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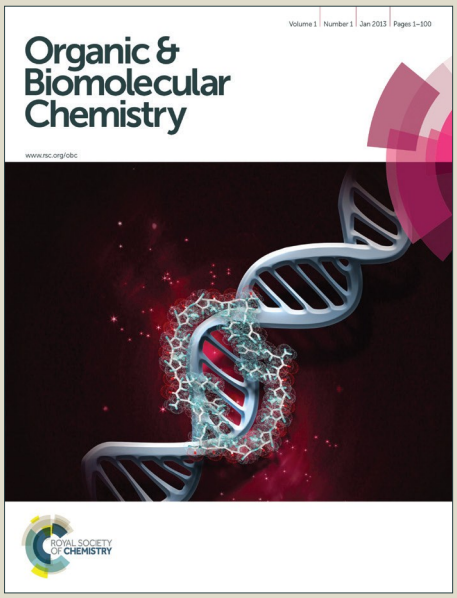

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ARTICLE

The design, synthesis, and biological evaluation of novel YC-1 derivatives as potent anti-Hepatic fibrosis agents

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1-benzyl-3-(substituted aryl)-5-methylfuro[3, 2-c]pyrazoles (**YC-1**) is a well-known synthetic compound with various satisfactory pharmacological activities, such as the activation of soluble gualylate cyclase (sGC) and the inhibition of hypoxia-induced factor-1α (HIF-1α). Recently, **YC-1** has been demonstrated to have a potent activity on anti-fibrotic. However, the mechanism underlying its anti-fibrotic activity is still largely unknown. To this end, we presented here the design and synthesis of **YC-1** and its novel derivatives, as well as the evaluation of their anti-fibrotic effects on activated human hepatic stellate cells (HSCs) LX-2. Moreover, the possible underlying mechanism of anti-fibrotic was also investigated for the first time by means of CCK-8 assay, cell apoptosis analysis, and western blot. Our study revealed that **YC-1** and its derivatives suppressed activated LX-2 cell viability and induced cell apoptosis in a time and dose-dependent manner. Western blot data demonstrated that these derivatives not only decreased the expression of α-smooth muscle actin (α-SMA), but also increased the expression of caspase-3, resulting in cell apoptosis. These findings strongly indicated that **YC-1** and its derivatives, especially **AC** could significantly inhibit LX-2 cell activation and induce LX-2 cell apoptosis by inhibiting α-SMA protein expression and promoting caspase-3 expression, respectively. In summary, our findings suggested that **YC-1** derivatives might be potential agents for hepatic fibrosis therapy.

Introduction

1-benzyl-3-(5'-hydroxymethyl-2'-furyl) indazole (**YC-1**) is a synthetic compound that has been demonstrated with various potent biological and pathological activities. For example, **YC-1** was first developed as an activator of soluble gualylate cyclase (sCG), and played an important role in inhibiting platelet aggregation, vascular contraction, and ATP release.[1, 2] After then, various biological functions and pharmacological actions of **YC-1** have been achieved to date. These discrete actions include antiplatelet activity,[3] sGC activity,[4] suppression of hypoxia-induced factor-1α (HIF-1α),[5-7] and anticancer activity.[8-11] In addition, **YC-1** also exhibited an anti-proliferative effect through arresting the cell cycle in the G_0-G_1 phase in hepatocellular carcinoma cells.[12] Many evidences suggested that **YC-1** attenuated hypoxia-induced pulmonary arterial hypertension in mice[13] and induced lipolysis through a PKA pathway.[14] Beside above mentioned features of **YC-1**, latest research from our collaborated team indicated that **YC-1** could decrease pro-casepase-3 and nuclear factor kB (NF-kB) expression. It means that **YC-1** may play an important role in controlling liver fibrosis by regulating the apoptosis of HSC. Nevertheless, the mechanism underlying its anti-fibrotic activity is still largely unknown.

Based on the previous studies, it is known that liver fibrosis is a dynamic process that results from an imbalance between the production and dissolution of the extracellular matrix. It is caused by a variety of chronic stimuli, including viral, autoimmune, drug induced, cholestatic and metabolic diseases.[15-17] Generally speaking, activation of hepatic stellate cells (HSCs) is recognized as the primary cellular source of matrix components in chronic liver disease as it plays a critical role in the development and maintenance of liver fibrosis.[18] Upon activation, HSCs change their phenotype into myofibroblast-like cells along with increasing production of extracellular matrix (ECM) components (such as α-SMA), and up-regulating expression of several plasma membrane receptors (such as PDGF-receptors).[19] Therefore, apoptosis of activated HSC could effectively control fibrosis in the liver. In such an apoptosis process, the mitochondrial-induced apoptosis members of the bcl-2 and bax families, and the casepase families (such as caspase-3), are always involved.[20-23]

Taking these factors into account, we hypothesized that **YC-1** and its analogues may also provide an effective effect for antifibrosis of HSCs through the similar pathways (inhibiting the activation or inducing activated apoptosis of HSCs). To verify our above mentioned hypothesis, we presented herein the design and synthesis of **YC-1** and its three novel derivatives (Fig. 1). For the first time, we explored their capability of anti-

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hepatic fibrosis and the corresponding underlying mechanism. Our findings indicated that all derivatives significantly inhibited LX-2 cell activation and induced LX-2 cell apoptosis by inhibiting α-SMA protein expression and promoting pro-apoptotic protein caspase-3 expression, respectively. Moreover, we also demonstrated the structure-activity relationship of **YC-1** analogues. It is found that bulky substituted benzyl group is of significant benefit for anti-hepatic fibrosis. Compared with **YC-1** and other derivatives, **AC** has the strongest capability of preventing the development of liver fibrosis, and thus could be treated as a potent anti-hepatic fibrosis agent.

Results and discussion

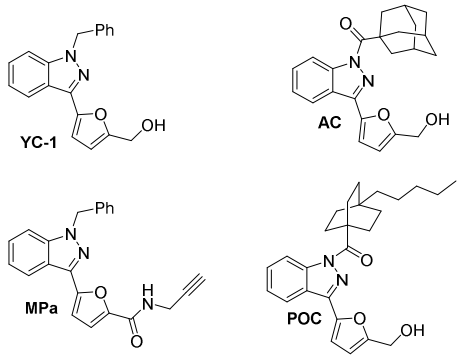


Figure 1. The structure of **YC-1** and its analogues.

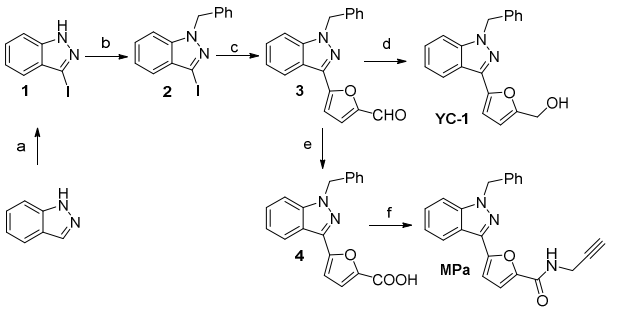
Design concepts

YC-1 has been well demonstrated with various biological activities.[24] To further investigate the structure-activity relationship of **YC-1**, its synthetic analogues through chemical modification are urgently needed. Generally, the structural modifications of **YC-1** are mainly focused on the benzyl or hydroxymethyl moiety.[25] Previous studies shown that furan moiety was considered as an optimal heterocyclic pharmacophore for its capability of potent HIF-1 inhibition.[7] Meanwhile, when the benzyl or hydroxymethyl group was altered appropriately, their activity toward sGC, antiplatelet activities and HIF-1 inhibitory activity were maintained or improved.[26-28] These **YC-1** analogues have guided us in developing activity-based skeleton for the investigation of the anti-fibrotic.

Considering these factors, we set to introduce bulky substituents containing carbonyl group in the benzyl position of **YC-1**. Their corresponding structures are documented in Fig.1. It is well known that adamantine is often used in modification of many bioactive compounds in which it is viewed as providing just the critical lipophilicity for known pharmacophores. The steric bulk of adamantane modifications were chosen to not only enhance lipophilicity and stability of the drugs; but also restrict or modulate intramolecular reactivity; and impede the access of hydrolytic enzymes, thereby improving their pharmacokinetics and plasma half-life.[29, 30] Hence, adamantane containing derivative **AC** was designed and synthesized. In addition, the introduction of proper alkyl chain can not only add drug bulk, but also increase drug's lipid solubility and membrane permeability, thereby improving drug absorption and bioavailability. To this end, 4-pentylbicycle[2.2.2]-octane containing derivative 1-(4'-pentylbicyclo[2.2.2]octan-1'-formoxyl)-3-(5'-hydroxymethyl-2'-furyl) indazole (**POC**) was synthesized. Moreover, the molecular shape and electrostatic distribution of **YC-1** play a crucial role in enzyme recognition and contribute extensively to binding affinity.[31] To further investigate the biological activity of hydroxymethyl moiety of **YC-1** and the electrostatic distribution of 3-furylindazole skeleton, we induced an acylamino group to substitute hydroxymethyl moiety. We expected that such a modification could not only increase interactions and hydrogen bondings, but also improve their water solubility. Therefore, acylamino group containing derivative 5-(1-benzyl-1H-indazol-3-yl)-N-(prop-2-yn-1-yl) furan-2-carboxamide (**MPa**) was synthesized. Moreover, **MPa** was designed with a premeditated terminal alkynyl group that can be used for tracking through cell imaging by click reaction.

Synthesis

The synthetic routes of **YC-1** and its derivatives are summarized as follows (Scheme 1-3). The commercially available material 1H-indazole reacted with iodine and potassium hydride in DMF to give the key intermediate compound 3-iodoindazole **1**. Then **1** was treated with benzyl bromide or appropriate acyl chloride in the presence of potassium tert-butoxide in absolute THF to give the corresponding 3-iodo-1-substituted-1H-indazoles **2** in high yields. And then Suzuki–Miyaura coupling of **2** with (5-formylfuran-2-yl)boronic acid proceeded in the presence of palladium catalyst ($Pd(PPh_3)_4$, 5 mM%) and Na_2CO_3 in dimethyl formamide (DMF) at 80 °C to give the corresponding 5-formylfuranyl-indazoles **3**. The formyl group of **3** was reduced by sodium borohydride in methanol to give **YC-1**. **AC** and **POC** were synthesized in the same methods. The formyl group of **3** was oxidized by potassium permanganate in acetone and water (v/v, 1:1) at 0°C to give oxidation product **4** which reacted with propynylamine could give **MPa** in 45% yield.



Scheme 1. The synthetic routes of **YC-1** and **MPa**. Reagents and conditions: (a) KOH, I_2, DMF, rt.; (b) benzyl bromide, t-BuOK, THF, rt.; (c) (5-formylfuran-2-yl) boronic acid, 5 mM% $Pd(PPh_3)_4$, Na_2CO_3, DMF, 80-90°C; (d) $NaBH_4$, MeOH, rt.; (e) $KMnO_4$, H_2O, Me_2CO, 0°C; (f) Propynylamine, EDC·HCl, HOBt, DIPEA, DMF, rt..

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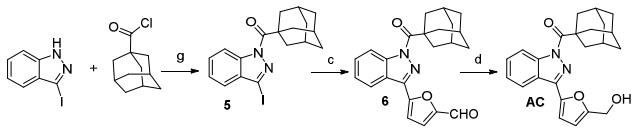
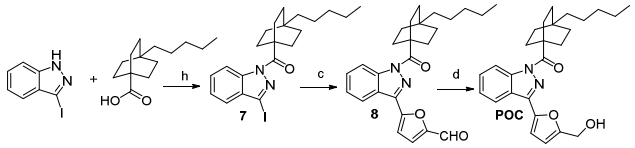


Scheme 2. The synthetic routes of **AC**. Reagents and conditions: (g) t-BuOK, THF, rt.; (c) (5-formylfuran-2-yl) boronic acid, 5 mM % $Pd(PPh_3)_4$, Na_2CO_3, DMF, 80-90 °C; (d) $NaBH_4$, MeOH, rt..



Scheme 3. The synthetic routes of **POC**. Reagents and conditions: (h) 1. $SOCl_2$, DCM, 0 °C, 100%; 2. t-BuOK, THF, rt.; (c) (5-formylfuran-2-yl)boronic acid, 5 mM % $Pd(PPh_3)_4$, Na_2CO_3, DMF, 80-90 °C; (d) $NaBH_4$, MeOH, rt..

Biological evaluation

Inhibitory effect on cellular viability of LX-2

To evaluate the anti-fibrotic effects of the **YC-1** analogues. LX-2 cells (a stable and unlimited source of human HSC) were employed as they have been extensively characterized as a valuable cell-based model for studies of human hepatic fibrosis.[32] Firstly, the viability of LX-2 cells following respective treatment with **YC-1**, **AC**, **POC**, and **MPa**, were investigated by CCK-8 assay. After direct pretreatment with different concentrations of **YC-1** and its analogues (0, 5, 25, 50, and 100 μM) for 24h, respectively, living LX-2 cells were found to be decreased in a dose-dependent manner (Fig. 2a). The viability rate at the concentration of 100 uM was measured as 42.2%, 32.0%, 33.5%, and 60.0%, respectively. This result suggested that **YC-1**, **AC**, **POC**, and **MPa** exhibited cytotoxicity to LX-2 cells and could induce LX-2 cells apoptosis. Moreover, in the case of **AC** and **POC**, The concentration at 25 μM exhibited a significant cytotoxicity towards LX-2 cells compared with **YC-1**. Next, in order to investigate whether the doses showing toxicity in HSC LX-2 cells are also toxic in non-liver cell types, we checked CCK-8 assay with Hela cells under the same conditions. As shown in Fig.2b, both **YC-1** and its analogues showed a certain but much weaker cytotoxicity towards Hela cells compared with LX-2 cells. The cell viability after treatment with **YC-1** and its analogs were 72.7%, 68.7%, 91.1%, 69.8%, respectively.

The effect on LX-2 apoptosis

HSC apoptosis is thought to be essential for the resolution phase of fibrosis. Thus, induction of HSC apoptosis would be expected to be with anti-fibrogenic effect by inhibiting the accumulation of the activated HSCs within the liver.[33] From the cell viability assay data, we observed that **YC-1**, **AC**, **POC** and **MPa** at a concentration level of 100 uM significantly induced cell death. Thus, we hypothesized that **YC-1** and its derivatives perhaps also induce cell apoptosis. To this end, we next examined the effects of **YC-1** analogues on LX-2 apoptosis by using PI and FITC conjugated annexin-V staining. PI-positive cells and FITC-positive/Pi-negative cells were measured by flow cytometry. First, LX-2 exhibited significant morphology alteration following treatment of 25 uM **YC-1** and its analogues at 18h. As shown in Fig. 3A inset, cell shrinkage and plasma membrane blebbing were distinctly observed. The cell death rate after treatment with 25 uM of **YC-1**, **AC**, **POC** or **MPa** was determined as 14.7%, 22.9%, 20.1% and 29.4%, respectively (Fig. 3B light gray bar). While lengthening the exposure time from 24 h to 48 h, the cell death rate was increased (32.7%, 53.9%, 36.0%, 32.2%, respectively, Fig. 3C dark gray bar). After FITC conjugated annexin-V/PI double staining, the average data of early apoptosis rate was measured as 29.5%, 32.1%, 28.9%, 28.4% and later apoptosis rate was detected as 5.5%, 11.8%, 7.6%, 7.4%, respectively (Fig.3C, the raw flow cytometry data shown in figure S1). These results were in keeping with the PI simple staining. Therefore, these data indicated that **YC-1**, **AC**, **POC**, and **MPa** could induced LX-2 apoptosis in a time-dependent manner.

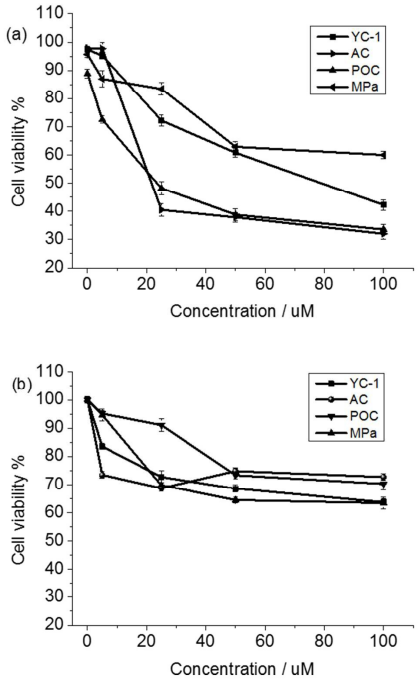


Figure 2. Inhibitory effect of **YC-1** and its analogues on LX-2 (a) or Hela (b) cell viability measured by the CCK-8 assays. Cells were incubated with **YC-1**, **AC**, **POC**, and **MPa** at different concentrations (0, 5, 25, 50, and 100 μM) in DMEM for 24 h, respectively. Data were presented as mean ±S.D. of three independent experiments with n = 3.

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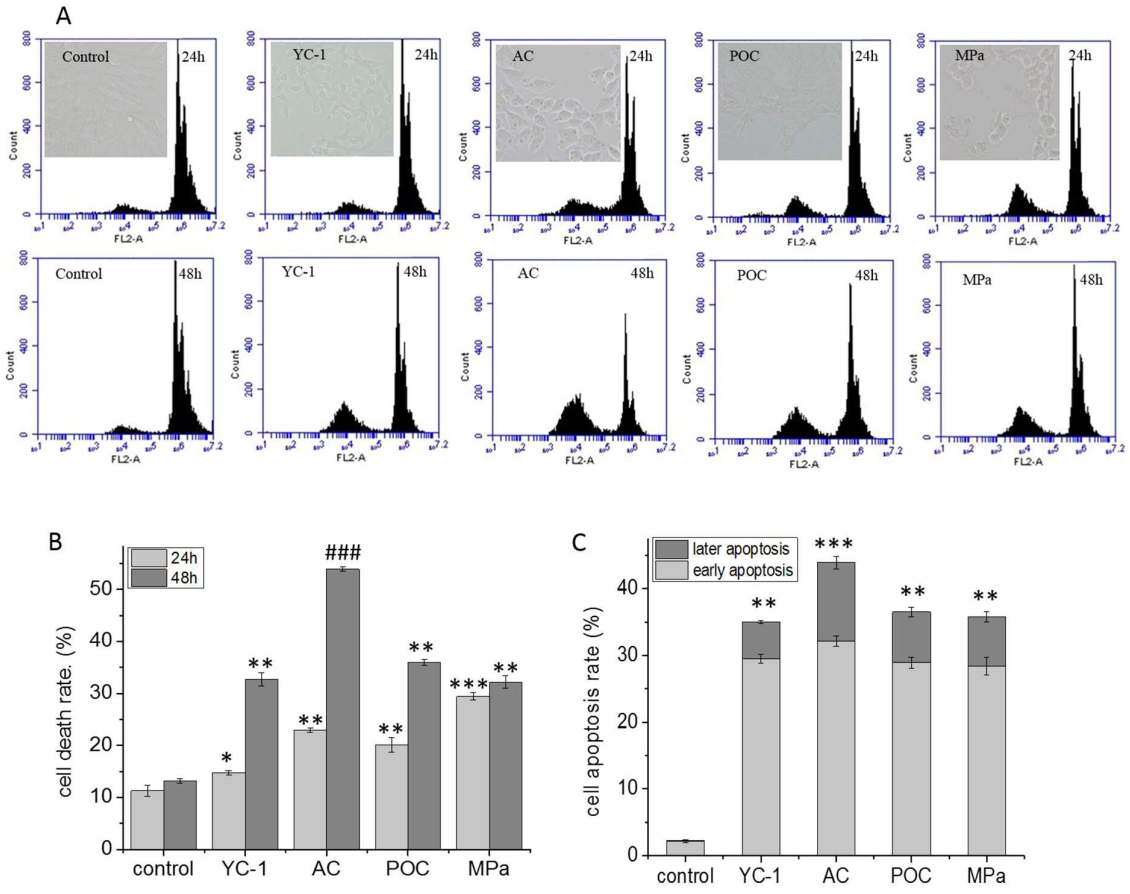

Figure 3. The effects of **YC-1**, **AC**, **POC** and **MPa** on LX-2 apoptosis. Activated LX-2 (2 ×10^5 cells/well in 6-well plate) were incubated with 25 uM **YC-1**, **AC** and **MPa** for 24 h or 48 h, respectively. Cellular morphology were observed by confocal microscope at 18 h (A inset). Cells were then harvested and incubated with propidium iodide (PI) (A) or FITC conjugated annexin-V and PI. PI-positive cells (B) and FITC-positive/PI-negative cell (C) were measured by flow cytometry. Data are presented as mean ± standard deviation (SD) (n = 3/group).*P < 0.05, **P < 0.01, ***P< 0.001, compared with the control group, ###P < 0.001, significantly different compared with **YC-1**.

The effect on the levels of α-SMA expression

The activation of HSC cells is considered as one of the central pathophysiological mechanisms of liver fibrosis, and HSC activation is characterized by the overexpression of a smooth muscle actin (α-SMA) and collagen I. Herein, we measured α-SMA expression by western blot assay. We found that the level of α-SMA protein underwent a significantly down-regulation after treatment with 25 uM **YC-1**, **AC**, **POC,** and **MPa** respectively for 3 h (Fig. 4). The results suggested that **YC-1** and its derivatives could inhibit α-SMA protein expression in a time-dependent manner. In summary, **YC-1** and its analogues may inhibit the activation of HSCs, then prevent the development of liver fibrosis.

The effect on the levels of caspase-3 expression

During the cell apoptosis assay, we found that **YC-1** and its analogues could induce LX-2 cell apoptosis. Such an apoptosis is believed to be caused by the pro-apoptosis protein-caspase-3.[34] Herein, we detected the expression of pro-apoptosis protein caspase-3 by western blot assay. Afther pretreatment of LX-2 cells with 25 uM **YC-1** and its analogues for 12 h, the cleaved substrate of caspase-3 (it was not observed when treated for 3 h or 6 h) was obviously detected (Fig. 5). Compared with the control group, the protein level of cleaved

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caspase-3 were obviously increased, especially for the compound **AC,** which indicated that **AC** was more potency than **YC-1**. These results indicated that **YC-1** and its analogues **AC**, **POC**, **MPa** could promote caspase-3 expression. And the effects of **YC-1** and its analogues on LX-2 cell apoptosis maybe depended on caspase-3 expression and cleavage.

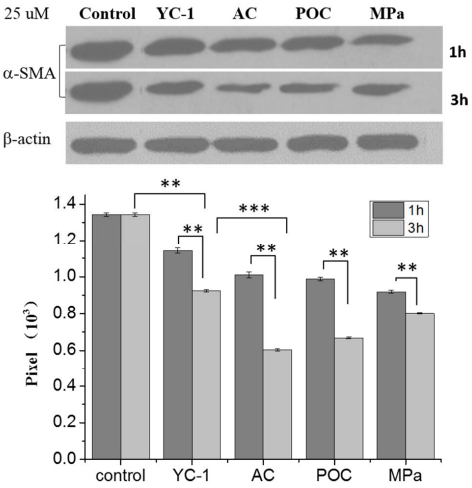

Figure 4. Effects of **YC-1**, **AC**, **POC**, and **MPa** on α-SMA expression. LX-2 cells were pretreated with or without 25 uM **YC-1**, **AC**, **POC**, and **MPa** for 1 h or 3 h, respectively. α-SMA proteins were detected via western blotting with specific antibodies. β-Actin was used as a loading control. Data are presented as mean ± standard deviation (SD) (n = 3/group). ** p < 0.01, ***p < 0.001.

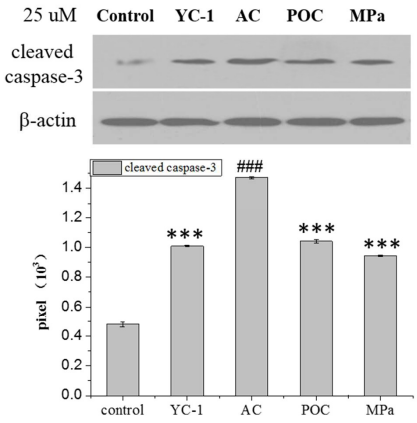

Figure 5. Effects of **YC-1**, **AC**, **POC**, and **MPa** on caspase-3 expression. LX-2 cells were pretreated with or without 25 uM **YC-1**, **AC**, **POC** and **MPa** for 12 h, respectively. Caspase-3 proteins were detected via western blotting with specific antibodies. β-Actin was used as a loading control. Data are presented as mean ± standard deviation (SD) (n = 3/group). ***p < 0.001, compared with control group, ###p < 0.001, compared with **YC-1**.

The relationship between chemical structures and bioactivity

With these bioactivity data in our hand, we further summarized and discussed here the structure-bioactivity relationship between **YC-1** and its derivatives. According to the results of CCK-8 assay and the cell apoptosis assay, it is clear that **YC-1**, **AC**, **POC**, and **MPa** could be useful for anti-hepatic fibrosis by inhibiting the activation and proliferation of HSCs or inducing activated HSCs apoptosis. In addition, from the cell apoptosis assay data we found that **AC** was more potent efficient than **YC-1** on inducing activate LX-2 cell apoptosis. This may attributed to the introduction of bulky substituents adamantane that has been found to increase drug-like qualities of a lead compound. From what has been discussed above, we can concluded that the introduction of bulky substituents to the benzyl group was benefit for inhibiting LX-2 cell activity which is closely associated with anti-liver fibrosis. Besides, hydroxymethyl moiety of **YC-1** was efficient but not prerequisite for anti-liver fibrosis. Moreover, the introduction of conjugated carbonyl may be useful for improving the analogues biological activity.

Conclusions

We designed and synthesized **YC-1** and its three derivatives **AC**, **POC**, and **MPa**. Their anti-fibrosis activity and the corresponding underlying mechanism were investigated in detail for the first time. Our findings indicated that all of them showed significant inhibition of LX-2 cell activation through inhibiting α-SMA protein, and induction of LX-2 cell apoptosis through promoting caspase-3 expression. More important, we also demonstrated that bulky substituted benzyl group is of significant benefit for anti-hepatic fibrosis. All **YC-1** derivatives especially **AC** has the capability of preventing the development of liver fibrosis, and thus could be treated as a potent anti-hepatic fibrosis agent. Further studies on the effects of **YC-1** analogues associated proteins is still undergoing in our lab. Such a research can supply further understanding about molecular and cellular mechanisms responsible for their inhibitory effects on liver fibrosis, which can provide importance basis for the development of new efficient anti-Hepatic Fibrosis agent.

Experimental section

Materials and general methods

All chemical reagents were purchased from commercial suppliers (Aladdin Industrial Corporation or J&K Scientific, Beijing, China); Human HSC LX-2 cell line was graciously provided by Professor Nan from college of pharmacy, Yanbian University (Jilin province, China). The biological reagents Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.25% Trypsin and penicillin streptomycin were purchased from Gibco (USA). Cell counting kit-8 (CCK-8), dimethyl sulfoxide (DMSO), Annexin V-FITC apoptosis detection kit, Prodium Iodide (PI), α-smooth muscle actin (α–SMA) antibody, caspase-3 antibody, anti-β-actin antibodies, anti-rabbit IgG antibody conjugated with horseradish peroxidase were obtained from Cell signaling technology Inc. (USA). Western blot detection reagents enhanced chemiluminescence (ECL) and nitrocellulose (NC) membrane were purchased from Millipore (USA). Proton nuclear magnetic resonances (^{1}H NMR) and ^{13}C NMR spectra were recorded in deuterated solvents on a Brucker (300 MHz) NMR

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spectrometer. Chemical shifts are reported in parts per million (ppm, δ). ^{1}H NMR splitting patterns are designated as singlet (s), doublet (d), triplet (t), and quartet (q). Splitting patterns that could not be interpreted or easily visualized were recorded as multiplet (m) or broad (br). MALDI-TOF mass spectra were recorded by using a Shimadzu MALDI AXIMA-CFR+ spectrometer. Analytical thin layer chromatography (TLC) was performed on a glass plates of silica gel 60 GF254 (Qingdao Haiyang chemical Co., Ltd, China) with visualization accomplished with phosphomolybdic acid, iodine, or with a UV-visible lamp. Column chromatography was conducted on silica gel (Qingdao Haiyang chemical Co., Ltd, 100-200 mesh). Most reagents were purchased from commercial suppliers and used without further purification. Some solvents were purified and dried by standard methods prior to use: tetrahydrofuran (THF) was distilled from sodium/benzophenoneketyl. All the end-products finally were further purified by reversed-phase HPLC.

Synthesis

3-iodoindazole (1): Iodine (0.86 g, 3.4 mM) and potassium hydroxide pellets (0.2 g, 3.4 mM) were successively added into a DMF solution (5 ml) of indazole (0.2 g, 1.7 mM) at room temperature under stirring. After 2 h, the reaction mixture was poured into 10% aqueous $NaHSO_3$ (100 ml) and extracted with Et_2O (3 x 150 ml). The combined organic layers were washed with water and brine, dried (anhydrous Na_2SO_4), filtration and the solvent evaporated under reduced pressure to give **1** as white solid (0.410 g, yield 99.2%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.12 (s, 1H), 8.25 (dd, J = 7.6, 1.8 Hz, 1H), 7.46 (dt, J = 7.8, 2.1 Hz, 1H), 7.41 (dd, J = 7.8, 2.1 Hz, 1H), 7.36 (dd, J = 7.0, 1.8 Hz, 1H); Calc. for $C_7H_5IN_2$: 244.03, Tof-MS found: 245.2.

General Procedure for Synthesis of 2, 5, 7: To a solution of 3-iodoindazole **1** (0.201 g, 0.82 mM) in anhydrous THF (6 ml) cooled at 0 °C was added potassium tert-butoxide (0.141 g, 1.30 mM). After 1 h at 0°C, benzyl bromide or appropriate acyl chloride (approximately 0.1 ml, 0.82 mM) was added drop wise. The resulting mixture was stirred 4 h at room temperature then evaporated. The residue was dissolved with EA (50 ml), washed with water and brine, dried ($MgSO_4$) , filtration and the solvent evaporated under reduced pressure to give **2** as a light yellow oil (0.260g, yield 95%). 1-Benzyl-3-iodoindazole (**2**): ^{1}H NMR (300 MHz, $CDCl_3$) δ7.51(dd, *J* = 8.1, 1.2Hz, 1H), 7.40 (td, *J* = 8.1, 1.2Hz, 1H), 7.36-7.29(m, 4H), 7.26 - 7.19 (m, 3H), 5.63 (s, 2H); Calc. for $C_{14}H_{11}IN_2$:334.16, Tof-MS found: 334.6.

1-Adamantaneformoxyl-3-iodoindazole (5): Yield 98%; a white solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (dd, *J* = 8.4, 1.2Hz, 1H), 7.61 (td, *J* = 8.4, 1.2Hz, 1H), 7.51 (dd, *J* = 7.5, 0.6Hz, 1H), 7.42 (td, *J* = 7.5, 0.6Hz, 1H), 2.38 (d, *J* = 2.4Hz, 6H), 2.15(s, 3H), 1.85(s, 6H); Calc. for $C_{18}H_{19}IN_2O$: 406.26, Tof-MS found: 406.8.

1-(4'-pentylbicyclo[2.2.2]octan-1'-formoxyl)-3-iodoindazole (7): Yield 95%; a white solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (dd, *J* = 8.4, 1.3 Hz, 1H), 7.61 (td, *J* = 8.4, 1.3 Hz, 1H), 7.50 (dd, *J* = 8.1, 0.9 Hz, 1H), 7.41 (td, *J* = 8.4, 0.9 Hz,1H), 2.29-2.16 (m, 6H), 1.60-1.46 (m, 6H), 1.33-1.15 (m, 8H), 0.91 (t, *J* = 6.9 Hz, 3H); Calc. for $C_{21}H_{27}IN_2O$: 450.36, Tof-MS found: 450.9.

General Procedure for Synthesis of 3, 6, 8: To a mixture of 1-benzyl-3-iodoindazole **2** or **5** or **7** (0.334 g, 1.0 mM) and $Pd(PPh_3)_4$ (0.08 g, 0.07 mM) in DMF (5 ml), then (5-formylfuran-2-yl) -boronic acid (0.168 g, 1.2 mmol) was added followed by the addition of sodium carbonate (0.318 g, 3.0 mM). The reaction mixture was refluxed with vigorous stirring under nitrogen atmosphere and the rate of the reaction was followed by TLC. After the starting materials were consumed, the reaction mixture was filtered, the filtrate was poured into water and extracted with DCM (3×50 ml). The combined DCM layers were washed with water and brine, dried (anhydrous Na_2SO_4), filtration and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (silica gel, ethyl acetate/Petroleum ether 1 : 4) to give 3 as an orange solid (0.182g, yield 60 %). 5-(1'-benzyl-1H-indazol-3'-yl) furan-2-carbaldehyde (**3**): ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.77 (s, 1H), 8.30 (d, *J* = 8.1Hz, 1H), 7.43 - 7.28 (m, 9H), 7.14 (d, *J* = 3.6Hz, 1H), 5.69 (s, 2H); Calc. for $C_{19}H_{14}N_2O_2$: 302.33, Tof-MS found: 302.3.

5-(1'-adamantaneformoxyl-1H-indazol-3'-yl)furan-2-carbaldehyde (6): Yield 49%; a light yellow solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.82 (s, 1H), 8.54 (dd, *J* = 8.4, 0.9 Hz, 1H), 8.35 (dd, *J* = 8.4, 0.9 HZ, 1H), 7.63 (td, *J* = 7.2, 0.9 Hz, 1H), 7.51(dd, *J* = 7.2, 0.9 Hz, 1H),7.46(d, *J* = 3.9 Hz, 1H), 7.29 (d, *J* = 3.9 Hz, 1H), 2.44 (s, 6H), 2.17 (s, 3H), 1.83 (s, 6H). Calc. for $C_{23}H_{22}N_2O_3$: 474.16, Tof-MS found: 374.2.

5-(4'-pentylbicyclo[2.2.2]octan-1'-formoxyl-1H-indazol-3'-yl)furan-2-carbaldehyde (8): Yield 54%; a light yellow solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.82 (s, 1H), 8.52 (dd, *J* = 8.7, 0.9 Hz, 1H), 8.34 (dd, *J* = 8.1, 0.9 Hz, 1H), 7.63 (td, *J* = 8.7, 0.8 Hz, 1H), 7.50 (dd, *J* = 8.1, 0.9 Hz,1H), 7.29 (d, *J* = 4.5 Hz, 1H), 2.40-2.19 (m ,6H), 1.60-1.46(m, 6H), 1.37-1.14 (m 8H), 0.91 (t, *J* = 6.9 Hz, 3H); Calc. for $C_{26}H_{30}N_2O_3$: 418.23, Tof-MS found: 418.2.

General Procedure for Synthesis of YC-1 and AC, POC: To a solution of **3** or **6** or **8** (0.12 g, 0.4 mM) dissolved in MeOH (8 ml) was added $NaBH_4$ (0.02 g, 0.5 mM). After stirred 1 h at room temperature, the resulting mixture was evaporated. The residue was dissolved with EA (20 ml), washed with water and saturated sodium bicarbonate, dried (anhydrous Na_2SO_4), filtration and the solvent evaporated under reduced pressure. Chromatography (silica gel, ethyl acetate/Petroleum ether 1:1) followed by recrystallization from hexane gave **YC-1** as a white solid (0.11 g, yield 92%). 1-benzyl-3-(5'-hydroxymethyl-2'-furyl)indazole (**YC-1**): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 (d, *J* = 8.4 Hz, 1H), 7.38-7.22 (m, 8H), 6.90 (d, *J* = 3.3 Hz, 1H), 6.51 (d, *J* = 3.3 Hz, 1H), 5.68 (brs, 2H), 4.77 (s, 2H), 1.92 (brs, 1H); Calc. for $C_{19}H_{16}N_2O_2$: 304.12, Tof-MS found: 304.2.

1-adamantaneformoxyl-3-(5'-hydroxymethyl-2'-furyl)indazole (AC): Yield 90%; a white solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.53 (dd, *J* = 8.4, 0.9 Hz, 1H), 8.20 (dd, *J* = 7.2, 0.9 Hz, 1H), 7.58 (td, *J* = 8.4, 0.9 Hz, 1H), 7.43 (td, *J* = 7.2, 0.9 Hz, 1H), 7.08 (d, *J* = 3.4 Hz, 1H), 6.55 (d, *J* = 3.4 Hz, 1H), 4.81 (s, 2H), 2.44 (d, *J* = 2.6 Hz, 6H), 2.16 (s, 3H), 1.94-1.72 (m, 7H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.64 (s), 155.26 (s), 148.11 (s), 141.39 (s), 140.07 (s), 129.48 (s), 124.69 (s), 122.57 (s), 121.51(s), 116.36(s), 110.17(s), 109.86(s), 57.76 (s), 44.56 (s), 38.79 (s, 3C), 36.79 (s, 3C), 28.41

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(s, 3C); Calc. for $C_{23}H_{24}N_2O_3$: 376.18, HRMS found: $[M+Na]^+$ 399.1677.

1-(4'-pentylbicyclo[2.2.2]octan-1'-formoxyl)-3-(5'-hydroxymethyl-2'-furyl)indazole (POC): Yield 67%; a white solid; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.51 (dd, *J* = 8.4, 1.2 Hz, 1H), 8.18 (dd, *J* = 7.8, 0.9 Hz, 1H), 7.58 (td, *J* = 8.4, 1.2 Hz,1H), 7.42 (td, *J* = 7.8, 0.9 Hz,1H), 7.07 (d, *J* = 3.3 Hz, 1H), 6.55 (d, *J* = 3.3 Hz, 1H), 4.81 (s, 2H), 2.36-2.22 (m, 6H), 1.88 (brs, 1H), 1.61-1.49 (m, 6H), 1.38-1.15 (m, 8H), 0.92 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.31 (s), 155.26 (s), 148.03 (s), 141.30 (s), 139.99 (s), 129.48 (s), 124.70 (s), 122.65 (s), 121.52 (s), 116.35 (s), 110.29 (s), 109.89 (s), 57.74 (s), 42.89 (s), 41.57 (s), 32.87 (s), 30.81 (s), 30.61(s, 3C), 28.64(s, 3C), 23.44 (s), 22.75 (s), 14.14 (s); Calc. for $C_{26}H_{32}N_2O_3$: 420.24, HRMS found: $[M+Na]^+$ 443.2299.

5-(1'-benzyl-1H-indazol-3'-yl)furan-2-carboxylic acid (4): To a solution of 3 (0.1 g, 0.33 mmol) in acetone (3 ml) was added a solution of $KMnO_4$ (0.06 g, 0.38 mM) in water (2 ml) at 0°C. The reaction mixture was stirred about 1 h and then filtered. The residue was washed and the filtrate was acidified with 1 M HCl, then extracted with EA (3×20 mL), the combined DCM layers were washed with water and brine, dried (anhydrous Na_2SO_4), filtration and the solvent was removed under reduced pressure. The crude product was purified by recrystallization from acetonitrile to give 4 as a light yellow solid (0.034 g, yield 32%). ^{1}H NMR (300 MHz, DMSO) δ 13.19 (s, 1H), 8.16 (d, *J* = 8.1 Hz, 1H), 7.82 (d, *J* = 8.4 Hz, 1H), 7.50 (td, *J* = 8.4, 0.9 Hz, 1H), 7.39 (d, *J* = 3.6 Hz, 1H), 7.36-7.25 (m, 6H), 7.19 (d, *J* = 3.6 Hz, 1H), 5.77 (s, 2H). ^{13}C NMR (75 MHz, DMSO) δ 159.76 (s), 151.82 (s), 144.50 (s), 140.84 (s), 137.52 (s), 134.78 (s), 129.12 (s, 2C)), 128.14 (s), 127.81 (s, 2C), 127.63 (s), 122.71 (s), 121.31 (s), 120.99 (s), 119.98 (s), 111.05 (s), 109.22 (s), 52.64 (s); Calc. for $C_{19}H_{14}N_2O_3$: 318.33, Tof-MS found: 318.8.

5-(1-benzyl-1H-indazol-3-yl)-N-(prop-2-yn-1-yl)furan-2-carboxamide (MPa): To a solution of 4 (0.030 g, 0.094 mM) in anhydrous THF (5 ml) was added EDC·HCl (0.022 g, 0.115 mM) under an argon atmosphere, followed by the addition of HOBT (0.013 g, 0.096 mM) and DIEA (10 ul). The reaction mixture was stirred for 30 min followed by addition of propynylamine (0.006 g, 0.1 mM). The resulting mixture was stirred 24 h at room temperature then saturated aqueous $NaHCO_3$ (20 ml) was added, and the mixture was extracted with EA (3×20 ml). The combined organic layers washed with water and brine, dried (Na_2SO_4), filtration and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (silica gel, ethyl acetate/ Petroleum ether 1 : 2) to give MPa as a white solid (0.047 g, yield 45%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.02 (d, *J* = 8.1 Hz, 1H), 7.49-7.39 (m, 2H), 7.38-7.23 (m, 6H), 7.02 (d, *J* = 3.6 Hz, 1H), 6.87 (d, *J* = 3.6 Hz, 1H), 5.69 (s, 2H), 4.31 (d, *J* = 2.5 Hz, 2H), 2.31 (t, *J* = 2.5 Hz, 1H), 1.98 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.93 (s), 150.22 (s), 146.59 (s), 140.62 (s), 136.32 (s), 135.15 (s), 128.85 (s, 2C), 128.00 (s), 127.18 (s), 127.12 (s, 2C), 122.10 (s), 121.48 (s), 120.98 (s), 116.77 (s), 109.97 (s), 109.40 (s), 79.40 (s), 71.77 (s), 53.40 (s), 28.88 (s); Calc. for $C_{22}H_{17}N_2O_2$: 356.39, HRMS found: 356.1382.

Biology

Cell Culture Conditions: LX-2 cells were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin and streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and the medium was changed every other day.

Cellular viability of LX-2: Cell viability was determined by CCK-8 assay. A total of 1×10^4 cells were seeded in 96 well plates for 24h. **YC-1**, **AC**, **POC** or **MPa** was dissolved in DMSO and reached the final culture concentration of 0.1% in 200 ul DMEM respectively. After treating with different concentrations (0, 5, 25, 50, and 100 μM) of **YC-1** (**AC**, **POC** or **MPa**) for 24h, 20 ul CCK-8 was added to the wells and incubated for additional 4h at 37 °C. The optical density of the dissolved material was measured at 450 nm. The assays were performed in three independent experiments with n = 3.

LX-2 apoptosis analysis: The apoptosis of cells were analyzed with Annexin V-FITC apoptosis detection kit and PI simple staining. Activated HSCs LX-2 cells were plate in 6-well plates 2×10^5 cells/well and cultured 24 h. Then treated with 25 uM **YC-1**, **AC**, or **MPa** respectively. Cellular morphology were observed by confocal microscope at 18 h. Cells were collected by trypsinization after 24 and 48 h, followed by repeated washes with PBS. Cells were then incubated with propidium iodide (PI) or FITC conjugated annexin-V and propidium iodide (PI) away from light for 20 min at room temperature. PI-positive cells and FITC-positive / Pinegative cells were analyzed using a Becton-Dickinson FAC Scan flow cytometer respectively.

Western blot analysis: Whole cell proteins were lysed on ice by RIPA lysis buffer with 1% phenylmethylsulfonyl fluoride (PMSF), centrifuged at 15,000 g for 15 min and the supernatant was collected. Protein concentration was measured using enhanced BCA protein assay kit. Equal amounts of the protein (60 μg/lane) were separated by SDS-PAGE and transferred to NC membrane. The membranes were blocked with 5% milk containing 0.1% Tween 20 for 1 h at room temperature. Blots were probed overnight at 4 °C with the following primary antibodies: anti-α–SMA and anti-β-actin antibodies at a dilution of 1:1000. This was followed by incubation with secondary antibody: anti-rabbit IgG antibody conjugated with horseradish peroxidase 1:5000 for 1 h. Immuno-detection was visualized using the ECL detection system according to the manufacturer's instructions.

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