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Original article

Novel liver-specific nitric oxide (NO) releasing drugs with bile acid as both NO carrier and targeting ligand

Xue-Yuan Jin [a], Shi-Yong Fan [b], Hong-Wu Li [b], Wei-Guo Shi [b], Wei Chen [b], Hui-Fen Wang [a,*], Bo-Hua Zhong [b,*]

[a] Chinese PLA General Hospital & Chinese PLA Medical School, Beijing 100853, China
[b] Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China

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ABSTRACT

Novel liver-specific nitric oxide (NO) releasing drugs with bile acid as both the NO carrier and targeting ligand were designed and synthesized by direct nitration of the hydroxyl group in bile acids or the 3-*O*-hydroxyl alkyl derivatives, with the intact 24-COOH being preserved for hepatocyte specific recognition. Preliminary biological evaluation revealed that oral administrated targeted conjugates could protect mice against acute liver damage induced by acetaminophen or carbon tetrachloride. The nitrate level in the liver significantly increased after oral administration of **1e** while nitrate level in the blood did not significantly change. Co-administration of ursodeoxycholic acid (UDCA) significantly antagonized the increase of nitrate in the liver resulted by administration of **1e**.

1. Introduction

Nitric oxide (NO) mediates multiple physiological and pathological processes [1]. Previous studies have demonstrated that NO can protect the liver from injury and ameliorate the progress of liver fibrosis or cirrhosis. NO-releasing drugs may represent an important therapy for liver damage, fibrosis, and cirrhosis [2,3]. However, non-specific NO-releasing drugs such as isosorbide-5-mononitrate induce significant reduction of arterial pressure, which limits their clinical application [4].

Liver targeted therapy can deliver chemotherapeutic drugs selectively to the liver to avoid side effects or toxicities. Bile acids are the only small molecules with high oral availability that are taken in specifically by the liver [5,6]. Conjugating NO releasing components with bile acids such as cholic acid (CA) or ursodeoxycholic acid (UDCA) can deliver NO selectively to the liver for the treatment of liver diseases without significant reduction of arterial pressure. NCX-1000 (Fig. 1) is the first liver-specific NO donor drug that can protect mice against acute liver injury induced by acetaminophen (APAP) or Con A [7,8]. However, NCX-1000 has not met the endpoints in phase 2 clinical trials.

NCX-1000 was prepared by conjugating the NO releasing moiety with the 24-COOH of UDCA by two ester bonds. However, the intact 24-COOH of bile acid is essential for liver specificity [5]. Conjugating with the 24-COOH may abolish the targeting of UDCA, and the ester bond in the conjugate is not stable in human blood.

Novel liver-specific nitric oxide (NO) releasing drugs were designed and synthesized by use of the hydroxyl group on the steroid nucleus at position 3, 7, or 12 as the conjugating group with the preserved 24-COOH for hepatocyte specific recognition. Compounds **1a–1d** were nitrates of 3-OH, 7-OH, or 12-OH. In these compounds, the bile acids are used as both the NO carrier and targeting ligand. Compounds **1e** and **1f** were nitrates of 3-O$(CH_2)_4$ OH and 3-O$(CH_2)_2$OH derivatives of UDCA, respectively. The linkage chain between nitrate and UDCA can modulate the release rate of NO from the conjugates.

Preliminary biological evaluation revealed that the targeted conjugates can protect mice against acute liver injury induced by APAP or carbon tetrachloride (CCl_4). Both the site and number of nitration could affect the biological activity. Compounds **1e** and **1f** are two most active compounds and show more potent protective effects than NCX-1000. Further *in vivo* nitrate distribution investigation revealed that the nitrate level in the liver significantly increased after oral administration of **1e**, while nitrate level

* Corresponding authors.
E-mail addresses: wanghf0501@163.com (H.-F. Wang), bohuazhong@yahoo.com (B.-H. Zhong).

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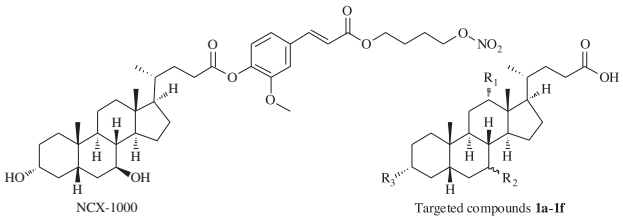


Fig. 1. Structure of NCX1000 and targeted compounds.

in the blood did not significantly changed. Co-administration of UDCA significantly antagonized the increase of nitrate in the liver resulted by **1e**, which demonstrated that the delivery of **1e** to the liver was mediated *via* the bile acid transport system.

2. Experimental

2.1. Chemistry

The synthesis of compounds **1a** and **1b** is outlined in Scheme 1. The 24-COOH of bile acids was first protected by methylation in HCl-methanol solution to give **2a**. Target compound **1a** was obtained by nitration of all three hydroxyl groups of **2a** with fuming nitric acid in Ac_2O and then deprotection with 5% KOH menthol solution. The 3-OH of **2a** was selectively reacted with acetyl chloride to give the 3-OH protected derivative **3b**. Compound **1b** was obtained by nitration of **3b** followed by deprotection of **4b**, similar to **1a**.

Compounds **1c** and **1d** were synthesized as depicted in Scheme 2. The methylated derivatives (**2a** and **2d**) of bile acids were reacted with Ac_2O to give the acetate derivatives **3c** and **3d**; **3c** and **3d** were selectively deprotected to give the 3-OH free derivative **4c** and **4d**. The target compounds **1c** and **1d** were obtained by nitration and then deprotection as that of **1a**.

Compounds **1e** and **1f** were prepared as outlined in Scheme 3. **4d** was reacted with methanesulfonyl chloride to obtain the methanesulfonate derivative **6**. Compound **6** was then reacted with 1, 4-butanediol or ethylene glycol to give the hydroxyl alkylated derivatives **7e** and **7f**, respectively. Compounds **7e** and **7f** were nitrated and then deprotected to afford target compounds **1e** and **1f**, respectively. NCX-1000 was synthesized according to the literature [9].

The structures of compounds **1a–1f** were characterized [10].

2.2. Pharmacology

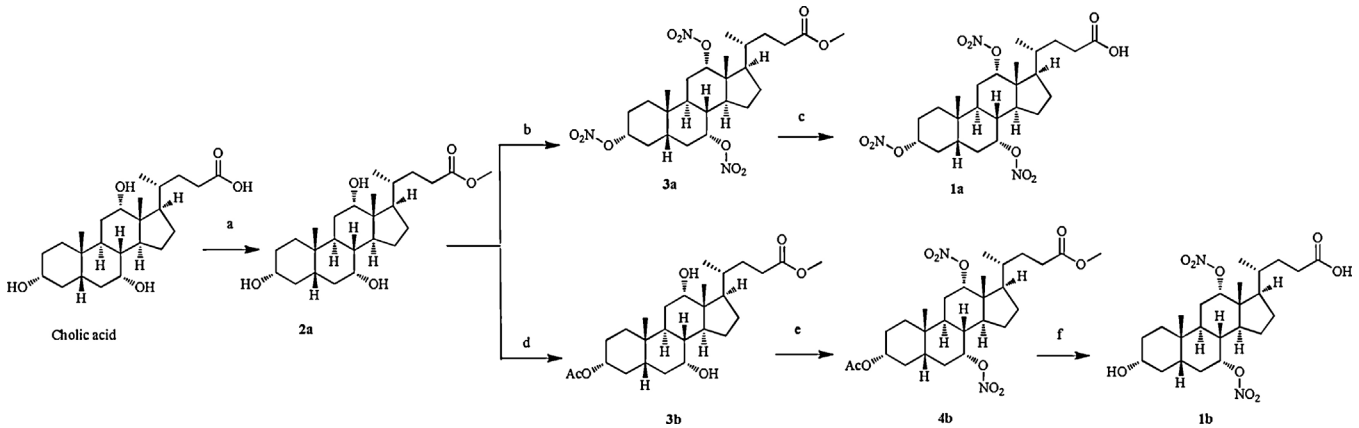
2.2.1. Protective effect against acute liver injury induced by CCl_4 or APAP

Baclb/c mice were randomly divided (n = 10, half male and half female). Compounds were suspended in 1% sodium methoxycellulose and administered by intragastric at dosage of 50 mg/kg. Sixty minutes after administration of the test compounds, 0.1% CCl_4 in olive oil at dose of 10 mL/kg or APAP at dose of 200 mg/kg was administrated by i.p. injection to induce the acute liver injury model. Saline was subcutaneous injected as normal control. Twelve hours after administration of carbon tetrachloride or APAP, same amount of the compounds was administered by intragastric. Twenty-four hours after the second administration of compounds, whole blood samples were taken from the suborbital sinus and the samples were centrifuged, the plasma was frozen and stored immediately at −70 °C. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was determined with the Olympus AU5400 automated biochemistry analyzer (Olympus Corporation, Tokyo, Japan).

2.2.2. NO distribution in vivo

After overnight fasting, male Baclb/c mice were randomly divided into 4 groups (n = 10). Compounds were suspended in 1% sodium methoxycellulose and administered by intragastric injection. The mice in the first group were treated with saline as control; the mice in the second group were treated with **1e** (10 mg/mL, 20 mL/kg); in the third group, mice were treated with UDCA (50 mg/mL, 20 mL/kg); in the fourth group, mice were treated with UDCA (50 mg/mL, 20 mL/kg) and **1e** (10 mg/mL, 20 mL/kg). Five mice from each group were sacrificed in 2 h or 4 h after administration of the compounds. Whole blood samples were taken from the suborbital sinus, and the samples were centrifuged. The plasma was frozen and stored immediately at −70 °C. The livers were collected at the same time and rapidly frozen in liquid nitrogen and maintained at −80 °C until analysis. Liver lysates were prepared by homogenization in Tris–HCl buffer (0.01 mol/L + NaCl 0.1 mol/L). The homogenates were centrifuged at 15,000 rpm for 15 min and the supernatant was taken for nitrate determination.

The nitrate concentration was measured by Griess assay using a colorimetric assay kit (Beyotime, China) [11]. Briefly, 50 μL of the sample solutions were mixed with 50 μL Griess reagent I and 50 μL Griess reagent II, and the mixture was then placed at room temperature for 10 min. The absorbance was measured at 540 nm, and sodium nitrate solutions at different concentrations were used as positive controls for the standard curve.



Scheme 1. Synthesis route of compounds **1a–1b.** (a) HCl–methanol, r.t., 12 h; (b, e) acetic anhydride, fuming nitric acid, −5 °C, 1 h; (c, f) 5% KOH menthol solution, reflux, 1 h; (d) acetyl chloride, pyridine, 0 °C, 1 h, then r.t., 2 h.