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2-Arylthio-5-iodo pyrimidine derivatives as non-nucleoside HBV polymerase inhibitors

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ABSTRACT

In this study, a series of 2-arylthio-5-iodo pyrimidine derivatives, as non-nucleoside hepatitis B virus inhibitors, were evaluated and firstly reported as potential anti-HBV agents. To probe the mechanism of active agents, DHBV polymerase was isolated and a non-radioisotopic assay was established for measuring HBV polymerase. The biological results demonstrated that 2-arylthio-5-iodo pyrimidine derivatives targeted HBV polymerase. In addition, pharmacophore models were constructed for future optimization of lead compounds. Further study will be performed for the development of non-nucleoside anti-HBV agents.

1. Introduction

The hepatitis B virus (HBV) belongs to Hepadnaviridae which can cause chronic liver diseases.[1] Despite the existence of vaccines against HBV, an estimated 257 million people worldwide are living with HBV infection now.[2] The Chinese Center for Disease Control and Prevention (China CDC) reported that 28 million people in China were involved in chronic HBV infection in 2017.[3] Currently, there are only seven anti-HBV drugs in clinical use, including two interferons (IFN-α and pegylated IFN-α) as immune system modulators and five nucleoside analogs (lamivudine, telbivudine, entecavir, adefovir dipivoxil and tenofovir) as HBV polymerase inhibitors.[4] Unfortunately, the IFN-α therapy is limited by low efficiency (30–40%).[5] Meanwhile, some nucleoside agents are also unsatisfactory due to the increasing risk of development of drug resistance and adverse effects associated with long-term use of these drugs.[6] Therefore, it is urgent to develop innovative, effective and low toxic anti-HBV drugs.

Currently, non-nucleoside anti-HBV agents have attracted more attention due to their favorable pharmacokinetic properties and wide range of chemical diversity. As shown in Fig. 1, non-nucleoside inhibitors have been involved in different stages of HBV life cycle. For the entry section, a series of benzoimidazole derivatives were reported to block virus entry which constituted a therapeutic approach to prevent primary HBV infection.[7] Recently, Cai et al. reported that disubstituted sulfonamides could interfere with the conversion of rcDNA into cccDNA.[8] For the transcription stage, Helioxanthin[9] and Rosiglitazone[10] derivatives have been identified as inhibitors of some transcription factors. Moreover, it was reported that heteroaryldihydropyrimidines (HAPs)[11] and phenopropenamides[12] could block pgRNA packaging and core assembly. For the secretion and ER-associated processing section, *N*-nonyl-deoxy-nojirimycin derivatives could misfold the HBV envelope proteins and therefore prevent HBV secretion.[13]

During these phases, HBV polymerase (HP) demonstrates an important role in HBV replication. HP is composed of four domains including an N-terminal protein (TP), a spacer region, a reverse transcription (RT) domain, and a C-terminal RNase H domain.[14] Particularly, the RT domain of HBV polymerase shows significant homology to HIV-1 RT.[15] As we know, HBV and HIV replicate via reverse transcriptase with RNA as a template. The nucleoside inhibitors of HBV polymerase, lamivudine and adefovir, were originally developed from HIV-1 RT inhibitors.[16] In addition, resistance to these nucleoside inhibitors occurred in similar RT regions of HP as HIV RT.[17]

Based on our previous study, dihydroalkylthio benzyloxopyrimidine (S-DABO) derivatives showed significant antiviral activity as

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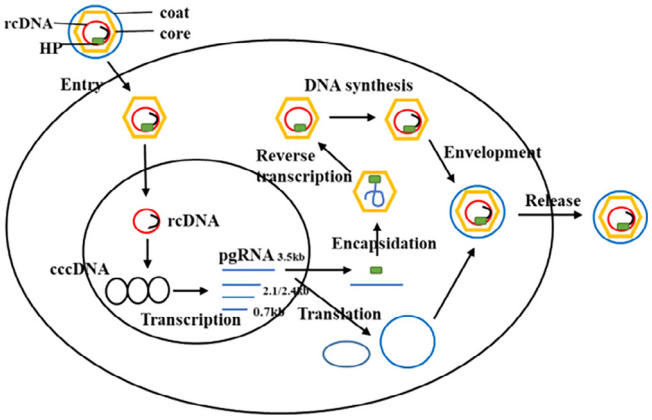


Fig. 1. The key steps of the hepatitis B virus life cycle.

HIV-1 RT inhibitors.[18] According to the structure-activity relationship (SAR), substituents on the side chain of C-2 and C-6, especially the iodine atom at the 5-position were important for antiviral activity. To validate our hypothesis, novel substituted 2-arylthio-5-iodo pyrimidine analogs with the structural diversity were prepared and evaluated for their anti-HBV activity. To further explore the mechanism of these compounds, the inhibitory activity of HBV polymerase was determined. The SAR was supported by pharmacophore models developed for the future optimization of lead compounds.

2. Results and discussion

2.1. Chemistry

The general procedures for synthesizing the target compounds were outlined in Scheme 1.[19] β-ketoesters **3a–d** were prepared via Hannick and Kishi reaction with benzyl cyanide and ethyl 2-bromoacetate as starting materials. Compound **3e** was prepared by an alternative method. Benzo[*d*]triazol-1-yl acetic acid **2** reacted with CDI, followed by treatment with ethyl potassium malonates. Condensation of β-ketoesters with thiourea in the presence of sodium ethoxide provided the 6-aryl thiouracil derivatives **4a–e**. S-alkylation of compounds **4a–e** with appropriate substituted alkyl halides could afford **5a–e,** which reacted with NIS to give S-DABO compounds **6a–e**.

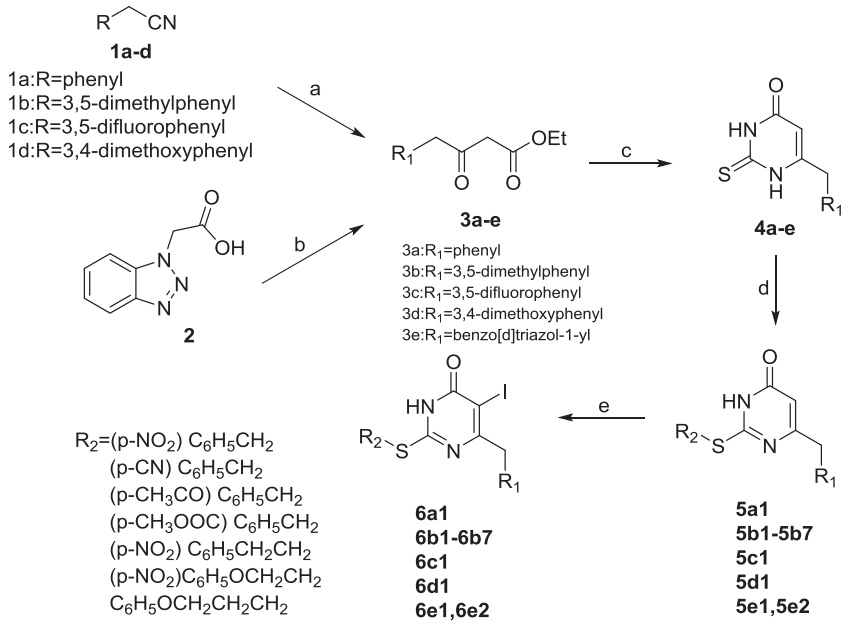
Moreover, target compounds **11** and **14** were synthesized with an alternative route (Scheme 2).[20] *p*-Cyanobenzyl bromide **7** was treated with thiourea to afford cyanobenzyl thiourea **8**, which was condensed with ethyl 4-chloroacetoacetate and diethyl oxaloacetate, respectively, to give the benzyl chloride analog **9** and 2-thioorotic acid derivative **12**. Iodination was performed on the C-5 via the reaction of compounds **9** and **12** with NIS. Subsequent treatment of compound **10** with the corresponding aliphatic amines provided target compounds **11a–b**. Meanwhile, compound **13** reacted with thionyl chloride and then was treated with appropriate amines to yield the target compounds **14a–c**.

2.2. Biological evaluation

2.2.1. Inhibitory activity on HBV DNA replication in HepG2.2.15 cells

All the target compounds were evaluated for their anti-HBV activity in HepG2.2.15 cells with lamivudine as a reference and cytotoxicity data was determined for the active compounds. The results were summarized in Table 1. Most of the target compounds displayed activity against HBV replication. Considering the different substituents at the C-2 position, compounds **6b** with electron withdrawing groups, particularly the nitro and cyano groups, would be beneficial for the inhibitory activity with EC_{50} values between 6.57 and 12 μM. In addition, compound **6b6** (EC_{50} = 6.88 μM) exhibited slightly better activity than that of **6b5** (EC_{50} = 9.8 μM), indicating that the introduction of an additional oxygen atom on an alkyl linker at the C-2 position would be beneficial for the antiviral activity. However, with the replacement of the substituted phenyl groups by the aliphatic cyclic amine groups on the C-6 side chain (compounds **11a–b**), the decreased anti-HBV activity was observed. Interestingly, bearing the amide moiety at C-6 position, compounds **14a–c** (EC_{50} < 3 μM) could effectively inhibit HBV virus.

The promising results were observed for our target compounds, especially **6b1**, **6b6**, **6c1** and **14a–14c** with EC_{50} values below 7 μM against HBV DNA replication. Therefore, the mechanism was



Scheme 1. Synthesis of target compounds **6**. Reagents and conditions: (a) ethyl bromoacetate, Zn/THF, reflux. (b) $MgCl_2$, Et_3N, CDI, $CH_2(COOEt)$ (COOK), r.t. for 12 h and then reflux for 2 h. (c) Thiourea, EtONa/EtOH, reflux. (d) Substituted alkyl halide, K_2CO_3, rt. (e) NIS, DMF, 0 °C.

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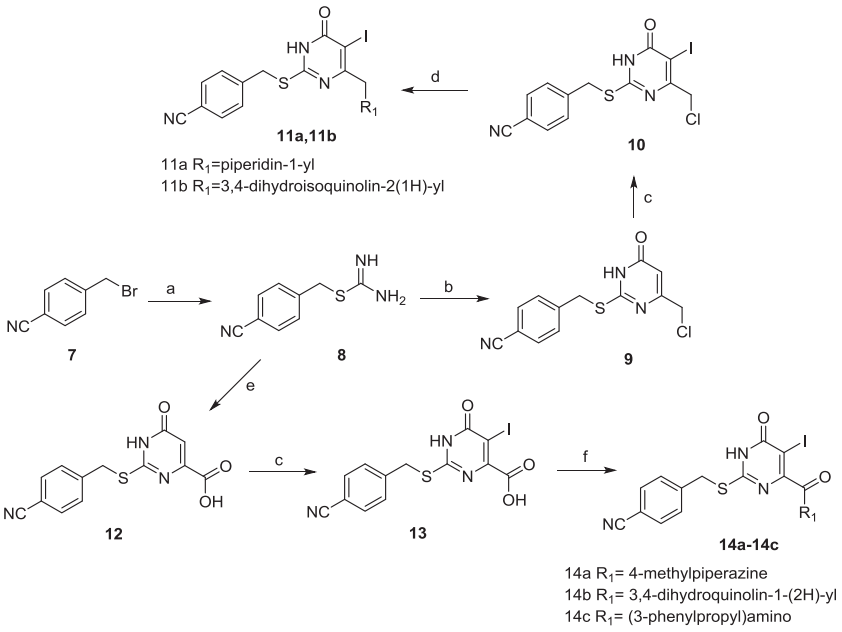
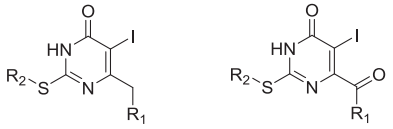


Scheme 2. Synthesis of target compounds **11** and **14**. Reagents and conditions: (a) Acetone, thiourea, r.t. (b) Ethyl 4-chloroacetoacetate, H_2O, Na_2CO_3, r.t. (c) NIS, DMF, 0 °C. (d) aliphatic amines, DMF, r.t. (e) diethyl oxaloacetate, H_2O, NaOH, r.t. conc. HCl. (f) $SOCl_2$, reflux; aliphatic amines, CH_2Cl_2, 0 °C.

Table 1
Inhibitory activity of target compounds against HBV DNA (μM).



Compd.	R_1	R_2	CC_{50}[a] (μM)	EC_{50}[b] (μM)	SI[c]
6a1	phenyl	(*p*-NO_2) $C_6H_5CH_2$	52.68	7.57	7.0
6b1	3,5-dimethylphenyl	(*p*-NO_2) $C_6H_5CH_2$	39.04	6.57	5.9
6c1	3,5-difluorophenyl	(*p*-NO_2) $C_6H_5CH_2$	>100	6.19	>16
6d1	3,4-dimethoxyphenyl	(*p*-NO_2) $C_6H_5CH_2$		>20	
6e1	Benzo[*d*]trazol-1-yl	(*p*-NO_2) $C_6H_5CH_2$		NA[d]	
6b2	3,5-dimethylphenyl	(*p*-CN) $C_6H_5CH_2$	43.19	7.03	6.1
6e2	Benzo[*d*]trazol-1-yl	(*p*-CN) $C_6H_5CH_2$		NA[d]	
6b3	3,5-dimethylphenyl	(*p*-CH_3CO) $C_6H_5CH_2$	78.34	12.15	6.4
6b4	3,5-dimethylphenyl	(*p*-CH_3OOC) $C_6H_5CH_2$	64.76	12.84	5.0
6b5	3,5-dimethylphenyl	(*p*-NO_2) $C_6H_5CH_2CH_2$	66.56	9.8	6.8
6b6	3,5-dimethylphenyl	(*p*-NO_2) $C_6H_5OCH_2CH_2$	96.61	6.88	14.0
6b7	3,5-dimethylphenyl	$C_6H_5OCH_2CH_2CH_2$	>100	8.87	>11
11a	piperidin-1-yl	(*p*-CN) $C_6H_5CH_2$		NA[d]	
11b	3,4-dihydroisoquinolin-2(1*H*)-yl	(*p*-CN) $C_6H_5CH_2$		>20	
14a	4-methylpiperazine	(*p*-CN) $C_6H_5CH_2$	>100	2.60	>38
14b	3,4-dihydroquinolin-1-(2*H*)-yl	(*p*-CN) $C_6H_5CH_2$	>100	1.61	>62
14c	(3-phenylpropyl)amino	(*p*-CN) $C_6H_5CH_2$	>50	1.62	>30
LAM			>100	0.61	>164

[a] 50% cytotoxicity concentration.
[b] Concentration inhibiting viral replication by 50%.
[c] Selectivity index, defined as CC_{50}/EC_{50}.
[d] No anti-HBV activity observed at the concentration of 10 μM.

explored further. Since HBV polymerase has structural homology with HIV-1 RT and S-DABO derivatives HIV-1 RT inhibitors, we speculated that 2-arylthio-5-iodo pyrimidine analogs might target HBV polymerase. Consequently, we prepared to isolate HBV polymerase and evaluate the inhibitory activity of our target compounds against HBV polymerase.

2.2.2. Inhibitory activity against HBV polymerase

Currently, there are two methods of isolating HBV polymerase which was expressed in insect cells by recombinant baculovirus system[21] and isolated from duck livers infected by DHBV[22] respectively. It was reported that the expression of HBV polymerase was unstable and inefficient. Especially, there are concerns about the

activity of the obtained protein. To obtain the HBV polymerase with satisfactory purity to evaluate the activity of target compounds, we decided to isolate the protein from duck livers and the following research was done.

DHBV polymerase was isolated from Ducklings infected with DHBV. The duck livers were collected and homogenized. The protein was purified by sucrose density gradient centrifugation. To determine the activity of HBV polymerase in each different fraction, the assay for measuring the activity should be established.

Considering the biosafety and radiosafety, we established a new enzyme-linked immunosorbent assay (ELISA) method for determining HBV polymerase activity.[23] As reported in our previous work[24], oligo(dT)$_{15}$ primer was immobilized to Covalink-NH microtiter plates. Then the inhibitors, DHBV polymerase and reaction buffer (containing 17.5 μg/ml poly (A), 8 μM dTTP and 2 μM biotin-11-dUTP) were mixed in each well, incubated for 1 h and then was washed. After coating with BSA, streptavidin-alkaline phosphatase (SA-ALP) solution was added and incubated. Finally, a chemiluminescent substrate, *p*-nitrophenyl phosphate, disodium (PNPP) was added. The reaction was terminated by the addition of 1N NaOH and the solution was measured at 405 nm.

The activity of DHBV polymerase in the different fractions was measured. As shown in Table 2, the parts of 35% and 40% exhibited better activity of DHBV polymerase. In addition, the results of gel electrophoresis, shown in Fig. 2, also displayed that the quantity of DHBV polymerase in 35% and 40% parts was higher than in the other fractions'. Therefore, we mixed the fractions of 35% and 40% for the following experiments.

To optimize the conditions in our experiment, we tested the activity of DHBV polymerase at different concentration. The quantity of DHBV polymerase from one duck liver was defined as "concentration of 1 unit". According to the results in Fig. 3, the concentration of "1/100" and time of 60 min were chosen for the following experiments.

Subsequently, we investigated the reproducibility and stability of the assay. As shown in Table 3, the coefficients of variation (CV) were 3.96% and 7.05% respectively. These results revealed that the reproducibility and stability were qualified for our experiments.

As a viral DNA polymerase inhibitor[25], foscarnet (PFA) was selected as the reference. The relationship of dose-dependent inhibition of DHBV polymerase was observed as shown in Fig. 4. The IC$_{50}$ value of PFA (1.96 ± 0.14 μg/ml) was consistent with the literature value.[26] The relevant experimental data as above-mentioned indicated that the isolated DHBV polymerase and the assay could be used for further screening of the inhibitory activity of the target compounds.

Thereafter, we detected the inhibitory activity of target compounds against DHBV polymerase. The biological results displayed that some compounds were active against DHBV polymerase (Table 4). Among them, compounds **6b1**, **6b2** and **6c1** exhibited moderate inhibitory activity with IC$_{50}$ value about 30 μM, which suggested that these compounds targeted HBV polymerase. While, to our surprise, compounds **14a–c** with amide link at C-6 position,

Table 2
The activity of DHBV polymerase in different parts.

Concentration of different fractions	OD value
20%	0.134
25%	0.265
30%	0.715
35%	1.306
40%	1.438
45%	1.105

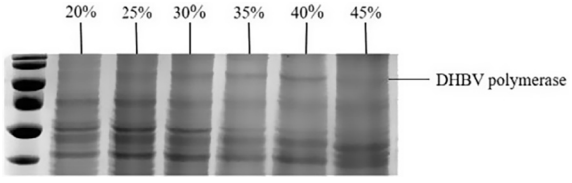


Fig. 2. The proteins in parts of different concentration.

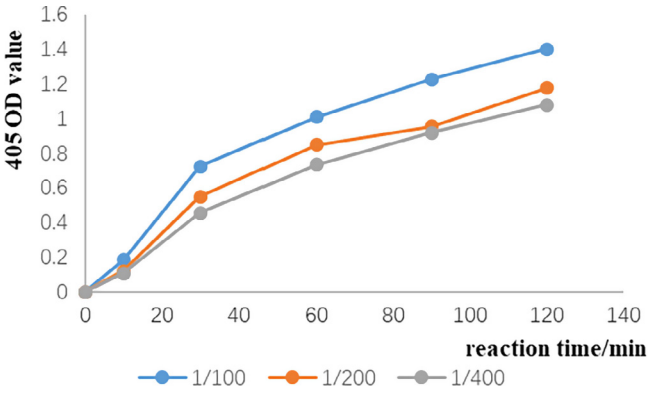


Fig. 3. The Time-OD value curve at different concentration of DHBV polymerase.

Table 3
The reproducibility and stability of DHBV polymerase assay.

	Repeats	Average	SD	CV/%
In a lot	10	1.2028	0.0476	3.96
Among the lots	10	1.0978	0.0774	7.05

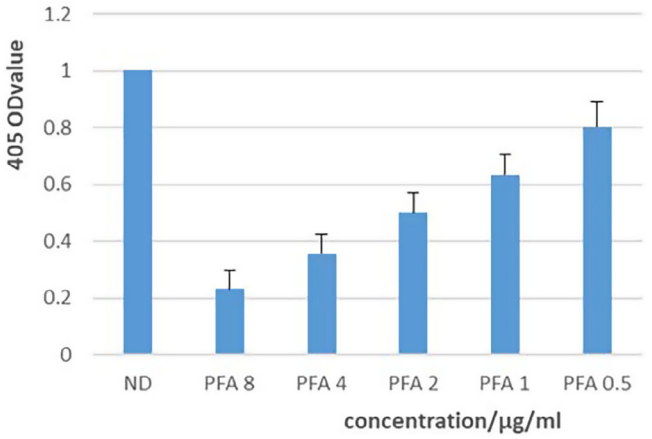


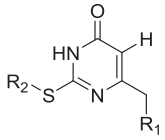
Fig. 4. Inhibitory activity of PFA against DHBV polymerase.

Table 4
Inhibitory activity of target compounds against DHBV polymerase.

Compd.	IC$_{50}$ (μM)	Compd.	IC$_{50}$ (μM)
6a1	42.84 ± 3.19	6b5	71.85 ± 3.07
6b1	33.02 ± 4.22	6b6	55.97 ± 3.42
6c1	29.50 ± 4.13	6b7	69.36 ± 2.13
6d1	70.96 ± 1.02	11a	NA*
6e1	103.81 ± 5.04	11b	60.53 ± 2.74
6b2	35.55 ± 1.98	14a	NA
6e2	128.06 ± 4.94	14b	>200
6b3	42.92 ± 4.98	14c	>200
6b4	38.84 ± 3.85	PFA	6.54 ± 0.45

* No anti-HBV polymerase activity observed at the concentration of 200 μM.

Table 5
Inhibitory activity of compounds **5c1** and **5b4** against DHBV polymerase and HBV DNA.



Compd.	DHBV P[a]	HepG2.2.15[b]
5c1	16.15%	32.92%
5b4	3.66%	13.35%

[a] Inhibitory rate at the concentration of 100 μM.
[b] Inhibitory rate at the concentration of 20 μM.

significantly inhibited HBV DNA replication, displayed no inhibition on DHBV polymerase.

To investigate the effect of the iodine atom at C-5 position, compounds **5c1** and **5b4** were also evaluated for their inhibitory activity both on DHBV polymerase and HepG2.2.15 cells. Compared to the corresponding compounds **6c1** and **6b4**, their activity disappeared as the iodine atom was removed (Table 5). These biological results were consistent with their inhibitory activity on HIV-1 RT, in which the iodine atom at the C-5 position might play a crucial role in the binding process with HBV polymerase.

2.3. Pharmacophore models

As we know, the high-resolution structure of HBV polymerase has not been analyzed. Therefore, construction of a pharmacophore model would be necessary to optimize the structure and identify lead compounds. Compounds **6a1**, **6b1, 6b2**, **6b6, 6b7** and **6c1**, which showed the best activity against HBV polymerase, were selected to generate pharmacophore models. As shown in Fig. 5, several pharmacophore points were identified for the 2-arylthio-5-iodo pyrimidine derivatives, including two hydrophobic, one hydrogen bond acceptor and one hydrogen bond donor points. Two phenyl groups (R8, R9) were recognized as aromatic rings. The π-π conjugated system of phenyl ring on position C-2 (R9) was consistent with the docking results of these compounds binding to HIV-1 RT.[18] The iodine atom on position C-5 was recognized as a hydrophobic point. As reported, the C-5 iodine atom of S-DABOs played an important role in improving anti-HIV-1 activity. It provided a steric hindrance role in the hydrophobic region and formed a bond with Tyr181 of HIV-1 RT.[27] We speculated that the C-5 iodine atom might have a similar effect on HP. These pharmacophore results suggested that the possible binding modes between target compounds with HBV polymerase and HIV-1 RT were similar.

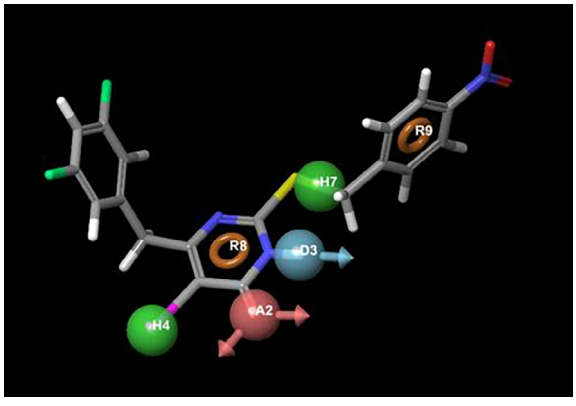


Fig. 5. Pharmacophore models of target compounds. H-bond acceptor: light red, arrows pointing in the direction of the lone pairs; H-bond donor: light blue, arrow pointing in the direction of the potential H-bond; hydrophobic is presented by green sphere, positive by blue sphere and aromatic ring by orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Conclusion

In summary, we have evaluated a series of 2-arylthio-5-iodo pyrimidine derivatives against HBV DNA replication. In order to find out the mechanism of these compounds, DHBV polymerase was isolated from duck livers. Using a chemiluminescent substrate, we firstly established a non-radioisotopic assay for measuring the inhibitory activity of our compounds on DHBV polymerase. The biological results demonstrated that 2-arylthio-5-iodo pyrimidine derivatives as non-nucleoside inhibitors could be against HBV DNA replication and targeted HBV polymerase. Further investigation of structural optimum is ongoing in our laboratories.

4. Experimental

4.1. In vitro cytotoxicity study of target compounds

The HepG2.2.15 cells were incubated in 96-well plates at 2×10^5 cells per mL (200 μL per well). 24 h later, the cells were treated with various concentrations of target compounds. 72 h later, the medium was replcaced by fresh one containing the compounds. Another 72 h later, 20 μL of MTT solution was added to each well and was incubated for 4 h. The culture medium was discarded then and 200 μL DMSO was added to each well to solubilize formazan. The plate was measured at an absorbance of 490 nm by an automatic plate reader.

4.2. Assays for measuring the inhibition activity of compounds against HBV DNA

The HepG2.2.15 cells were incubated in 96-well plates at 2×10^5 cells per mL (200 μL per well). 24 h later, the cells were treated with various concentrations of target compounds. 72 h later, the medium was replcaced by fresh one containing the compounds. Another 72 h later, the medium was removed and the cells was washed by phosphate-buffered saline (PBS). The cells was harvested and the intracellular HBV DNA was extracted from cell lysis. The levels of HBV DNA was determined by quantitative real-time PCR (qRT-PCR).

4.3. Isolation DHBV polymerase from duck livers

Ducklings aged 1 day were infected with DHBV at a dose of 0.5 ml DHBV (+) serum per duckling (containing DHBV DNA). Seven days later, the duck livers were collected. The liver homogenate was prepared with 1:1 NEB buffer (Tris-HCl pH 7.5, 20 mM; NaCl 50 mM; $MgSO_4$ 7 mM; DTT 0.1%; NP-40 0.5%; Sucrose 8.57%). Most of the tissue fragments in the homogenate were removed by centrifugation at 11,000 rpm at 4 °C for 1 h. The supernatant portion was centrifuged at 45,000 rpm at 4 °C for 3 h and the sediment was collected to separate the DHBV polymerase. The protein was purified through sucrose density gradient centrifugation. Sucrose of 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10% was added to the tube from bottom to the top. The diluted sediment was added on the top of the sucrose. The mixture was centrifuged at 27,000 rpm at 4 °C for 4 h. After the gradient centrifugation, per fraction was collected according to different concentration of sucrose. The fractions of 35%

and 40% were mixed and centrifuged at 45,000 rpm at 4 °C for 7 h and the pellets were diluted and preserved at −80 °C.

4.4. Assays for measuring the inhibition activity of compounds against DHBV polymerase

Oligo(dT)$_{15}$ primer (TaKaRa Co., Otsu, Japan) was immobilized via its 5′-terminal phosphate to Covalink-NH microtiter plates (NUNC Co., Roskilde, Denmark). Then 10 Âμl of inhibitors, 20 Âμl of PBS containing enzyme and 70 Âμl of buffer were mixed in each well on the plate. The mixture contained 17.5 μg/ml poly (A), 8 μM dTTP, 2 μM biotin-11-dUTP, 5 mM dithiothreitol, 17 mM $MgCl_2$, 65 μg/ml Albumin from bovine serum (BSA), 50 mM Tris-HCl (pH 8.3), 75 mM KCl. After incubation at 37 °C for 1 h and washing, 100 μL 1% BSA was added and incubated for 1 h at room temperature. After washing again, 50 μL of alkaline phosphatase streptavidin (SA-ALP, 100 ng/ml) solution was added to the well, incubated for 1 h at 37 °C and then was washed. After SA-ALP reaction, a chemiluminescent substrate, *p*-nitrophenyl phosphate, disodium (PNPP) was added and finally 1N NaOH was added to stop the reaction. The plate was measured at an absorbance of 405 nm.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2018.02.003.

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