA-10 decreased to a very low level at 12 h postdosing in all of the examined tissues.

Excretion results showed that up to 48 h, the total unchanged DHA-10 excreted in urine and faeces was only 10.62 ± 0.25% of the oral dose, suggesting that most of DHA-10 may excrete as metabolite forms.

The drug in bound form to plasma protein is not available for interaction with its biological targets such as enzyme, receptor transporter, etc. The ratio of free and

Table 2 Urine, faeces and biliary cumulative excretion of DHA-10 in rats during each time interval after single-dose administration of 140 mg/kg ($n = 6$)

Matrix	Time (h)	Excretion amount (μg, mean ± SD)	Excretion accumulation (μg, mean ± SD)	Excretion accumulation (% of oral dose)
Urine	0–2	57.27 ± 15.10	57.27 ± 15.10	0.205 ± 0.054
	2–4	73.83 ± 34.28	131.10 ± 21.86	0.468 ± 0.078
	4–8	85.41 ± 32.14	216.51 ± 51.49	0.773 ± 0.184
	8–12	143.44 ± 62.55	359.95 ± 88.63	1.286 ± 0.317
	12–24	49.36 ± 51.70	412.18 ± 74.05	1.462 ± 0.264
	24–36	10.31 ± 6.87	424.91 ± 74.75	1.499 ± 0.267
	36–48	0.22 ± 0.12	425.13 ± 74.67	1.500 ± 0.262
Faeces	0–12	800.47 ± 285.61	800.47 ± 285.61	2.86 ± 1.02
	12–24	1574.17 ± 772.77	2374.64 ± 759.13	8.48 ± 2.71
	24–36	179.19 ± 138.95	2553.83 ± 708.05	9.12 ± 2.53
	36–48	0.56 ± 0.28	2554.38 ± 708.00	9.12 ± 2.53
Bile	0–2	24.70 ± 8.26	24.70 ± 8.26	0.078 ± 0.030
	2–4	54.04 ± 17.33	78.75 ± 23.28	0.27 ± 0.083
	4–6	62.53 ± 12.07	141.28 ± 25.50	0.49 ± 0.091
	6–8	49.68 ± 11.89	190.96 ± 26.34	0.67 ± 0.094
	8–10	38.78 ± 5.42	229.74 ± 26.89	0.81 ± 0.096
	10–12	35.95 ± 14.22	265.69 ± 27.63	0.94 ± 0.099
	12–24	138.39 ± 20.43	404.08 ± 34.02	1.43 ± 0.12
	24–36	0.91 ± 0.12	405.00 ± 34.01	1.45 ± 0.12

plasma protein bound drugs will have an impact on pharmacokinetics, pharmacodynamics and safety margins of the compound. The rates of DHA-10 binding to plasma protein were less than 80% in a concentration-independent manner has a certain reference value to guide the dosage of administration.

Conclusions

In summary, this is the first report for the synthesis of DHA-10 and studies of the preclinical pharmaceutics, tissue distribution, excretions and plasma protein binding of DHA-10 in rats. The results showed linear disposition of DHA-10 at the examined dose range, with C_{max} and $AUC_{0-\infty}$ being proportional to the administered dosages in rats. The oral bioavailability of the drug was calculated to approximately 69.09 ± 3.9%. In tissue distribution studies, the highest level in small intestine, followed by the liver suggests that DHA-10 was mainly absorbed in the small intestine and metabolism mainly in the liver. Very little DHA-10 crossed the blood–brain barrier (BBB). Only 10.62 ± 0.25% of the administered DHA-10 was excreted as the unchanged form from urine (1.50 ± 0.26%) and faeces (9.12 ± 2.53%). The information from the synthesis and pharmacokinetic of DHA-10 supported further drug design optimization in the development of both current and future series' as potential antifungal agents for the effective treatment of invasive fungal infection diseases.

Declarations

Conflict and interest

All Authors declare that they have no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. HPLC-MS/MS method for the determination of DHA-10 in rat plasma, tissue, faeces, urine and bile.

Appendix S2. The characterization of DHA-10.