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Design, synthesis and biological evaluation of benzamide derivatives as novel NTCP inhibitors that induce apoptosis in HepG2 cells

Shuangmei Zhao[a], Yongqi Zhen[a], Leilei Fu, Feng Gao, Xianli Zhou, Shuai Huang[*], Lan Zhang[*]

School of Life Science and Engineering, Southwest Jiaotong University, Chengdu, PR China

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ABSTRACT

Sodium taurocholate cotransport polypeptide (NTCP) plays an important role in the development of hepatitis and acts as a switch to allow hepatitis virus to enter hepatic cells. As the entry receptor protein of hepatitis virus, NTCP is also an effective target for the treatment of hepatocellular carcinoma. Herein, twenty-five benzamide analogues were synthesized based on the virtual screening design and their anti-proliferative activities against HepG2 cells were evaluated *in vitro*. Compound **35** was found to be promising, with an IC_{50} value of 2.8 μM. The apoptosis induced by **35** was characterized by the regulation of markers, including an increase in Bax, cleaved-caspase 3, and cleaved-PARP proteins, and a decrease in Bcl-2 protein. Molecular docking and molecular dynamics (MD) simulation confirmed that compound **35** can bind tightly to NTCP. Western blot analysis also showed that NTCP was inhibited. Altogether, these results indicate that compound **35** acts as a novel NTCP inhibitor to induce apoptosis in HepG2 cells.

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer-related death worldwide.[1,2] To date, various treatments have been used in HCC, including mainly radiotherapy, chemotherapy, biological immunotherapy, surgical resection and liver transplantation.[3,4] However, poor prognosis and high recurrence rates are still the main causes of death in HCC patients.[5] The occurrence and development of HCC are related to various factors, including aflatoxin intake and smoking,[6] alcoholic liver disease and non-alcoholic fatty liver disease,[7] hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, or other causes of cirrhosis.[8,9] Studies have shown that there is a close relationship between hepatitis virus infection and HCC, and in approximately 40% of patients, HCC was caused by viral hepatitis C.[10] Therefore, development and application of hepatitis virus-related targeted drugs may be an effective means for HCC treatment.

The sodium taurocholate cotransport polypeptide (NTCP) is the cardinal liver transporter that binds bile acids and is a functional receptor for human HBV and hepatitis D viruses (HDVs).[11] Jia and Xie et al. found that NTCP could mediate the uptake of bile acids in hepatoma cells, and the bile acid-microbial axis is formed when the bile acid ingested by NTCP increases, which regulates the occurrence and development of HCC.[12] When the S267F mutation occurs in NTCP, the HBV infection capacity is decreased, and the incidence of HBV infection-associated cirrhosis and HCC is reduced.[13] Taken together, NTCP is an important target for the treatment of HCC.

Currently, many old drugs have been identified as NTCP inhibitors and applied to the study of NTCP (Fig. 1). For instance, rosiglitazone, zafirlukast, TRIAC and sulfasalazine have shown good inhibitory activity against NTCP in 1280 screenings of clinically approved drugs.[11] Fluvastatin is an NTCP inhibitor, which interacts with NTCP at the level of the bile acid binding pocket.[14] Macitentan[15], bosentan[16] and irbesartan[17] were also shown to act as NTCP inhibitors.[18] Studies have indicated that fasiglifam could inhibit NTCP and affect HBV infection,[17] which also has severe hepatotoxicity.[19] Despite their NTCP inhibitory activities and anti-neoplastic properties in HCC, these inhibitors are associated with many side effects.[20–22] Thus, the development of efficient NTCP inhibitors is the primary goal for the treatment of HCC.

In this study, we discovered a novel NTCP inhibitor (compound **35**) that inhibits the expression of NTCP. Compound **35** exhibited excellent anti-proliferative activity and induced apoptosis in HepG2 cells. Our study shows that compound **35**, as an NTCP inhibitor, could be used as a candidate drug for future treatment of HCC.

To discover novel NTCP inhibitors, homology modeling was performed to obtain the structure of NTCP. We first screened for leading candidate compounds from the ZINC database according to Lipinski's Rule of Five (Fig. 2). Then, 10,000 compounds were selected using the LibDock protocol. Subsequently, the top 200 hits were further screened

* Corresponding authors at: School of Life Science and Engineering, Southwest Jiaotong University, NO. 111, North Second Ring Road, Chengdu 610031, Sichuan, PR China.

E-mail addresses: shuaih@swjtu.edu.cn (S. Huang), zhanglanx_9@126.com (L. Zhang).

[a] These authors contributed equally to this work and should be considered co-first authors.

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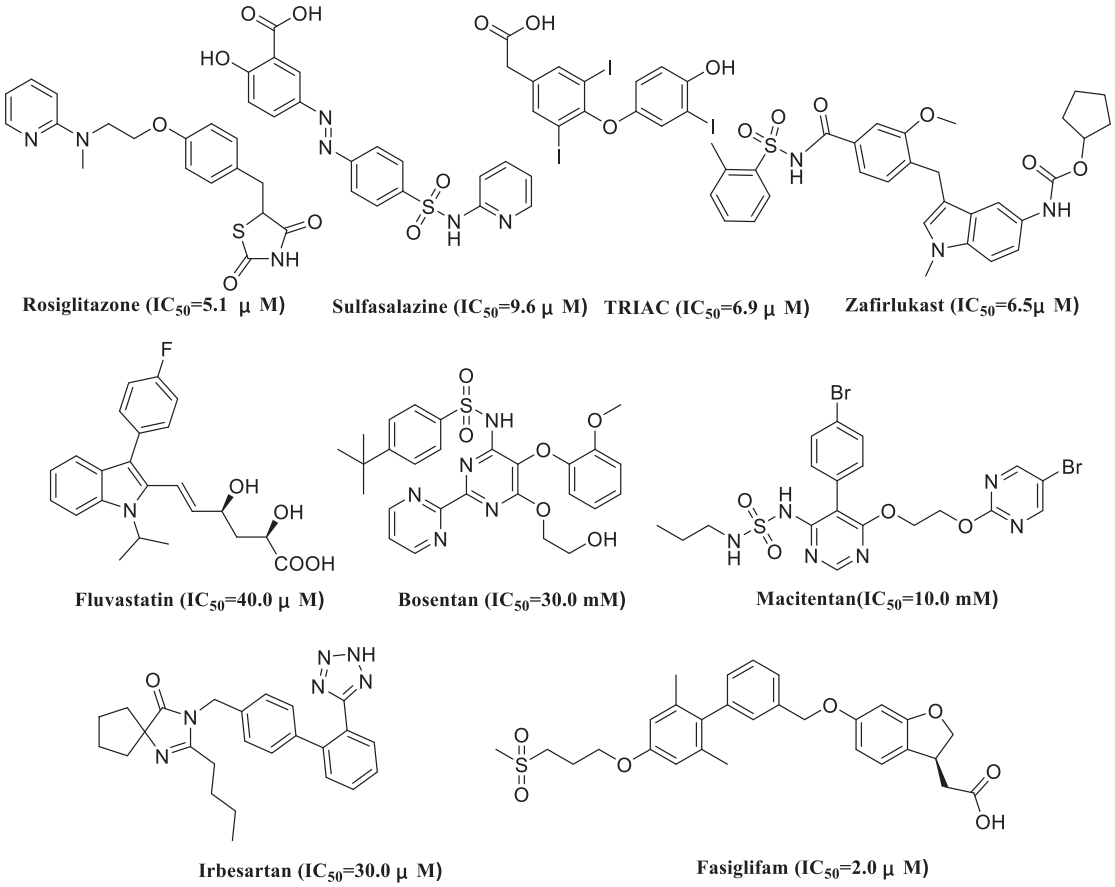


Fig. 1. The structures and activities of several NTCP inhibitors.

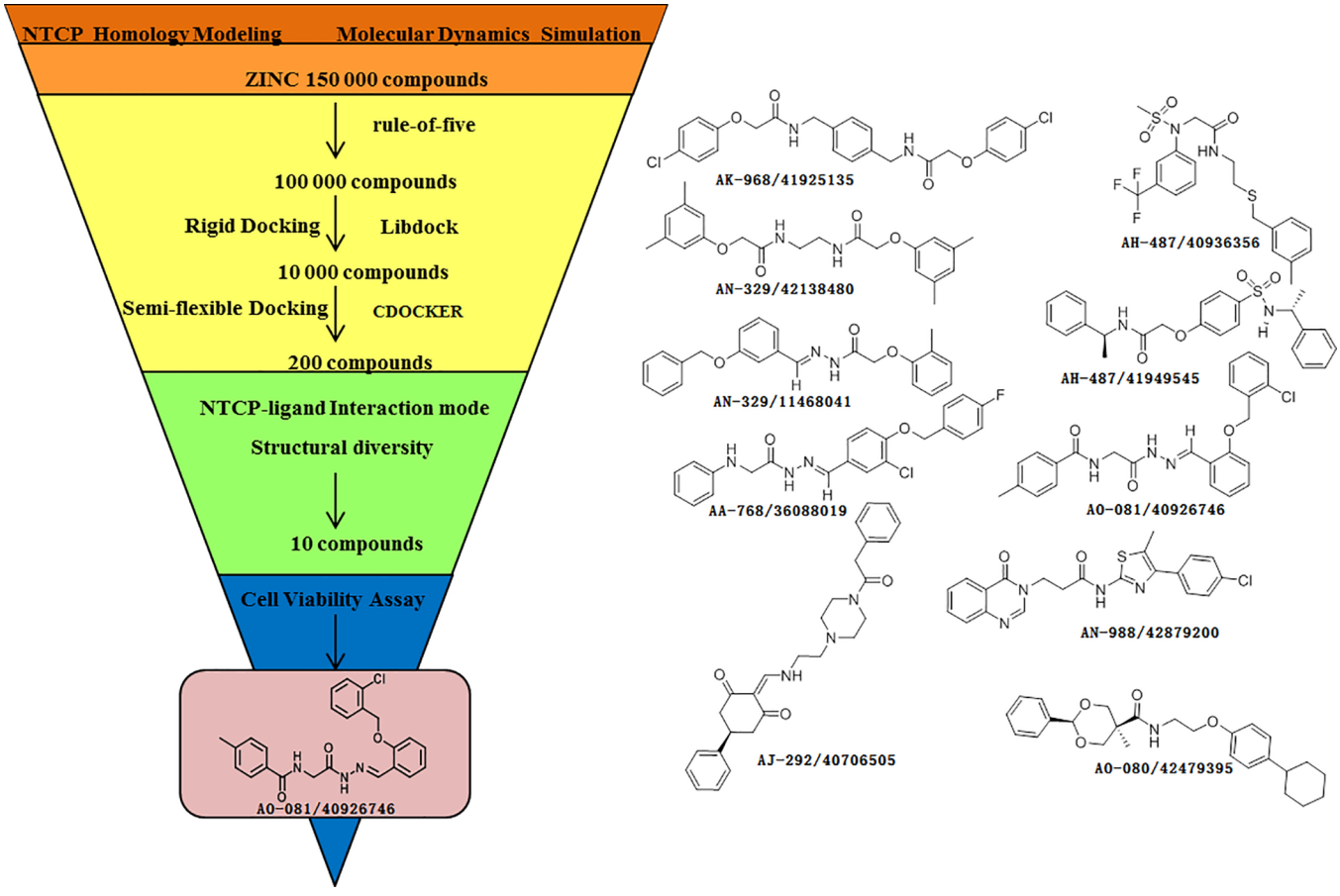


Fig. 2. Virtual screening protocol for identifying NTCP inhibitors.

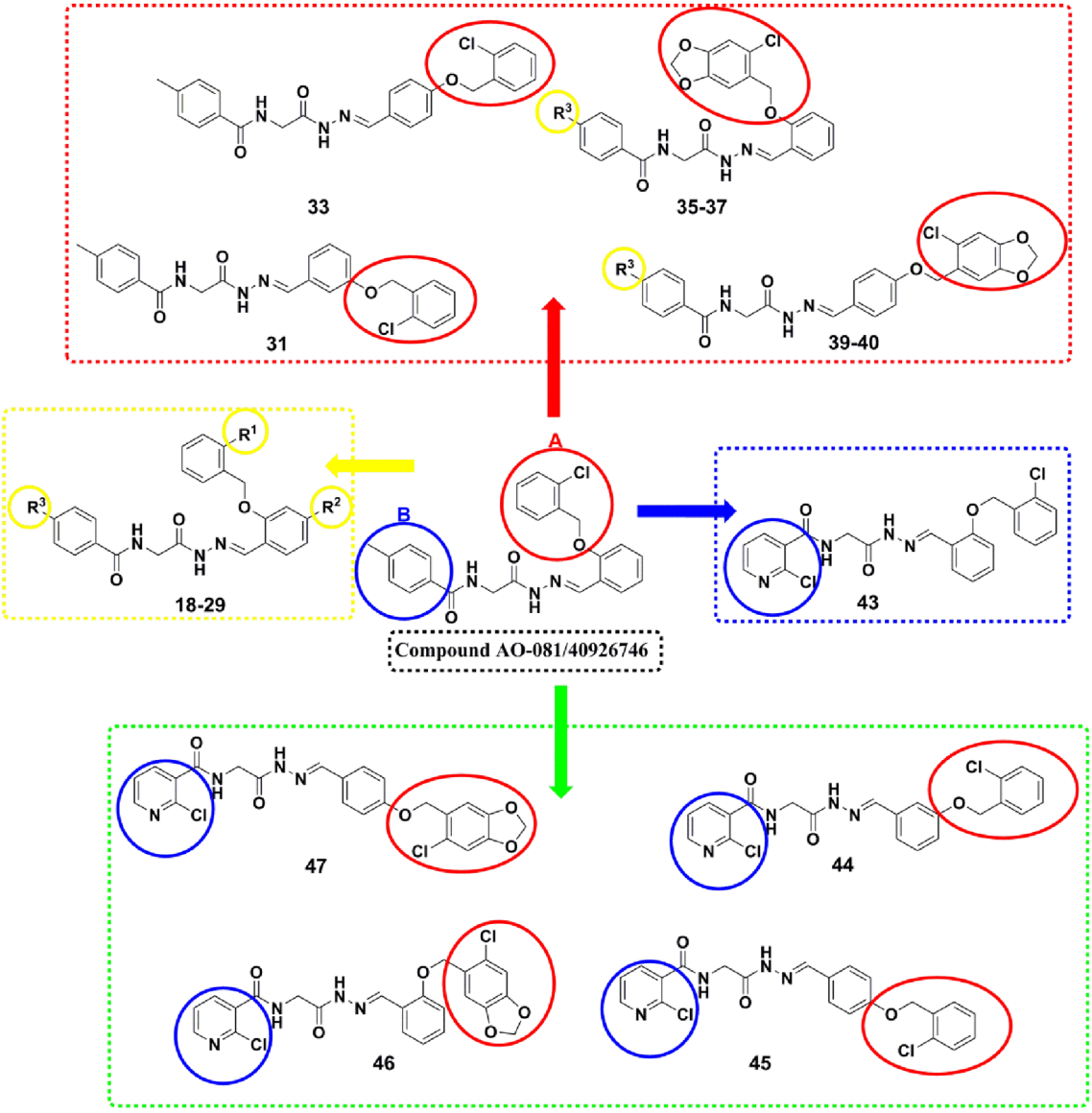
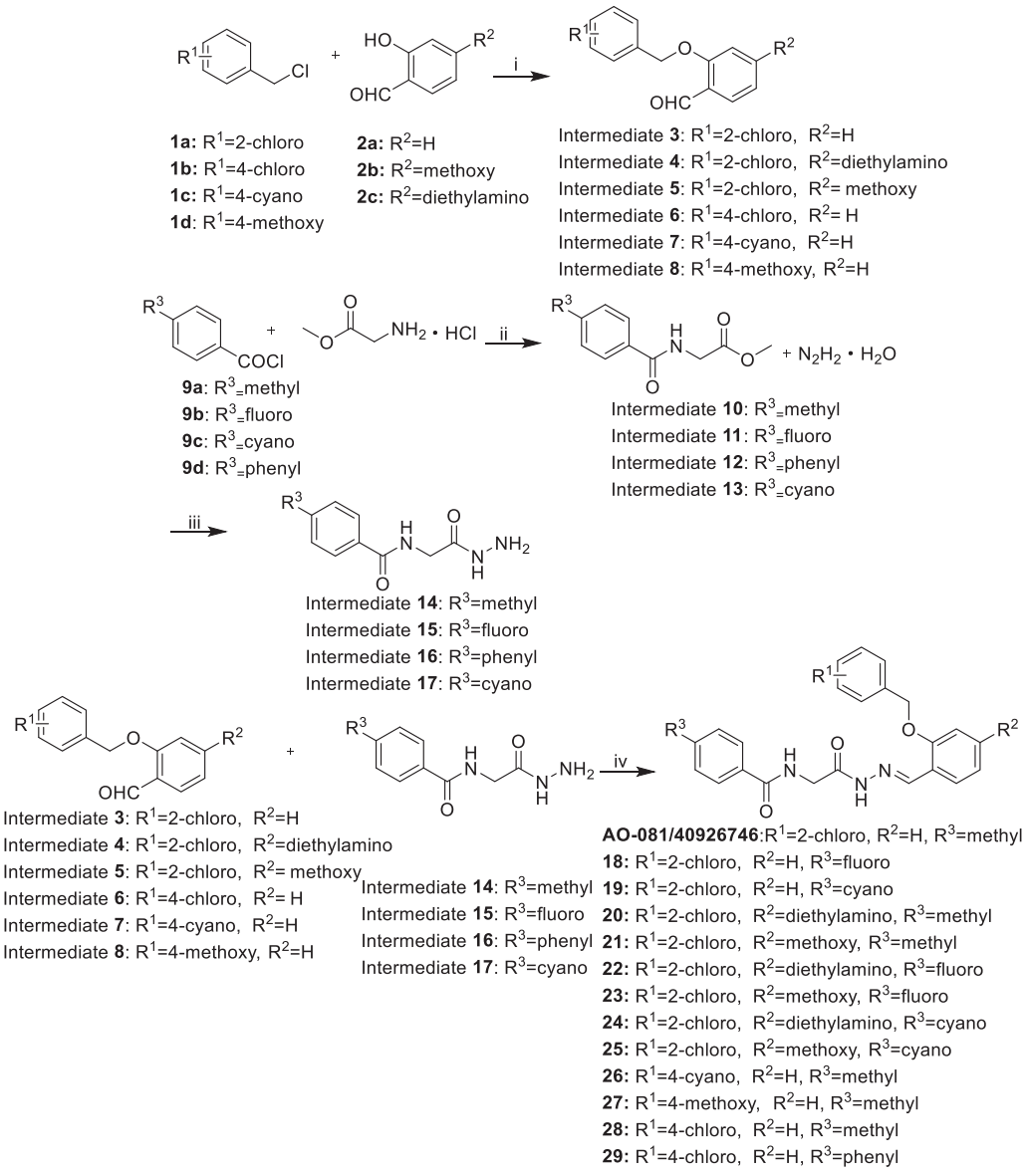


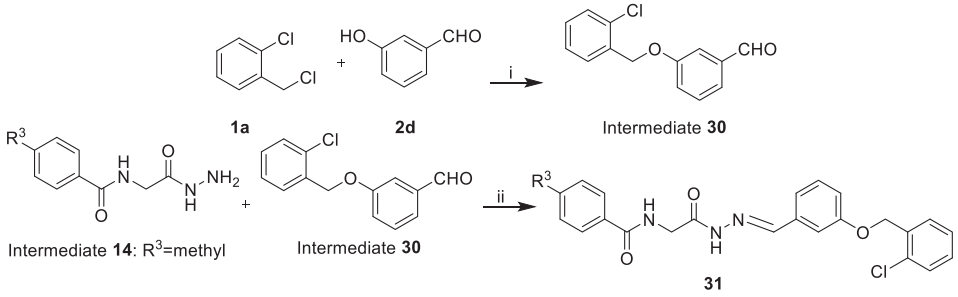
Fig. 3. Structure-activity relationships of the compounds.

using the CDOCKER protocol. As a result, we discovered 10 hits bearing better CDOCKER interaction energies and LibDock scores (Table S1). The anti-proliferative activities against HepG2 cells of these 10 compounds were determined by MTT assays (Fig. S1). Because of its excellent activity, **AO-081/40926746** was selected as a leading compound with significant potential for further optimization.

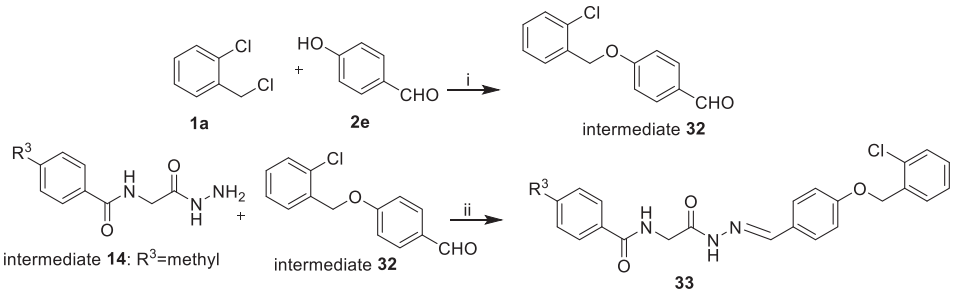
Based on the leading compound, we focused our attention on varying parts A and B and retained the chain of N-(2-(2-benzylindenyl-2-oxoethyl) acetamide (Fig. 3). The electron donating group and an electron withdrawing group (R^1, R^2, R^3) were initially introduced into parts A and B, and the derivatives **18–29** were synthesized (Scheme 1). Subsequently, part A was replaced by meta and para, and derivatives **31** and **33** were synthesized (Scheme 2–3). Introducing oxygen heterocycles in part A and substituting R^3, the derivatives **35–37** were synthesized (Scheme 4), and the *para*-substituted derivatives **39** and **40** were synthesized (Scheme 5). 2-Chloropyridine was substituted for part B, and derivative **43** was synthesized (Scheme 6). Finally, parts A and B were replaced at the same time, and derivatives **44–47** were synthesized (Scheme 7–10). According to the biological activity results (Table 1), we discovered compounds **23**, **36**, **39**, **43** and **47** exhibited moderate anti-proliferative activity, while compounds **35**, **44** and **45** exhibited superior biological activity. The remaining derivatives exhibited lower anti-proliferative activity. The anti-HBs and anti-HBe antigen activities of all compounds were tested in HepG2.2.15 cells at 5 μM, and Tenofovir was used as a positive control. These compounds had similar anti-HBV potency in terms of their anti-proliferative activities, among which compounds **35**, **44** and **45** showed the most potent anti-HBV activities (Table 1). To further evaluate the HBV inhibitory effects of compounds **35**, **44** and **45**, we detected the expression of HBV DNA in cells by using real-time PCR and found that HBV DNA levels in HepG2.2.15 cells were also markedly reduced after treatment with compounds **35**, **44** and **45** (Fig. 4).



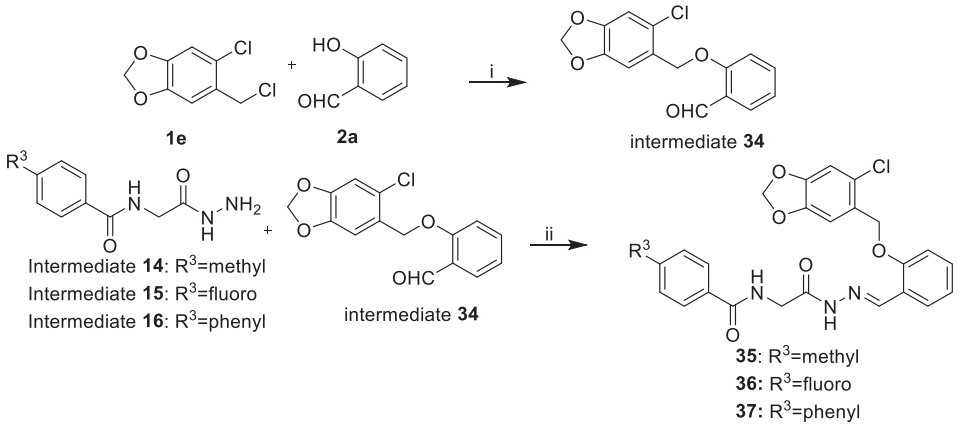
Scheme 1. General synthesis of compounds **18–29**. Reagents and conditions: (i) ethanol, K_2CO_3, KI, 85 °C, reflux, 4–8 h; (ii) acetonitrile; K_2CO_3, r.t, overnight; (iii) methanol, 80% hydrazine hydrate solution, 85 °C, reflux, overnight; (iv) ethanol, acetic acid, 85 °C, reflux, 6–8 h.



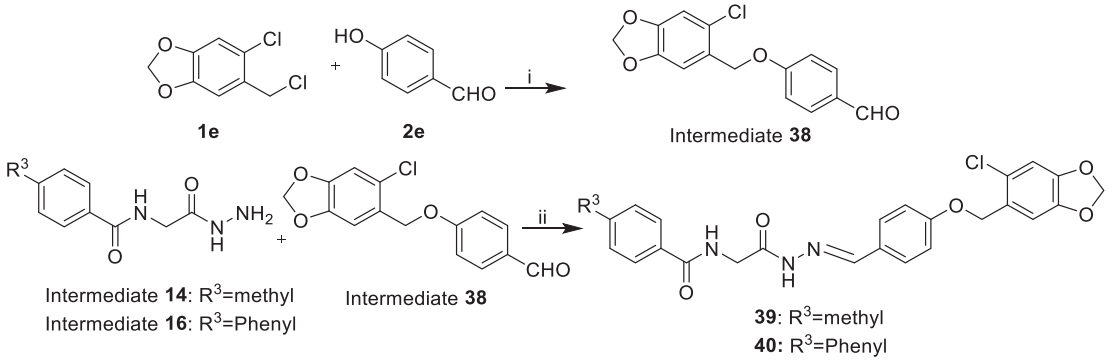
Scheme 2. General synthesis of compounds **31**. Reagents and conditions: (i) ethanol, K_2CO_3, KI, 85 °C, reflux, 4–8 h; (ii) ethanol, acetic acid, 85 °C, reflux, 6–8 h.



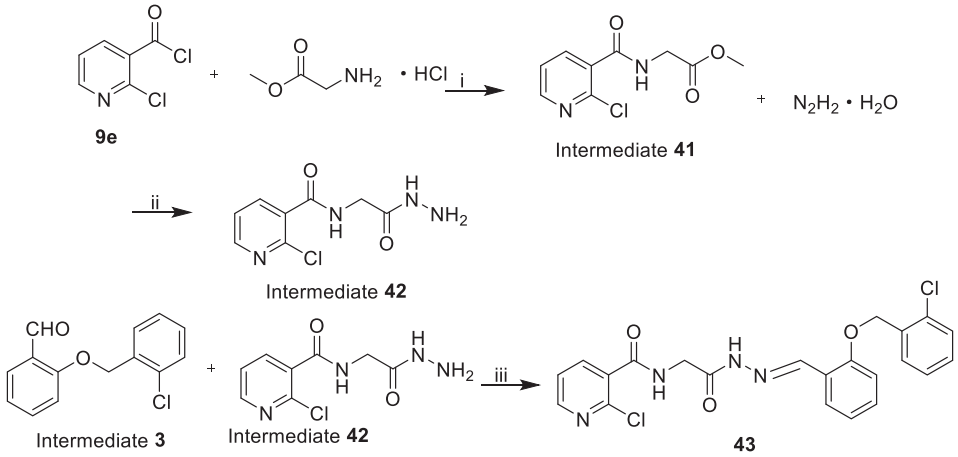
Scheme 3. General synthesis of compounds **33**. Reagents and conditions: (i) ethanol, K_2CO_3, KI, 85 °C, reflux, 4–8 h; (ii) ethanol, acetic acid, 85 °C, reflux, 6–8 h.



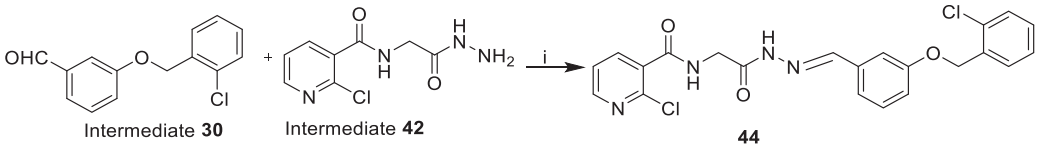
Scheme 4. General synthesis of compounds **35–37**. Reagents and conditions: (i) ethanol, K_2CO_3, KI, 85 °C, reflux, 4–8 h; (ii) ethanol, acetic acid, 85 °C, reflux, 6–8 h.



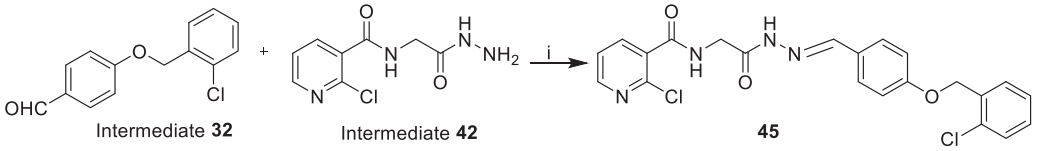
Scheme 5. General synthesis of compounds **39** and **40**. Reagents and conditions: (i) ethanol, K_2CO_3, KI, 85 °C, reflux, 4–8 h; (ii) ethanol, acetic acid, 85 °C, reflux, 6–8 h.



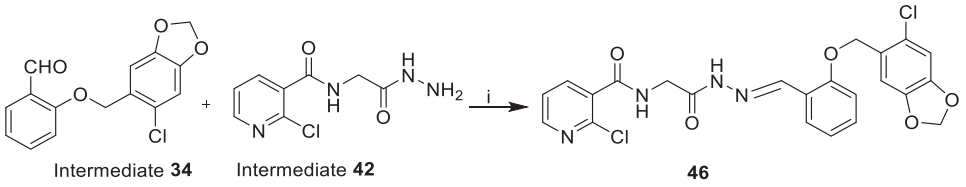
Scheme 6. General synthesis of compounds **43**. Reagents and conditions: (i) acetonitrile; K_2CO_3, r.t, overnight; (ii) methanol, 80% hydrazine hydrate solution, 85 °C, reflux, overnight; (iii) ethanol, acetic acid, 85 °C, reflux, 6–8 h.



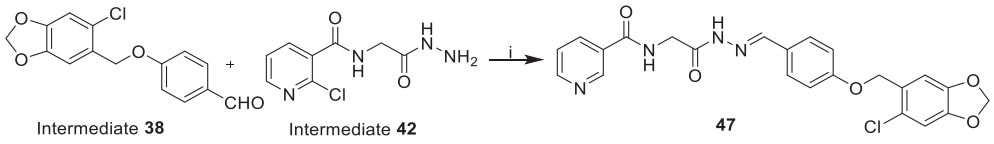
Scheme 7. General synthesis of compounds **44**. Reagents and conditions: (i) ethanol, acetic acid, 85 °C, reflux, 6–8 h.



Scheme 8. General synthesis of compounds **45**. Reagents and conditions: (i) ethanol, acetic acid, 85 °C, reflux, 6–8 h.



Scheme 9. General synthesis of compounds **46**. Reagents and conditions: (i) ethanol, acetic acid, 85 °C, reflux, 6–8 h.



Scheme 10. General synthesis of compounds **47**. Reagents and conditions: (i) ethanol, acetic acid, 85 °C, reflux, 6–8 h.

Table 1
Anti-proliferative activities and anti-HBV activities of the compounds.

No.	Anti-proliferative activity (IC_{50}, μM)[a]	HBsAg (ng/ml)[b]	HBeAg (ng/ml)[b]
Control	–[d]	8.41 ± 0.54	25.13 ± 1.93
Tenofovir	–[d]	1.72 ± 0.27	5.60 ± 0.58
Cisplatin(DDP)[c]	3.8 ± 1.1	–[d]	–[d]
AO-081/40926746	13.4 ± 1.6	3.95 ± 0.43	15.01 ± 0.97
18	24.9 ± 0.5	5.03 ± 0.58	14.53 ± 2.20
19	> 50	8.33 ± 0.71	25.22 ± 1.39
20	> 50	8.37 ± 0.62	24.55 ± 2.56
21	> 50	7.94 ± 0.80	23.53 ± 2.48
22	> 50	8.04 ± 0.55	24.34 ± 2.23
23	15.7 ± 3.8	4.23 ± 0.59	13.07 ± 1.53
24	> 50	8.17 ± 0.69	24.86 ± 1.75
25	> 50	8.29 ± 0.60	25.47 ± 2.32
26	> 50	8.25 ± 0.77	25.25 ± 1.94
27	> 50	7.66 ± 0.71	23.04 ± 1.69
28	> 50	7.79 ± 0.87	22.99 ± 2.03
29	> 50	8.31 ± 0.73	24.88 ± 2.16
31	47.5 ± 2.5	5.74 ± 0.68	18.09 ± 2.12
33	28.1 ± 1.8	4.83 ± 0.57	14.29 ± 2.09
35	2.8 ± 2.3	1.93 ± 0.18	7.43 ± 0.85
36	17.1 ± 1.0	3.98 ± 0.44	11.84 ± 1.42
37	> 50	7.72 ± 0.75	24.07 ± 2.86
39	14.9 ± 2.4	3.91 ± 0.30	11.47 ± 1.10
40	> 50	7.66 ± 0.68	23.80 ± 1.66
43	16.9 ± 3.2	4.08 ± 0.37	12.55 ± 1.55
44	3.1 ± 0.6	2.05 ± 0.20	6.43 ± 1.33
45	4.7 ± 1.3	2.48 ± 0.23	7.36 ± 1.01
46	> 50	8.39 ± 0.43	25.47 ± 2.23
47	10.1 ± 3.1	3.71 ± 0.26	11.45 ± 0.97

[a] Each compound was tested in triplicate.
[b] Each compound was tested at 5 μM for HBV antigen activity by ELISA.
[c] Cisplatin was used as a positive control for anti-proliferative activity.
[d] Data were not determined.

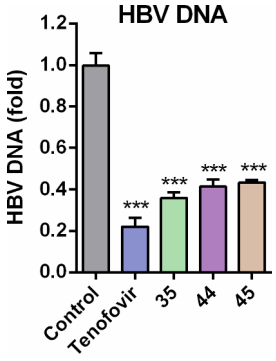
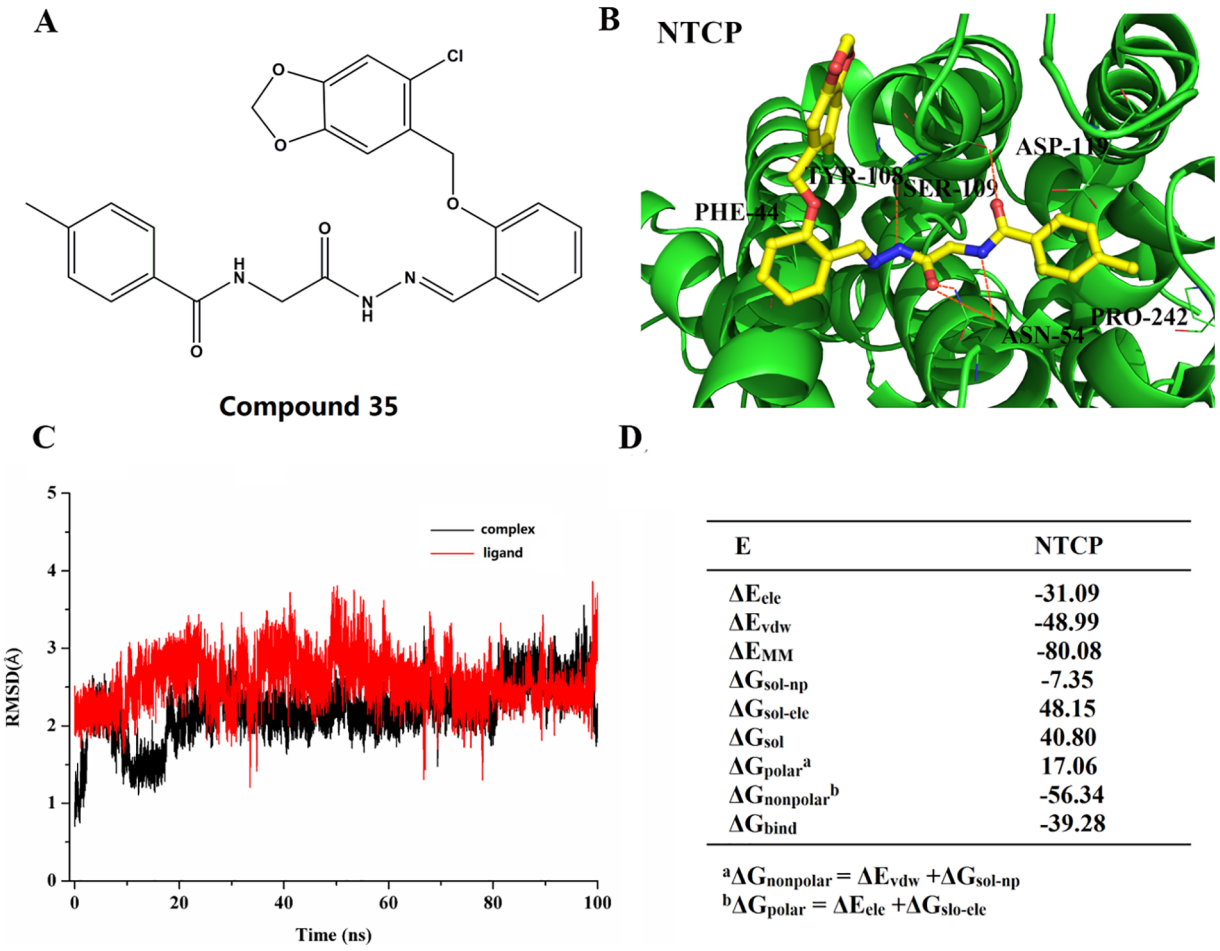


Fig. 4. HBV DNA inhibitory efficacies of NTCP inhibitors. HepG2.2.15 cells were treated with or without 10 μM Tenofovir or 10 μM **35**, **44**, or **45**, and HBV DNA in the supernatants were detected using real-time PCR.

To explore the binding mode of compound **35** to NTCP (Fig. 5A), a molecular docking simulation was performed using Discovery Studio 3.5 (Fig. 5B). Compound **35** could form three hydrogen bonds with Ser109, Asn54 and Tyr108. Additionally, the benzene ring of compound **35** could interact with Phe44 to form π-π interaction. Whereas the benzyl group at the other end of compound **35** could penetrate the hydrophobic cavity of NTCP. To further demonstrate the stability of the compound **35**-NTCP complex, a molecular dynamics simulation of 100 ns was established. The low root-mean-square deviation (RMSD) and the low binding free energy indicated that the system was stable (Fig. 5C and D). These results indicate that compound **35** could stably bind to NTCP.

Next, we investigated the effect of compound **35** on HepG2 proliferation, and with cisplatin as the positive control. It was found that



E	NTCP
ΔE_{ele}	-31.09
ΔE_{vdw}	-48.99
ΔE_{MM}	-80.08
$\Delta G_{sol\text{-}np}$	-7.35
$\Delta G_{sol\text{-}ele}$	48.15
ΔG_{sol}	40.80
ΔG_{polar}[a]	17.06
$\Delta G_{nonpolar}$[b]	-56.34
ΔG_{bind}	-39.28

[a] $\Delta G_{nonpolar} = \Delta E_{vdw} + \Delta G_{sol\text{-}np}$
[b] $\Delta G_{polar} = \Delta E_{ele} + \Delta G_{slo\text{-}ele}$

Fig. 5. Molecular docking and simulation of molecular dynamics. (A) Chemical structure of compound **35**. (B) Molecular docking of compound **35** and NTCP. (C) Molecular dynamics (MD) simulation of the compound **35**-NTCP complex. (D) The binding free energy of complex.

compound **35** inhibited the proliferation of HepG2 cells in a dose- and time-dependent manner (Fig. 6A and B). When the cells were treated with compound **35**, condensed chromatin and bright fluorescence was observed by fluorescence microscopy after Hoechst 33258 staining (Fig. 6C). In addition, apoptosis was further evaluated by Annexin-V/PI double staining. Compound **35** significantly increased the rate of apoptosis in HepG2 cells (Fig. 6D). Meanwhile, western blot analysis showed compound **35** inhibited NTCP expression, upregulated Bax, downregulated Bcl-2, and increased the cleavage of caspase 3 and PARP (Fig. 6E). These results indicate that compound **35** acts as an NTCP inhibitor to induce HepG2 cells apoptosis.

As a major cause of hepatocellular carcinoma induced by hepatitis, NTCP is one of the main targets for the treatment of HCC. In conclusion, we synthesized twenty-five structural analogs as candidate compounds. Molecular docking and MD simulation show that compound **35**-NTCP is a stable complex system. The MTT assay demonstrated that compound **35** caused time-dependent and concentration-dependent inhibition of the proliferation of HepG2 cells, and this anti-proliferative effect appeared to be due to its ability to induce apoptotic cells. In addition, the induction of apoptosis by compound **35** was confirmed using fluorescence microscopy, flow cytometry and western blot analysis. These findings will provide valuable guidelines for the development of targeted NTCP small molecule drugs for the treatment of HCC.

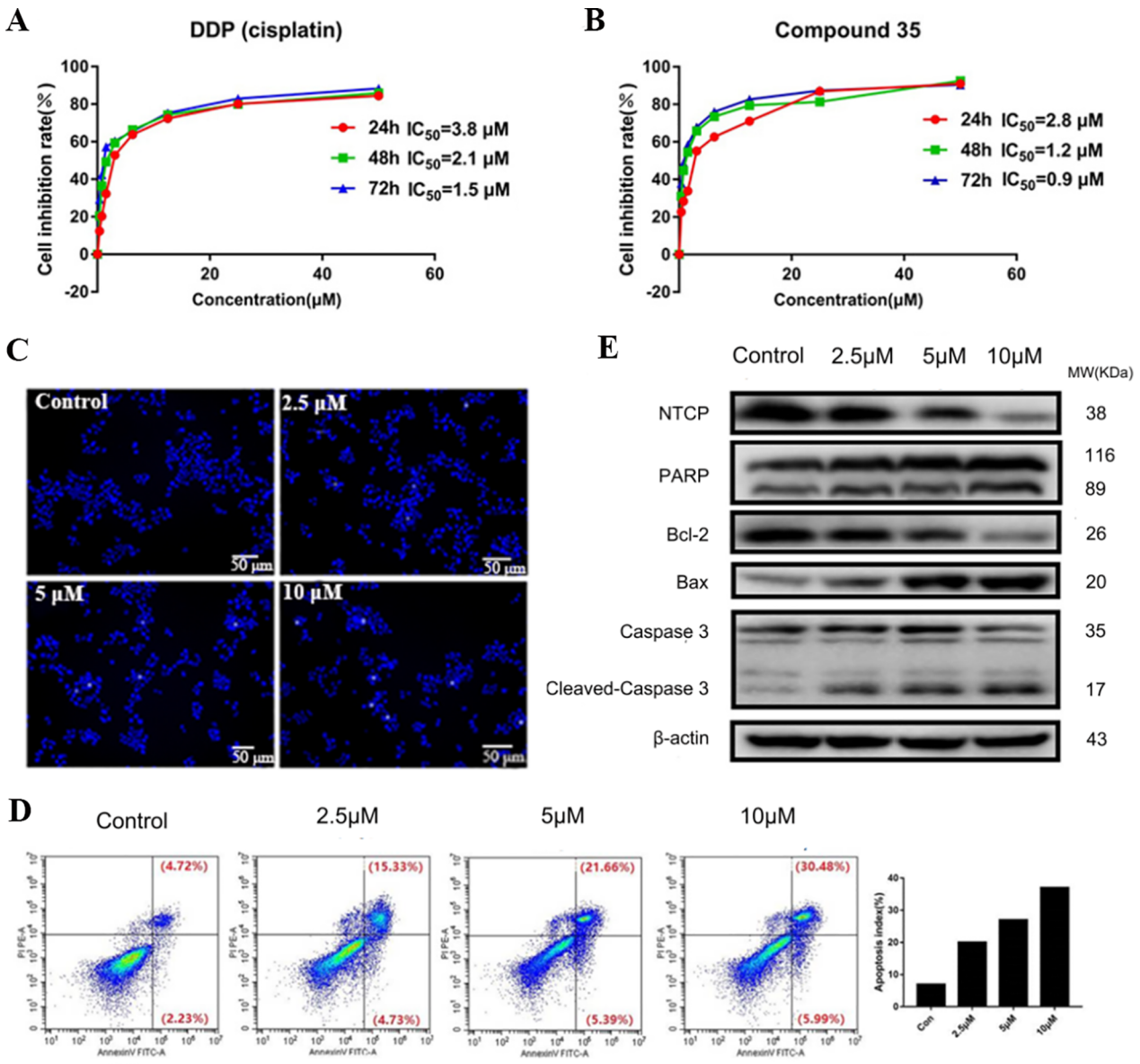


Fig. 6. Compound **35** induced apoptosis in HepG2 cells. (A, B) The cells were treated with different concentrations of compound **35** for indicated times, and then the cell inhibition rates were measured using MTT assay; (C) The cells were treated with different concentrations of compound **35** for 24 h. Morphologic changes were observed by fluorescence microscopy after Hoechst 33258 staining. Scale bar = 50 μm; (D) The cells were treated with different concentrations of compound **35** for 24 h, and the apoptosis ratios were determined by flow cytometry analysis after Annexin-V/PI double staining; (E) The cells were treated with different concentrations of compound **35** for 24 h and then the expressions of NTCP, PARP, Bax, Bcl-2 and caspase-3 were detected by western blot analysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.126623.

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