

Synthesis and antihepatotoxic activity of dihydropyrimidinone derivatives linked with 1,4-benzodioxane

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Purpose: To evaluate the antihepatotoxic activity of dihydropyrimidinone derivative linked with 1,4-benzodioxane.

Methods: A series of novel dihydropyrimidinone derivatives linked with 1,4-benzodioxane moiety were synthesized in good yield. Modern spectroscopic techniques and elemental analysis were used for the identification of the synthesized compounds. The hepatoprotective properties of compound **2**, 4-(4-nitrophenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one, was evaluated in a carbon tetrachloride (CCl₄)-induced hepatotoxicity rat model.

Results: Administration of compound **2** prior to CCl₄ exposure produced a dose-dependent decrease in the levels of elevated biochemical parameters compared with the standard drug silymarin. CCl₄ induced oxidative stress, increased lipid profile, and decreased high-density lipoprotein (HDL) levels. Compound **2** (20 mg/kg) significantly reduced the lipid profile and significantly improved HDL levels in a dose-dependent manner. CCl₄ treatment increased malondialdehyde (MDA) level and decreased nonprotein thiol (NP-SH) and total protein (TP) in liver tissues. Pretreatment of rats with compound **2** (20 mg/kg) decreased MDA level and increased NP-SH and TP in liver tissues. Histopathological examination of liver tissues also confirmed the hepatoprotective activity of compound **2**.

Conclusion: These results demonstrate the antihepatotoxic activity of compound **2** in CCl₄-induced hepatotoxicity model.

Keywords: antihepatotoxic activity, dihydropyrimidinone, 1,4-benzodioxane, carbon tetrachloride, silymarin

Introduction

Liver disease is a foremost cause of death worldwide. It is caused by excessive alcohol intake, malnutrition, exposure to pollutants, drugs, chemicals, viruses, and pathogens.¹ The liver is continuously exposed to various xenobiotics, which in turn lead to various liver disorders. To date, there are no effective measures available for the treatment of liver diseases, except for certain naturally occurring medicinal plants.² Silymarin has been used as an effective antihepatotoxic agent. It is obtained from the seeds of (milk thistle) *Silybum marianum*.³ It consists of three isomers namely, silychristin, silydianin and silybin.⁴ The most potent component is silybin, which contains the 1,4-benzodioxane ring system. The silychristin and silydianin do not possess 1,4-benzodioxane moiety, and therefore, do not display significant antihepatotoxic activity. 1,4-benzodioxane derivatives have been screened for various biological activities, including

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antihepatotoxic,^{5,6} D₂ antagonist/5-HT_{1A} partial agonist,⁷ anti-inflammatory,⁸ presynaptic 2-adrenoreceptor antagonist,⁹ antioxidant¹⁰ and hypolipidemic¹¹ activities.

Pyrimidines have displayed a very important role in the area of medicinal chemistry and are important moiety in the area of medicinal chemistry because of their significant biological activities such as antitumor, antiviral, and antibacterial activities.^{12–14} Pyrimidine derivatives have been used to lower blood pressure. Dihydropyridines have been used for the treatment of various cardiovascular diseases as calcium channel modulators.¹⁵ Dihydropyrimidines, popularly known as Biginell compounds, are reported with various biological activities.^{16–20} Pyrimidines are used as stimulants of protein synthesis as well as cellular recovery in the liver in cases of toxicity or infectious damage. Xymedon, (1-(2-hydroxyethyl)-4,6-dimethyl pyrimidin-2-one) have demonstrated hepatoprotective and stimulating effects on liver tissue recovery on experimental toxic liver damage in study in rats. It is a dihydropyrimidinone derivative, which was developed as a tissue regeneration stimulator, and it enhanced the activity of microsomal oxidases in human liver. It is regarded as a potent

hepatoprotective agent against carbon tetrachloride (CCl₄)-induced liver toxicity.^{21–23}

The literature review above suggested that compounds containing the two important scaffolds, 1,4-benzodioxane and dihydropyrimidinone, may have a significant potential in the management of liver diseases. We considered that 1,4-benzodioxane moiety plays an important role in demonstrating antihepatotoxic activity and, therefore, synthesized new dihydropyrimidinone derivatives possessing 1,4-benzodioxane ring system (Figure 1). The compounds synthesized were of low molecular weight and can be simply prepared in the laboratory in comparison to silybin.

Materials and methods

Solvents were procured from Merck. Thin layer chromatography (TLC), was done on Silica gel 60 F₂₅₄ coated plates (Merck Millipore, Billerica, MA, USA). For performing FT IR, Perkin Elmer FT-IR instrument was used (PerkinElmer Inc., Waltham, MA, USA). Gallenkamp melting point apparatus was used for the determination of melting point. Proton and carbon NMR were recorded in Bruker NMR 700/500

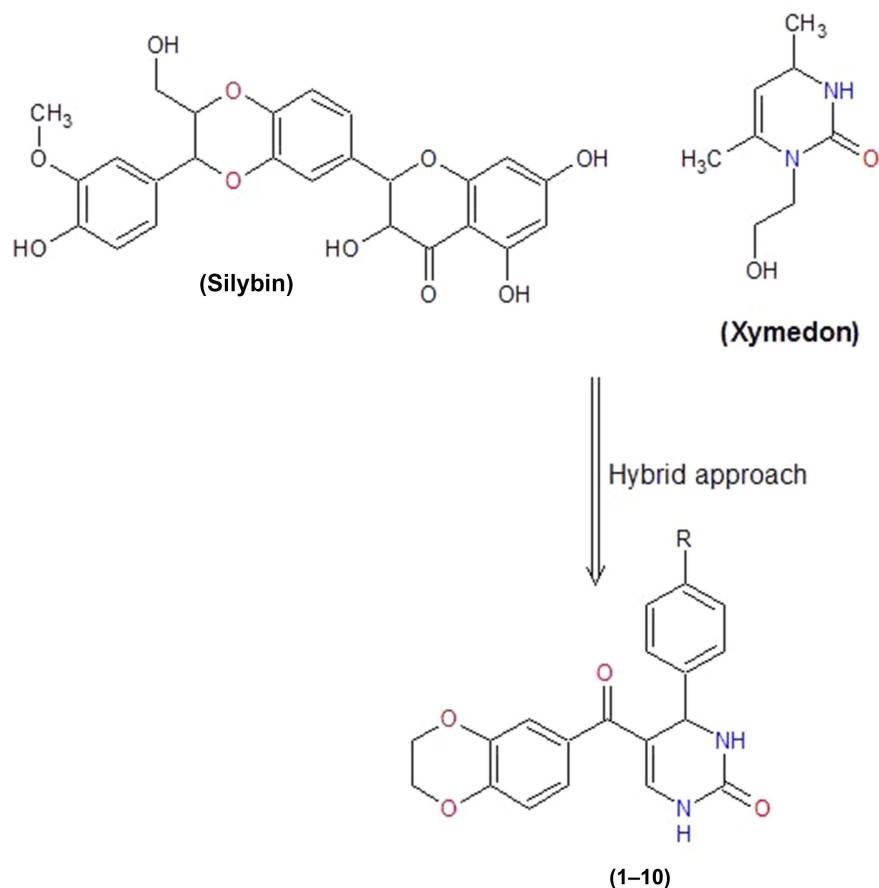


Figure 1 Structures of silybin, xymedon and dihydropyrimidinone derivatives (1–10).

MHz and 176.0/125 MHz spectrophotometer (Bruker Corporation, Billerica, MA, USA). All the samples were dissolved in deuterated dimethyl. Molecular ions of compounds were detected by GC/MS mass spectroscopy. The elemental analysis of the compounds was performed by CHN Elementar (Analysensysteme GmbH, Langensfeld, Germany).

Enaminone (**III**), 1-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-(dimethylamino)prop-2-en-1-one, was synthesized by refluxing 1-(2,3-dihydro-1,4-benzodioxin-6-yl)ethan-1-one (**I**) with dimethylformamide dimethyl acetal (DMF-DMA) (**II**) under solvent-free conditions for 10 hours.²⁴

Synthesis of 1-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-(dimethylamino)prop-2-en-1-one (**III**)

A mixture of 1-(2,3-dihydro-1,4-benzodioxin-6-yl)ethan-1-one (**I**) (0.02 mol) and dimethylformamide-dimethylacetal (DMF-DMA) (**II**) (0.023 mol) was refluxed for 10 hours in solvent-free conditions on a heating mantle, then the mixture was left to cool slowly at room temperature. Diethyl ether was added to the reaction mixture to precipitate it. Filtration was performed to collect the product. Absolute ethanol was used for the recrystallization. Yield: 75%; m.p.: 175–177°C; IR (KBr) cm^{-1} : 1700 (C=O), 1620 (C=C), 1118 (C-O); ¹H NMR (700 MHz, DMSO-d₆) δ ppm: 2.69 (3H, s, N-CH₃), 3.12 (3H, s, N-CH₃), 4.27 (4H, s, 2×OCH₂), 5.78–5.77 (1H, d, *J*=7 Hz, =CH), 6.85–7.67 (3H, m, Ar-H), 7.82 (1H, d, *J*=7 Hz, =CH); ¹³C NMR (176.0 MHz, DMSO-d₆): δ 14.9, 37.5, 64.4, 91.0, 115.3, 116.9, 121.2, 129.9, 132.3, 134.1, 143.3, 146.3, 154.2, 164.0, 184.8, 191.7; MS; *m/z* =233.10 [M]⁺

General synthesis of 4-(substituted phenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one (**1-10**)

A mixture of enaminone, 1-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-(dimethylamino)prop-2-en-1-one (**III**) (0.01 mol), urea (0.01 mol), different substituted benzaldehyde (0.01 mol) was refluxed in glacial acetic acid (10 mL) for 3 hours. The reaction mixture was poured into the cold water to get the precipitates (**1-10**). The compounds were obtained by filtration and were washed many times with cold water. The compounds obtained were recrystallized from glacial acetic acid and ethanol mixture.

4-(4-Chlorophenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one (**1**): Yield:

80%; m.p.: 220–222°C; IR (KBr) cm^{-1} : 3412 (NH str.), 1700 (C=O), 1654 (C=O), 1618 (C=C), 1196 (C-O); ¹H NMR (500 MHz, DMSO-d₆): δ =4.27 (4H, s, 2×-OCH₂), 5.41 (1H, d, *J*=3.0 Hz, H-4), 6.90–7.30 (7H, m, Ar-H), 7.85 (1H, s, =CH), 9.33 (1H, bs, -NH, D₂O exchg.), 10.0 (1H, bs, -NH, D₂O exchg.); ¹³C NMR (125.76 MHz, DMSO-d₆): δ =53.5 (OCH₂), 64.8 (OCH₂), 112.2, 117.3, 117.6, 122.3, 128.8, 128.9, 131.9, 132.3, 141.3, 143.5, 146.6, 151.6, 190.4 (C=O); MS: *m/z* =370.77 [M]⁺; analysis: for C₁₉H₁₅ClN₂O₄, calcd. C 61.55, H 4.08, N 7.56%; found C 61.31, H 4.09, N 7.58%.

4-(4-Nitrophenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one (**2**): Yield: 85%; m.p.: 198–200°C; IR (KBr) cm^{-1} : 3244 (NH str.), 1699 (C=O), 1617 (C=O), 1588 (C=C), 11,286 (C-O); ¹H NMR (500 MHz, DMSO-d₆): δ =4.27 (4H, s, 2×-OCH₂), 5.54 (1H, d, *J*=3.0 Hz, H-4), 6.90–7.96 (7H, m, Ar-H), 8.21 (1H, s, =CH), 9.45 (1H, bs, -NH, D₂O exchg.), 10.2 (1H, bs, -NH, D₂O exchg.); ¹³C NMR (125.76 MHz, DMSO-d₆): δ =53.8 (OCH₂), 64.8 (OCH₂), 111.6, 117.6, 117.3, 117.7, 122.3, 124.3, 128.3, 131.8, 141.8, 143.4, 146.7, 147.2, 151.4, 151.6, 190.4 (C=O); MS: *m/z*=381.34 [M]⁺; analysis: for C₁₉H₁₅N₃O₆, calcd. C 59.84, H 3.96, N 11.02%; found C 59.61, H 3.95, N 11.04%.

4-(2-Nitrophenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one (**3**): Yield: 85%; m.p.: 180–182°C; IR (KBr) cm^{-1} : 3411 (NH str.), 1700 (C=O), 1654 (C=O), 1611 (C=C), 1285 (C-O); ¹H NMR (500 MHz, DMSO-d₆): δ =4.2 (4H, s, 2×-OCH₂), 5.5 (1H, d, *J*=2.5 Hz, H-4), 6.9–7.9 (7H, m, Ar-H), 8.1 (1H, s, =CH), 9.5 (1H, bs, -NH, D₂O exchg.), 10.2 (1H, bs, -NH, D₂O exchg.); ¹³C NMR (125.76 MHz, DMSO-d₆): δ =64.4 (OCH₂), 64.8 (OCH₂), 111.5, 117.3, 121.6, 122.9, 130.7, 131.8, 133.7, 142.1, 143.4, 146.6, 148.2, 151.4, 190.4 (C=O); MS: *m/z* =381.35 [M]⁺; analysis: for C₁₉H₁₅N₃O₆, calcd. C 59.84, H 3.96, N 11.02%; found C 59.65, H 3.97, N 11.05%.

4-(3-Nitrophenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one (**4**): Yield: 80%; m.p.: 193–195°C; IR (KBr) cm^{-1} : 3412 (NH str.), 1699 (C=O), 1617 (C=O), 1587 (C=C), 1286 (C-O); ¹H NMR (500 MHz, DMSO-d₆): δ =4.2 (4H, s, 2×-OCH₂), 5.5 (1H, d, *J*=2.5 Hz, H-4), 6.9–7.9 (7H, m, Ar-H), 8.1 (1H, s, =CH), 9.5 (1H, bs, -NH, D₂O exchg.), 10.2 (1H, bs, -NH, D₂O exchg.); ¹³C NMR (125.76 MHz, DMSO-d₆): δ =53.6 (OCH₂), 64.8 (OCH₂), 111.5, 117.7, 122.3, 130.7, 131.8, 133.7, 142.1, 143.4, 146.7, 148.2, 151.4, 190.4 (C=O); MS: *m/z* =381.30 [M]⁺; analysis: for C₁₉H₁₅N₃O₆, calcd.

C 59.84, H 3.96, N 11.02%; found C 59.67, H 3.94, N 11.03%.

4-(4-Methoxyphenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one (**5**): Yield: 65%; m.p.: 225–227°C; IR (KBr) cm^{-1} : 3412 (NH str.), 1700 (C=O), 1654 (C=O), 1617 (C=C), 1287 (C-O); ^1H NMR (500 MHz, DMSO- d_6): δ =3.7 (3H, s, -OCH₃), 4.2 (4H, s, 2 \times -OCH₂), 5.3 (1H, d, J =2.5 Hz, H-4), 6.8–7.2 (7H, m, Ar-H), 7.8 (1H, s, =CH), 9.2 (1H, bs, -NH, D₂O exchg.), 10.0 (1H, bs, -NH, D₂O exchg.); ^{13}C NMR (125.76 MHz, DMSO- d_6): δ =53.4 (OCH₃), 55.5 (OCH₂), 64.8 (OCH₂), 112.9, 114.2, 117.2, 123.3, 129.1, 132.1, 136.7, 140.7, 143.4, 146.5, 151.7, 159.0, 190.5 (C=O), 206.9 (C=O); MS: m/z =366.35 [M]⁺; analysis: for C₂₀H₁₈N₂O₅, calcd. C 65.57, H 4.95, N 7.65%; found C 65.79, 4.96, 7.68%.

4-(4-Ethoxyphenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one (**6**): Yield: 66%; m.p.: 210–212°C; IR (KBr) cm^{-1} : 3420 (NH str.), 1700 (C=O), 1654 (C=O), 1617 (C=C), 1218 (C-O); ^1H NMR (500 MHz, DMSO- d_6): δ =1.3 (3H, s, -CH₃), 4.0 (2H, s, -OCH₂), 4.2 (4H, s, 2 \times -OCH₂), 5.3 (1H, d, J =3.0 Hz, H-4), 6.7–7.3 (7H, m, Ar-H), 7.7 (1H, s, =CH), 9.2 (1H, bs, -NH, D₂O exchg.), 9.9 (1H, bs, -NH, D₂O exchg.); ^{13}C NMR (125.76 MHz, DMSO- d_6): δ =19.0, 56.5 (OCH₂), 63.4 (OCH₂), 64.8 (OCH₂), 114.3, 116.6, 119.2, 122.3, 128.0, 132.1, 136.6, 139.6, 143.3, 146.5, 151.7, 158.2, 190.5 (C=O); MS: m/z =380.40 [M]⁺; analysis: for C₂₁H₂₀N₂O₅, calcd. C 66.31, H 5.30, N 7.36%; found C 66.57, H 5.31, N 7.38%.

5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-4-phenyl-3,4-dihydropyrimidin-2(1H)-one (**7**): Yield: 70%; m.p.: 250–252°C; IR (KBr) cm^{-1} : 3412 (NH str.), 1706 (C=O), 1654 (C=O), 1617 (C=C), 1288 (C-O); ^1H NMR (500 MHz, DMSO- d_6): δ =4.2 (4H, s, 2 \times -OCH₂), 5.4 (1H, d, J =2.0 Hz, H-4), 6.8–7.3 (8H, m, Ar-H), 7.8 (1H, s, =CH), 9.2 (1H, bs, -NH, D₂O exchg.), 10.0 (1H, bs, -NH, D₂O exchg.); ^{13}C NMR (125.76 MHz, DMSO- d_6): δ =56.5 (OCH₂), 64.8 (OCH₂), 112.7, 117.3, 117.6, 122.3, 126.8, 127.8, 128.9, 132.0, 141.0, 143.4, 144.5, 146.6, 151.7, 190.0 (C=O); MS: m/z =396.42[M]⁺; analysis: for C₁₉H₁₆N₂O₄, calcd. C 67.85, H 4.79, N 8.33%; found C 67.59, H 4.80, N 8.36%.

4-(2-Methoxyphenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one (**8**): Yield: 50%; m.p.: 200–202°C; IR (KBr) cm^{-1} : 3412 (NH str.), 1700 (C=O), 1654 (C=O), 1617 (C=C), 1286 (C-O); ^1H NMR (500 MHz, DMSO- d_6): δ =3.7 (3H, s, -OCH₃), 4.3

(4H, s, 2 \times -OCH₂), 5.6 (1H, d, J =2.5 Hz, H-4), 6.8–7.3 (7H, m, Ar-H), 7.8 (1H, s, =CH), 9.2 (1H, bs, -NH, D₂O exchg.), 10.3 (1H, bs, -NH, D₂O exchg.); ^{13}C NMR (125.76 MHz, DMSO- d_6): δ =49.7 (OCH₃), 55.9 (OCH₂), 64.7 (OCH₂), 111.2, 117.3, 121.1, 122.3, 128.0, 129.3, 131.1, 141.5, 143.4, 146.5, 152.3, 157.3, 190.4 (C=O); MS: m/z =366.35 [M]⁺; analysis: for C₂₀H₁₈N₂O₅, calcd. C 65.57, H 4.95, N 7.65%; found C 65.70, H 4.94, N 7.67%.

4-(3-Methoxyphenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one (**9**): Yield: 55%; m.p.: 230–232°C; IR (KBr) cm^{-1} : 3336 (NH str.), 1710 (C=O), 1654 (C=O), 1617 (C=C), 1287 (C-O); ^1H NMR (500 MHz, DMSO- d_6): δ =3.7 (3H, s, -OCH₃), 4.3 (4H, s, 2 \times -OCH₂), 5.4 (1H, d, J =3.0 Hz, H-4), 6.8–7.5 (7H, m, Ar-H), 7.8 (1H, s, =CH), 9.2 (1H, bs, -NH, D₂O exchg.), 9.9 (1H, bs, -NH, D₂O exchg.); ^{13}C NMR (125.76 MHz, DMSO- d_6): δ =53.8 (OCH₃), 55.8 (OCH₂), 64.8 (OCH₂), 113.4, 116.2, 118.9, 121.4, 129.8, 132.0, 138.1, 141.1, 143.4, 146.0, 151.8, 159.7, 160.2, 172.4 (C=O), 190.5 (C=O); MS: m/z =366.31 [M]⁺; analysis: for C₂₀H₁₈N₂O₅, calcd. C 65.57, H 4.95, N 7.65%; found C 65.72, H 4.96, N 7.63%.

4-(3,4-Dimethoxyphenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one (**10**): Yield: 60%; m.p.: 240–242°C; IR (KBr) cm^{-1} : 3413 (NH str.), 1710 (C=O), 1654 (C=O), 1617 (C=C), 1255 (C-O); ^1H NMR (500 MHz, DMSO- d_6): δ =3.7 (6H, s, -OCH₃), 4.3 (4H, s, 2 \times -OCH₂), 5.4 (1H, d, J =3.0 Hz, H-4), 6.9–7.6 (6H, m, Ar-H), 7.8 (1H, s, =CH), 9.2 (1H, bs, -NH, D₂O exchg.), 9.9 (1H, bs, -NH, D₂O exchg.); ^{13}C NMR (125.76 MHz, DMSO- d_6): δ =52.5 (OCH₃), 53.8 (OCH₃), 55.8 (OCH₂), 64.8 (OCH₂), 113.0, 116.0, 118.0, 121.1, 129.2, 132.0, 138.9, 141.0, 143.2, 146.1, 151.2, 159.3, 160.0, 172.2 (C=O), 190.0 (C=O); MS: m/z =396.39 [M]⁺; analysis: for C₂₁H₂₀N₂O₆, calcd. C 63.63, H 5.09, N 7.07%; found C 63.53, H 5.07, N 7.05%.

In vitro hepatoprotective assay

The in vitro hepatoprotective assay was based on the protection of human liver-derived HepG2 cells against CCl₄-induced damage. We procured HepG2 cell line from American Type Culture Collection no. (ATCC#HB-8065) (American Type Culture Collection (ATCC), Manassas, VA, USA). Human liver hepatocellular carcinoma cells (HepG2) were grown in DMEM media supplemented with 10% bovine serum, 0.1% antimycotic solution from (Sigma-Aldrich Co., St Louis, MO, USA)

at 37°C in a humidified chamber with 5% CO₂. The cells were then treated with medium containing 1% CCl₄ along with or without the test compounds. Silymarin was used as the reference drug. Stocks of all compounds (1.0 mg/mL) were made in DMSO and further dilutions (100 µg/mL) were prepared in culture media. Cells were treated with test compounds, separately; with dose of 25 µg/mL of each compound. Treated cells were incubated in complete growth media for 48 hours. As a positive control, cells were also tested with the reference drug, silymarin. Cytotoxicity was assessed by calculating the viability of the HepG2 cells by MTT reduction assay.²⁵

Cytotoxicity of compound 2 against noncancer cell line, MCF-10A

MCF-10A cells were grown in DMEM/F-12 (GIBCO; Thermo Fisher Scientific, Waltham, MA, USA). Cells transferred into 96-well plates at 0.6×10⁴/well and kept for overnight incubation. The medium was changed with a fresh one containing the required concentration of the compound. The WST-1 reagent was added to each well after 48 hours. The plates were re-incubated for 4 hours at 37°C. By using ELISA reader at 450 nm, the amount of formazan formed was quantified.

Animals and study design

Male Wistar albino rats (180–200 g), 5–6 weeks were procured from the animal house, College of Pharmacy, King Saud University, Riyadh. The animals were adapted to laboratory conditions. The animals were kept under standard conditions and housed in a hygienic environment. Six randomly assigned animals were kept in each treatment group. All animals received care in compliance with the guidelines of the ethics committee of the experimental animal care center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The research was prospectively reviewed and approved by a duly constituted animal care and use committee and adheres to the guide for the care and use of laboratory animals; 8th edition, National Academies Press, Washington, DC [https://oacuir.nih.gov/regulations-standard] (Approval number C.P.R. 7571 dated 20 September 2017).²⁶

Acute toxicity test

Female BALB/c mice, 6–7 weeks old, weighing 20–22 g were used for evaluation of acute toxicity. Each treatment and vehicle group consists of six randomly assigned

animals. Compound 2 was dissolved in 0.5% CMC (carboxy methyl cellulose) in water and administered at several doses, ranging from (10–1,000 mg/kg), to different groups by oral route. The animals were monitored continuously for 1 hour and then for 4 hours on the first day for symptoms of toxicity and additionally up to 72 hours followed by 14 days for any mortality.

Carbon tetrachloride-induced liver toxicity

After a 14-day acclimatization period, animals were randomly distributed into five groups each containing six animals and treated as follows: group (1), untreated as normal group; Carbon tetrachloride (0.25 mL) in liquid paraffin (1:1) 1.25 mL/kg body weight (IP) was given to groups (2, 3, 4, and 5). Group 2, control group, was administered CCl₄ only. Groups 3 and 4 received test compound 2 (10 mg/kg/day) and (20 mg/kg/day) by oral route for 14 days respectively. Rats in group 5 were given silymarin (10 mg/kg/day) by oral route for similar days. After 24 hours following the administration of CCl₄, blood was collected. The animals were sacrificed after the blood collection. For the biochemical estimations and histopathology assessment, the liver tissues were taken out.²⁷

Estimation of marker enzymes

Aspartate transaminase (AST), alanine aminotransferase (ALT), ALP, gamma-glutamyl transferase (GGT) and bilirubin were measured using Reflotron Plus Analyzer and Roche kits (Roche Diagnostics GmbH, Mannheim, Germany).²⁸

Determination of malondialdehyde (MDA)

The MDA content was estimated according to Utley et al. The tissue was extracted and each tissue was normalized in 0.15 M KCL to give a 10% w/v homogenate. The absorbance of the solution was recorded at 535 nm. By the standard curve of MDA solution, the content of MDA (nmol/g) was then calculated.

Estimation of nonprotein sulfhydryls (NP-SH)

Hepatic nonprotein sulfhydryls (NP-SH) was measured according to the reported method. Total of 2 mL supernatant was mixed with 4 mL of 0.4 mmol/L tris buffer (pH 8.9). A total of 0.1 mL of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was added and the sample was shaken. The

absorbance was measured at 412 nm against reagent blank within 5 minutes of addition of DTNB.²⁹

Determination of total protein (TP)

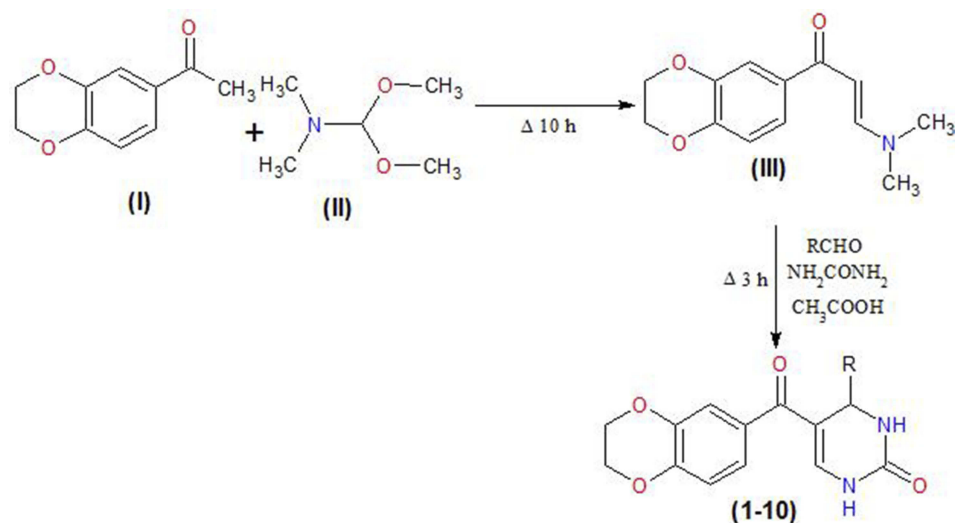
Total protein was measured by using the Lawry et al, method. Reagents: A: 2% Na₂CO₃ in 0.1 N NaOH; B: CuSO₄·5H₂O (final conc. 0.01%); C: Na or K tartrate (final conc. 0.02%); D: Folin reagent (200x dilution) in water; E: Mixture of reagents A, B, and C (50:1:1) respectively. The tissue homogenate was diluted (1:9) with distilled water, 50 µL of diluted homogenate was mixed with 1 mL of reagent “E” and added 100 µL phenanthrene I reagent and mixed very rapidly. After 30 minutes the sample was read on a spectrophotometer at the wavelength of 600 nm. Concentrations of unknown sample were estimated using the standard curve.

Histopathological evaluation

For the histopathological evaluation, the animals were sacrificed and the liver was removed. The liver tissue was stored in 10% formalin. Thick sections of liver tissue were cut. Paraffin-embedded rat liver was dewaxed in xylene. H&E stained slides were observed under a light microscope.

Results

A solution of enaminone (**II**) (0.01 mol) different substituted benzaldehyde (0.01 mol), urea (0.01 mol), dissolve in glacial acetic acid (10 mL) was heated under reflux for 3 hours to obtain dihydropyrimidinone derivatives (**Scheme 1**). The compounds were precipitated by adding the reaction mixture to ice-cold water. The product formed was collected by filtration, washed several times with cold water. The collected compounds were recrystallized from a mixture of glacial acetic acid and ethanol. The spectra of all compounds showed D₂O exchangeable singlet at δ 7.80–9.55 ppm corresponding to NH protons. The four protons (OCH₂) of 1,4-benzodioxane group were observed at δ 4.0–4.30 ppm. H-4 and =CH protons of dihydropyrimidinone moiety were observed at δ 5.3–5.60 and 7.7–8.21 ppm, respectively. The OCH₂ carbons of 1,4-benzodioxane were obtained at δ 53.5 and 64.8 ppm. The carbonyl group (C=O) peak was observed at 190.4 ppm. All the synthesized compounds were identified by molecular ion peak corresponding to their molecular weights. The experimental section contains the detailed results of ¹H NMR, ¹³C NMR spectra, and mass spectra. The analytical and spectral data established the composition of the synthesized compounds (**1–10**).



Compound	R
1	4-Cl-C ₆ H ₄
2	4-NO ₂ -C ₆ H ₄
3	2-NO ₂ -C ₆ H ₄
4	3-NO ₂ -C ₆ H ₄
5	4-OCH ₃ -C ₆ H ₄
6	4-OCH ₂ CH ₂ -C ₆ H ₄
7	C ₆ H ₅
8	2-OCH ₃ -C ₆ H ₄
9	3-OCH ₃ -C ₆ H ₄
10	3,4-(OCH ₃) ₂ -C ₆ H ₃

Scheme 1 Route for the synthesis of compounds (**1–10**).

Initial screening of the compounds showed that only compound **2**, 4-(4-nitrophenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one, had significant antihepatotoxic activity in HepG2 cells by using CCl₄ as a toxicant. The HepG2 cells exposed to CCl₄ showed a percentage viability of 20.13%. When treated with different synthesized compounds, these exposed cells showed increase in percentage viability. The percentage viability ranged between 32.72 and 68.43% at 25 µg/mL concentration (Figure 2). Compound **2** was found to be highly potent when compared to standard drug silymarin at 25 µg/mL concentration.

The compound **2** was also evaluated against noncancer cell line MCF-10A for its cytotoxic property using WST-1 assay. The compound did not show any cytotoxicity (IC₅₀>300 µM).

Acute toxicity studies were performed in mice with several doses of the test compound **2**. No toxicity symptoms and morbidity were observed up to 1,000 mg/kg in the rats. Based on this observation, 10 and 20 mg/kg were used as test doses for estimation of antihepatotoxicity activity in rats. CCl₄ is commonly used as a toxicant for hepatoprotective drug evaluation.

The results showed that rats treated with CCl₄ demonstrated a significant increase in all the tested biochemical parameters of liver function. However, rats treated with compound **2** (20 mg/kg) for 2 weeks before intoxication

with CCl₄ showed a significant decrease in AST, ALT, ALP, gamma GGT, and bilirubin levels (Table 1). The results also showed that CCl₄ increased the lipid profile, including cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol, and very low-density lipoprotein (VLDL) cholesterol, and decreased high-density lipoprotein (HDL) cholesterol levels in serum.

Fourteen-day pretreatment of rats with compound **2** (20 mg/kg/day) significantly lowered cholesterol, triglycerides, LDL cholesterol, VLDL cholesterol and significantly enhanced HDL cholesterol level (Table 2). Silymarin significantly decreased the CCl₄-induced increased levels of liver function enzymes and lipid profile. Treatment with CCl₄ caused a significant increase in MDA and a significant decrease in nonprotein thiol (NP-SH) and TP concentration in liver tissues (Table 3). Treatment of rats with compound **2** (20 mg/kg) resulted in a significant reduction in MDA levels and significantly enhanced NP-SH and TP levels in liver tissue. Thus, compound **2** afforded protection against CCl₄-induced increase in serum levels of liver function enzymes. The rats in the normal group displayed normal hepatocytes, sinusoidal spaces, and central vein (Figure 3A). The rats in the control group and those treated with CCl₄ presented centrilobular necrosis, vacuolization, sinusoidal congestion, infiltration of Kupffer cells, and loss of cell boundaries (Figure 3B). The rats treated with test compound **2** (10 mg/kg/day) exhibited

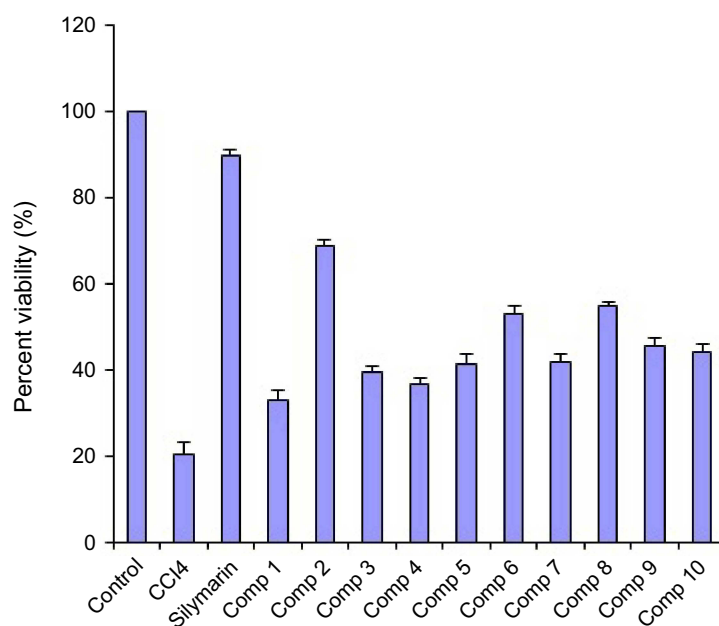


Figure 2 Hepatoprotective activity of the synthesized compound on CCl₄ intoxicated HepG2 cells.

Abbreviations: Comp, compound; HepG2, hepatocellular carcinoma cells.

Table 1 Effect of compound 2 on serum marker enzymes of control and experimental animals

Treatments (N=6)	Dose (mg/kg)	AST (U/L)		ALT (U/L)		ALP (U/L)		GGT (U/L)		Bilirubin (mg/dL)	
		Mean \pm SEM	% Change	Mean \pm SEM	% Change	Mean \pm SE	% Change	Mean \pm SEM	% Change	Mean \pm SEM	% Change
Normal		111.76 \pm 3.72		28.78 \pm 1.69		385.16 \pm 14.19		4.68 \pm 0.23		0.60 \pm 0.01	
CCl ₄	1.5 mL	386.83 \pm 9.95	↑	335.16 \pm 6.04	↑	601.83 \pm 7.98	↑	17.00 \pm 0.67	↑	3.01 \pm 0.10	↑
Silymarin +CCl ₄	10	144.00 \pm 7.44 ^{***a}	62.77↓	102.10 \pm 6.71 ^{***a}	69.53↓	442.16 \pm 13.51 ^{***a}	26.53↓	6.70 \pm 0.24 ^{***ab}	60.58↓	1.03 \pm 0.05 ^{***a}	65.75↓
2+ CCl ₄	10	383.33 \pm 6.05 ^a		294.00 \pm 10.76 ^{***a}	12.28↓	576.16 \pm 15.74 ^{ab}	4.26↓	13.50 \pm 0.59 ^{***ab}	20.28↓	2.83 \pm 0.06 ^a	6.02↓
2+ CCl ₄	20	206.83 \pm 7.34 ^{***a}	46.53↓	144.00 \pm 3.86 ^{***a}	57.03↓	452.16 \pm 13.79 ^{***a}	24.86↓	7.81 \pm 0.31 ^{***ab}	54.01↓	1.49 \pm 0.07 ^{***a}	50.49↓

Notes: All values represent \pm SEM. ** p <0.01, *** p <0.001; ANOVA, followed by Dunnett's multiple comparison test. ^aCompared with CCl₄ group. ↑, increased; ↓, decreased.

Abbreviations: AST, aspartate transaminase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; SEM, standard error of the mean.

Table 2 Effect of compound 2 on metabolism and serum lipoproteins of control and experimental animals

Treatments (N=6)	Dose (mg/kg)	Cholesterol (mg/dL)		Triglycerides (mg/dL)		HDL (mg/dL)		LDL (mg/dL)		VLDL (mg/dL)	
		Mean \pm SEM	% Change	Mean \pm SEM	% Change	Mean \pm SEM	% Change	Mean \pm SEM	% Change	Mean \pm SEM	% Change
Normal		89.66 \pm 1.68		71.63 \pm 1.83		57.58 \pm 2.28		17.75 \pm 1.13		14.32 \pm 0.36	
CCl ₄	1.5 mL	189.66 \pm 3.95	↑	183.50 \pm 4.63	↑	23.61 \pm 0.93	↓	129.35 \pm 4.74		36.70 \pm 0.92	
Silymarin+ CCl ₄	10	130.00 \pm 3.72 ^{***a}	31.45↓	116.76 \pm 7.39 ^{***a}	36.36↓	51.06 \pm 2.75 ^{***a}	116.23↑	55.58 \pm 2.07 ^{***a}	57.03↓	23.35 \pm 1.47 ^{***a}	36.36↓
2+ CCl ₄	10	175.33 \pm 4.91 ^{**a}	7.55↓	172.33 \pm 2.92 ^{ab}	6.08↓	26.11 \pm 0.62 ^{ab}	10.58↑	114.75 \pm 4.86 ^{ab}	11.28↓	34.46 \pm 0.58 ^{ab}	6.08↓
2+ CCl ₄	20	141.66 \pm 2.36 ^{***a}	24.25↓	136.83 \pm 3.80 ^{***a}	25.97↓	48.26 \pm 2.27 ^{***ab}	104.37↑	68.23 \pm 1.52 ^{***a}	47.24↓	27.16 \pm 0.76 ^{***a}	25.97↓

Notes: All values represent mean \pm SEM. ^{**}p<0.05; ^{***}p<0.001; ANOVA, followed by Dunnett's multiple comparison test. ^aCompared with CCl₄ group. [↑], increased; [↓], decreased.

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; SEM, standard error of the mean.

Table 3 Effect of compound **2** on MDA, NP-SH and total protein in liver tissue

Treatments (N=6)	Dose (mg/kg)	MDA (nmol/g)	NP-SH (nmol/g)	Total protein (g/L)
Normal	–	0.46±0.01	7.28±0.28	126.43±7.81
CCl ₄	1.5 mL	8.56±0.43	3.78±0.19	30.85±1.28
Silymarin+ CCl ₄	10	1.09±0.05***ba	7.36±0.67***ba	88.45±5.53***ba
2 + CCl ₄	10	6.84±0.52*ba	4.30±0.33 ^a	38.11±3.09 ^a
2 + CCl ₄	20	1.59±0.13***ba	6.16±0.31*** ^a	74.03±4.36***ba

Notes: All values represent mean ±SEM. *P<0.05; ***P<0.001; ANOVA, followed by Dunnett's multiple comparison test. ^aCompared with CCl₄ group.

Abbreviations: MDA, malondialdehyde; NP-SH, nonprotein sulfhydryl.

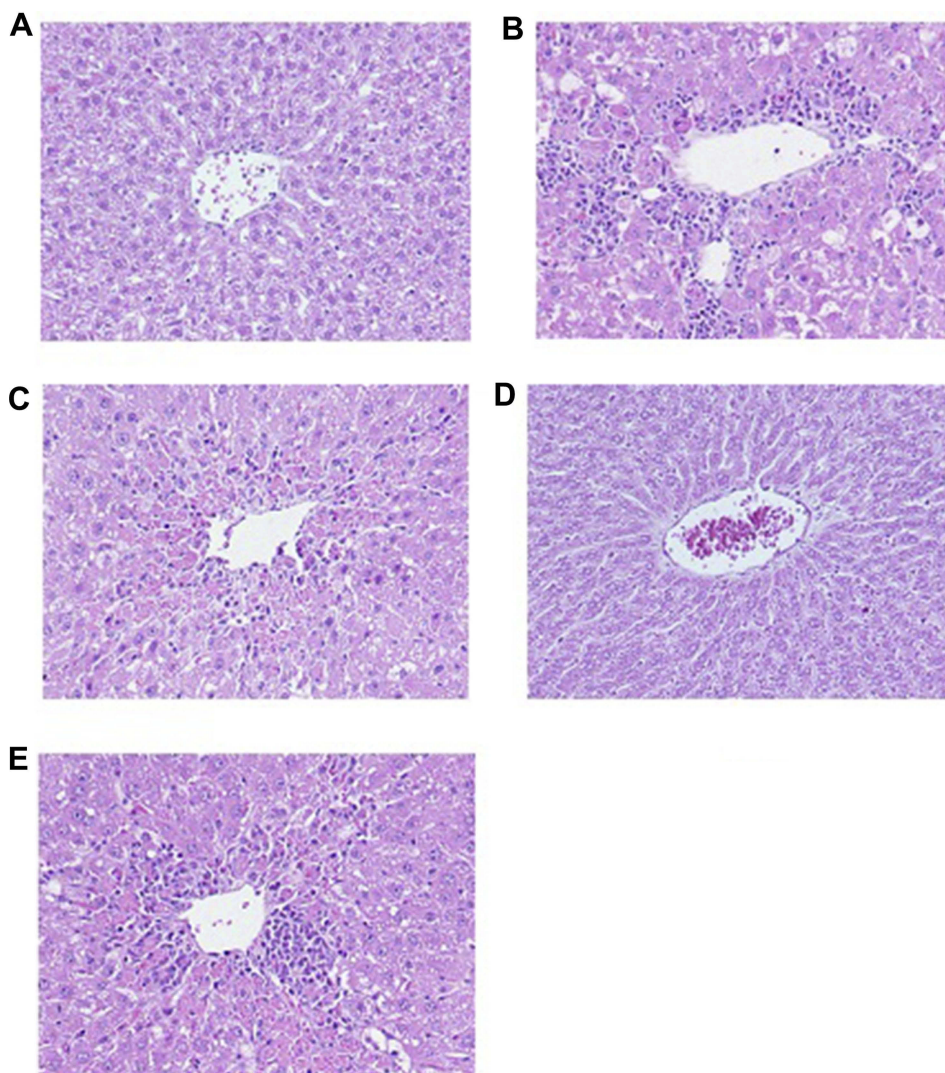


Figure 3 Light micrographs showing the effect of compound **2** on CCl₄-induced hepatotoxicity in rats. (A) Normal hepatocytes. (B) CCl₄-induced severe necrosis and inflammation. (C) Pretreatment of rats with compound **2** (10 mg/kg). (D) Pretreatment of rats with compound **2** (20 mg/kg). (E) Pretreatment of rats with silymarin (10 mg/kg). Magnification × 40.

dose-dependent protection of the liver against CCl₄ (Figure 3C and D). The rats treated with silymarin demonstrated normal hepatocytes (Figure 3E). The rats that

received compound **2** (10 and 20 mg/kg/day) as oral pretreatment exhibited a marked development in liver hepatocytes.

Structure activity relationship (SAR)

All the compounds were found to be active in antihepatotoxic activity. The substitutions at the phenyl ring of the dihydropyrimidinone moiety plays a significant role for the antihepatotoxic activity. The compounds **2** (R=4-NO₂-C₆H₄), **8** (R=2-OCH₃-C₆H₄) and **6** (R=4-OC₂H₅-C₆H₄) were found to be potent antihepatotoxic agents, while as the compounds **5** (R=3-OCH₃-C₆H₄), **7** (R = C₆H₅), **10** (R=3,4-(OCH₃)₂-C₆H₃) and **5** (R=3-OCH₃-C₆H₄) were found to be moderately active as antihepatotoxic agents. The least active compounds were **1** (R=4-Cl-C₆H₄), **3** (2-NO₂-C₆H₄) and **4** (R=3-NO₂-C₆H₄). The compound **2** having nitro substitution at the para position of the phenyl ring of the dihydropyrimidinone moiety was found to be the most highly potent compound of the series. From the in vitro data of antihepatotoxic activity of these compounds, it was found that both the moieties ie, dihydropyrimidinone and 1,4-benzodioxane are necessary for the activity.

Discussion

A series of compounds containing dihydropyrimidinone and 1,4-benzodioxane hybrid (**1–10**) were synthesized with high yield and purity. The compounds were synthesized from the enamionone, which is the preparatory material for the synthesis of various heterocyclic compounds of the pharmaceutical interest. The synthesized compounds were characterized by the modern spectral data which include FT IR, ¹H NMR, ¹³C NMR and mass spectroscopy. Initially all the synthesized compounds were evaluated for hepatoprotective activity against HepG2 cells. Compound **2** was found to be highly effective compared to the standard drug silymarin. The compound **2** was also checked for cytotoxicity against noncancer cell line MCF-10 A and was found to be safe. No toxicity was reported up to 1,000 mg/kg in acute toxicity in vivo test of the compound **2**. The compound was further evaluated in vivo for antihepatotoxic activity. For the evaluation of antihepatotoxic activity a CCl₄-induced hepatotoxicity model was used. CCl₄ is a toxic agent metabolized by the liver NADPH-cytochrome p450 enzyme to trichloromethyl free radical [CCl₃][•], which is highly reactive and produces damage to the liver.³⁰ It showed significant decrease in all the enzyme levels in animals intoxicated with CCl₄. It also significantly lowered the different types of glycerides and cholesterol. Reductions in MDA levels and increase in the NP-SH and total protein were observed. Levels of liver enzymes SGPT and SGOT were also decreased. Compound **2** produced dose dependent protection of the liver hepatocytes against CCl₄. Both biochemical parameters and histopathological study

results confirm the antihepatotoxic activity of test compound **2**, in CCl₄-induced hepatotoxicity rat model. Further studies are needed to establish the mechanism underlying the hepatoprotective activity of the test compound.

Conclusion

In conclusion, a series of novel dihydropyrimidinone derivatives containing 1,4-benzodioxane moiety were designed and synthesized in good yield as pure compounds. All the synthesized compounds were identified and confirmed by elemental analysis and modern spectroscopic methods. Compound **2**, 4-(4-nitrophenyl)-5-(2,3-dihydro-1,4-benzodioxin-6-ylcarbonyl)-3,4-dihydropyrimidin-2(1H)-one, was found to be a highly potent antihepatotoxic in CCl₄-induced hepatotoxicity rat model.

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Disclosure

The authors report no conflicts of interest in this work.

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