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Non-nucleoside anti-HBV agents: advances in structural optimization and mechanism of action investigations

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Hepatitis B is an infectious inflammatory disease of the liver, which is caused by the hepatitis B virus (HBV). Nowadays, the dramatic development of new HBV inhibitors is focused on discovering diverse non-nucleoside compounds with either novel structures or new mechanisms of action. In this review, we focus on the recent advances in discovery, structural modifications and biological activities studies of several distinct classes of synthetic non-nucleoside small molecular compounds with new mechanisms.

Introduction

Hepatitis B virus (HBV) is one important member of the *Hepadnaviridae* family. Chronic HBV infection is one major source of liver diseases like chronic hepatic insufficiency, cirrhosis, and hepatocellular carcinoma (HCC).[1] Currently, more than 240 million individuals worldwide are chronically infected with HBV according to the database of the World Health Organization (WHO), of whom 780 000 people will eventually die of acute or chronic liver diseases or HCC caused by HBV every year. An effective vaccine has been successfully developed and used for inoculations worldwide, but for those patients already infected with HBV, novel inhibitors against HBV are urgently in need.

Three types of particles are produced during the HBV infection, and the infectious one of the three is referred to as the *Dane* particle. This virion is comprised of 3.2 kb partially double-stranded relaxed circular DNA (rcDNA).[2,3] Infection of hepatocytes with HBV results in the formation of covalently closed circular DNA (cccDNA) in the nuclei of the cells. The genome of HBV consists of four overlapping open reading frames (ORFs): P, C, S and X. The polymerase translated from the P gene is the key protein for genome replication and consists of four domains: terminal protein, spacer, reverse transcriptase (RT) and Ribonuclease H (RNase H). The hepatitis B core antigen (HBcAg) and the hepatitis B e antigen (HBeAg) are both encoded by the C ORF. The S gene encodes for three surface proteins: large, medium and small hepatitis B surface antigens (HBsAg). Lastly, the X ORF encodes protein X (transcriptional co-activator). The P protein is the most attractive target in current anti-HBV chemotherapy, due to the indispensable role that RT plays in the HBV replication.[2–4]

Currently, clinically available drugs against HBV infection approved by the FDA comprise interferons (Interferon α and Pegylated Interferon α-2a) and nucleos(t)ide analogues (Lamivudine, Clevudine, Adefovir, Entecavir, Telbivudine, and Tenofovir, Fig. 1).[5,6] Interferons can develop the immune ability of patients, but are not ideal due to their adverse side effects and low cure rate. Nucleos(t)ide analogues are reverse transcriptase inhibitors (NRTIs), which target the HBV polymerase and effectively restrain the most important progress of the life cycle of the virus particle: HBV DNA replication. Regrettably, NRTIs are far from perfect because of the mutation domain of the reverse transcriptase. The emergence of drug-resistant virus strains and the appearance of severe side effects[7–9] encourage the development of novel non-nucleoside analogues targeting non-polymerase viral or host proteins. After years of effort, much progress has been achieved in the discovery of natural products and the synthesis of compounds for use as potent anti-HBV agents.[10,11] Natural product-derived compounds can be used with existing anti-HBV agents to reduce the possibility of drug resistance and show a synergetic effect. In contrast, the ease of synthesis of small synthetic heterocyclic molecules and their drug-like characteristics, offer a distinct advantage over natural compounds. Recent work about the application of heterocyclic compounds such as pyrimidines, benzimidazoles, phenylpropenamides, thiazolides, quinolines, 2-pyridones and sulfanilamides have achieved promising approaches, and further research in this area may lead to more efficacious and safer anti-HBV drugs. In this review, these representative small

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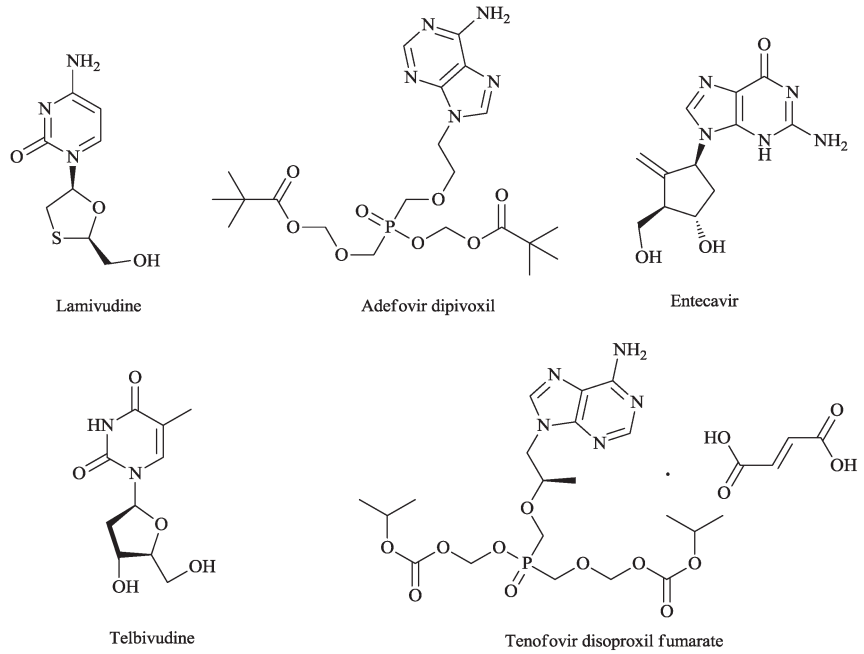

Fig. 1 Nucleos(t)ide drugs approved by the US FDA for the treatment of HBV.

synthetic heterocyclic molecules with potent anti-HBV activity will be discussed.

Dihydropyrimidines

Heteroaryldihydropyrimidines (HAPs, Fig. 2), first developed by Bayer investigators in a cell culture-based screening, were identified as a novel class of HBV inhibitors, *in vitro* and *in vivo* (animal models), with potency at nanomolar range. Assembly of the HBV capsid is a critical step in virus production and an attractive target for new antiviral therapies. HAPs target the life cycle of core assembly and RNA packaging. Moreover, HAP compounds prevent the proper formation of viral core particles (nucleocapsids), which are sites for viral DNA replication, and thus suppress HBV replication. Core particles are stable, high molecular weight aggregates assembled from HBV core protein subunits, which are sites for viral DNA replication.[12–14] Subsequent studies have demonstrated that HAPs can attach to HBV core proteins and disturb the normal assembly process of the capsid *in vitro*, rather than affecting the protein synthesis.[15,16]

Compound **1** (HAP1) is a variant of the original HAP structure.[14,17] Compound **2** (BAY41-4109, IC_{50} = 0.05 μM, TC_{50} = 7 μM) and its congeners **3** (BAY38-7690, IC_{50} = 0.15 μM, TC_{50} = 50 μM) and **4** (BAY39-5493, IC_{50} = 0.03 μM, TC_{50} = 25 μM) were discovered as highly potent inhibitors of HBV replication.[13] In HBV transgenic mouse models, the candidate BAY41-4109 has a favourable efficacy and a suitable pharmacokinetic and toxicological profile.[12] This compound also displayed antiviral properties against HBV in humanized Alb-uPA/SCID mice.[16] Currently, BAY41-4109 is undergoing a phase I study by AiCuris in Germany, but the clinical result is not clear.

Compound **5** (GLS4, IC_{50} = 0.012 μM), a new member of the HAP family, was developed from BAY41-4109. It is a potent inhibitor of both wild-type and adefovir-resistant HBV mutant strains in cell culture. Due to the good pharmacokinetic behaviors and tolerance GLS4 is currently undergoing phase I clinical trials in China.[18–20]

In order to gain insight and to correlate *in vitro* assembly with HBV replication in cell culture, Zlotnick *et al.*[21] prepared a closely related series of HAPs and studied their structure–activity relationships (SARs) in 2008. Larger aryl motifs at the 2-position on the pyrimidine ring could decrease the anti-HBV activity and the position of the pyridyl nitrogen atom was an important factor. The antiviral activity was susceptible to alterations in the 2-chloro-4-fluoroaryl moiety at the 4-position on the pyrimidine ring. As revealed by the complexed crystal structure, the 6-methyl group of the HAP1 core faces a hydrophobic tunnel.[21,22] The isopropyl congener was less potent, while replacement of a phenyl, chloro or substituted amino group with the 6-methyl group, was well tolerated. These derivatives with various lengths of amine substituents at position 6 were evaluated *in vitro* for their effects on the thermodynamics and kinetics of assembly. In 2013, Zlotnick *et al.* indicated that both kinetics and thermodynamics were critical to preventing formation of virions, but aberrant assembly was not required.[23] A SAR study revealed that having nonpolar or unprotonated amine groups near the dihydropyrimidine core and polar groups farther away was favorable because of the hydrophobic environment of the HAP pocket.[21]

Based on the SAR results of HAPs, in 2009 Yang's group[17] designed and synthesized a series of novel 2,4-diaryl-4,6,7,8-tetrahydroquinazolin-5(1*H*)-one derivatives with potent activity towards inhibiting HBV capsid assembly. It was consistently shown that the activity of these derivatives was sensitive to

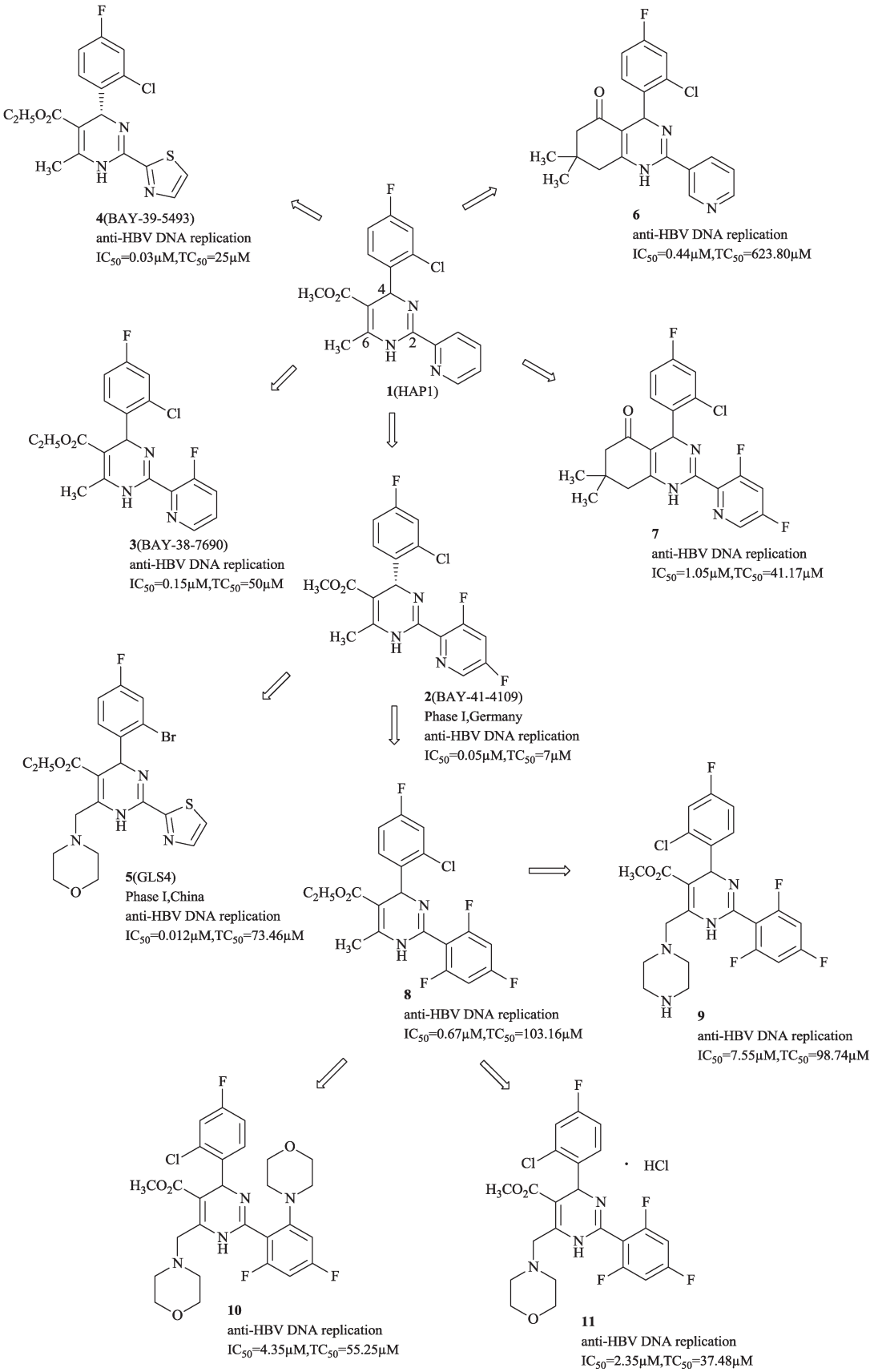

Fig. 2 Structures of dihydropyrimidine derivatives.

the positioning of the nitrogen atom at the 2-position on the pyridyl ring in the compound. Only the compounds containing pyrazin-2-yl and 3-fluoropyridin-2-yl rings showed increased activities against HBV replication. The activities had little tolerance to the changes made to the 4-phenyl rings and the 2-chloro-4-fluoroaryl group showed the best activity. Yang and coworkers found that compounds **6** (IC_{50} = 0.44 μM, TC_{50} = 623.80 μM) and **7** (IC_{50} = 1.05 μM, TC_{50} = 41.17 μM) could be used as lead compounds for further modification.

To improve the poor water solubility of HAPs, in 2014, Li *et al.*[24] designed and synthesized a series of novel HAP analogues with both good water solubility and high antiviral activities based on preliminary SARs. The new HAPs showed potent inhibition of HBV DNA replication at submicromolar concentrations and much higher activity than the control drug Lamivudine (IC_{50} = 14.17 ± 1.43 μM, TC_{50} = 5.75 μM). In their earlier report, owing to the 2,4,6-trifluorophenyl substitution, compound **8** (IC_{50} = 0.67 μM, TC_{50} = 103.16 μM) was the most potent. 2,4,6-trifluorophenyl substitution and 4-(2-chloro-4-fluoro-phenyl) substitution were reserved, and a medium or long nitrogenous heterocyclic group was introduced on the HAP core to orient the hydrophobic pocket. A SAR study highlighted that anti-HBV activity largely depends on the size, length and character of substituents at the 5-position of the core. Among this series, compounds **9** (IC_{50} = 7.55 μM, TC_{50} = 98.74 μM), **10** (IC_{50} = 4.35 μM, TC_{50} = 55.25 μM) and **11** (IC_{50} = 2.35 μM, TC_{50} = 37.48 μM) displayed the most potent activity.[24]

Thus, overall, Bay 41-4109 and GLS4 may be used as candidates to develop additional antiviral compounds that can target virus-encoded proteins other than polymerase, and will likely result in the establishment of combination therapies that will limit the appearance of virus resistance during chronic infections.

Benzimidazoles

Benzimidazoles can act in a similar manner to purines, and thus have the therapeutic potential to elicit some biological responses. Benzimidazole derivatives are of wide interest because of their diverse biological activities and clinical applications.[25] In a random screening, compound **12** (Fig. 3) was identified as an inhibitor of HBV, with a moderate antiviral potency (IC_{50} = 14.2 μM) against HBV DNA and low cytotoxicity (CC_{50} = 200 μM) *in vitro* using Lamivudine (IC_{50} = 0.38 μM, SI > 2632) and Adefovir (IC_{50} = 1.3 μM, SI = 156) as positive controls.

In order to further study the SARs of the related derivatives, Lu *et al.*[26] synthesized a series of novel benzimidazole derivatives and evaluated their anti-HBV activities and cytotoxicities in the HepG2.2.15 cell line in 2006.

This subseries has different patterns of substitution on the fused phenyl ring of the benzimidazole pharmacophore. A SAR study revealed that the chloro analogue was much less toxic than the corresponding hydrogen and fluoro analogues. The *N*-1 substituent is an important determinant of antiviral activity and was compatible with a broad range of substituents (nitro, methyl, trifluoromethyl, methoxyl, or isopropyl) with different lipophilic, electronic, and steric characters. Among them, compound **13** (IC_{50} = 2.9 μM, SI = 299) was selected as the benchmark compound for further optimization. Removal of the *N*-1 substituent resulted in a tremendous loss of activity. Replacement of the sulfonyl group with alkyl substituents at the *N*-1 position could dramatically increase antiviral activity and selectivity. The *N*-methyl derivative **14** (IC_{50} = 0.9 μM, SI > 1111) and the 4-methylbenzyl analogue **15** (IC_{50} = 0.7 μM, SI > 714) exhibited more highly potent anti-HBV activities and much higher SI values than those of compound **13** (IC_{50} = 2.9 μM, SI = 299) or Adefovir (IC_{50} = 1.3 μM, SI = 156). Besides, introducing 4-methylbenzoyl to the *N*-1 position of the benzimidazole core could decrease the cytotoxicity dramatically. The alkyl linker between the benzimidazole and phthalimide moieties was important for potent antiviral activity, and the methylene and propylene analogues were more toxic. But compound **16** (IC_{50} = 0.5 μM, SI = 180), with good potency, is the exception. The results of replacing the phthalimide group of the compound with phthalimidine, maleimide and different types of functional groups, suggested that the carbonyl group of the phthalimide and the presence of amides at the end of the alkyl linker were important features in conferring relatively low cytotoxicity, and that the phenyl moiety in the phthalimide group plays a less important role in its potent inhibitory activity. Compound **17** (IC_{50} = 0.68 μM, SI = 49) exhibited favourable antiviral activity.[26]

Unfortunately, further research indicated that compounds **14** and **15** had very low water-solubility and low oral bioavailability. In another series of benzimidazole derivatives that were screened, compound **18** (IC_{50} = 1.37 μM, SI = 131.8), with a pyridine moiety showed better water-solubility and oral bioavailability than its previous counterparts. In 2010, Lu *et al.*[27] chose compound **18** as the new benchmark compound for subsequent optimization. Then numerous compounds were prepared and assessed for their anti-HBV activity and cytotoxicity *in vitro*. The results further proved that derivatives with relatively good activities should bear a flexible two-carbon alkyl linker. Consistently, the *N*-1 position of the benzimidazole core tolerated a broad range of substituents with different steric characteristics. The 2-pyrrolyl analogue, compound **19** (IC_{50} = 0.41 μM, SI = 81.2), exhibited significant increased antiviral activity, and a nearly three-fold higher selectivity than Lamivudine (IC_{50} = 0.16 μM, SI = 31.3).

To further identify active compounds, Lu's group continuously screened their in-house compound library. Compound **20** (IC_{50} = 4.0 μM, SI = 27.0) was chosen as the lead compound, which was then mainly modified at positions 5- and 6- on the fused phenyl ring of the benzimidazole core. A SAR study showed that the substituent on the benzoyl ring was important at the 6-position. The halide groups were more active than the methyl group and no substituent on the phenyl resulted in a complete loss of activity. The safety profiles and potent anti-HBV activity of

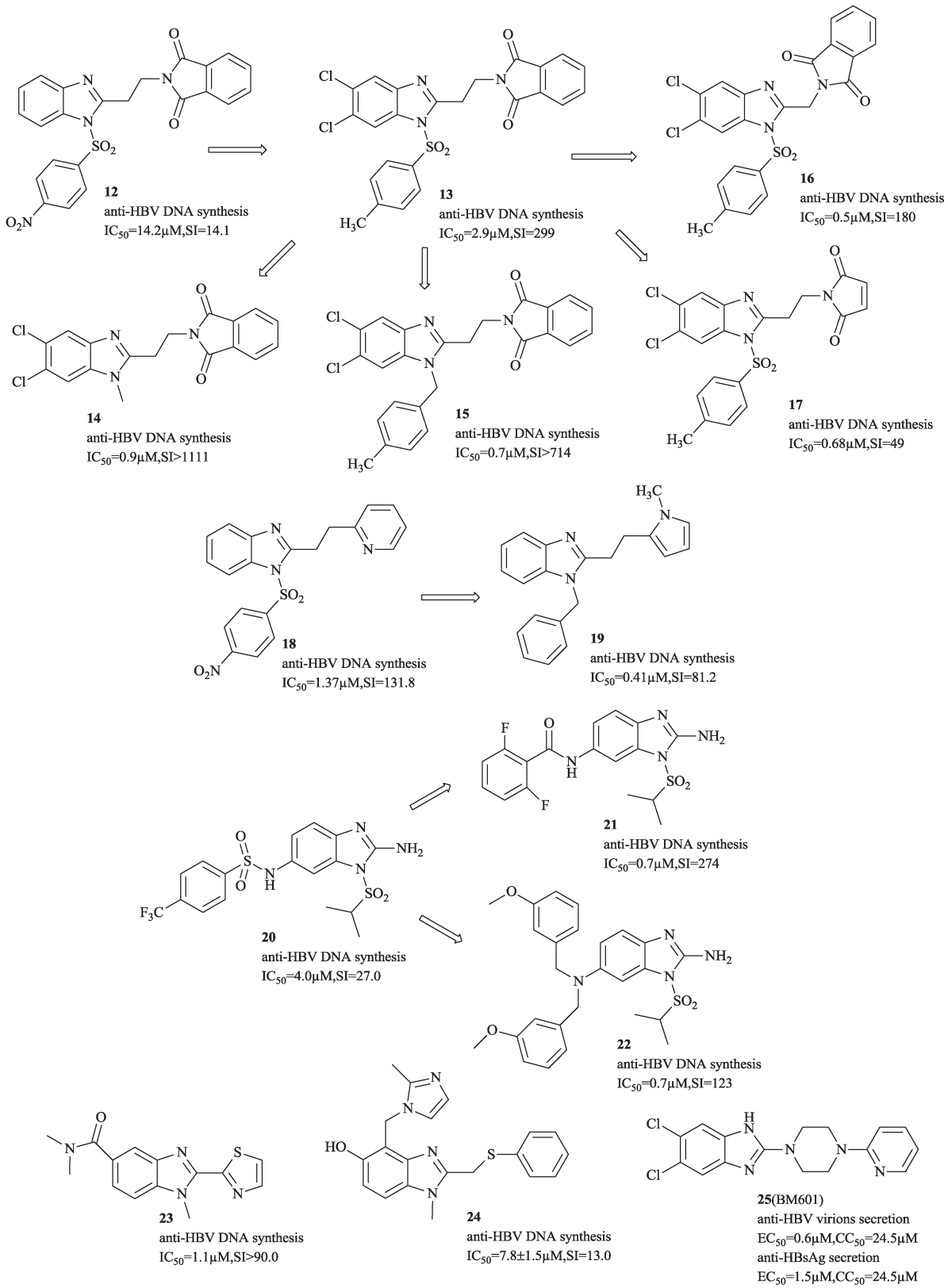

Fig. 3 Structures of benzimidazole derivatives.

the most promising compounds **21** (IC_{50} = 0.7 μM, SI = 274) and **22** (IC_{50} = 0.7 μM, SI = 123) demonstrated the potential of this series of benzimidazoles for the development of new anti-HBV drugs.[28]

In Lu's group, another series of novel thiazolyl-benzimidazole derivatives was synthesized and evaluated for their anti-HBV activity and cytotoxicity on the HepG2.2.15 cell line in 2011. Finally, the most promising compound **23** (IC_{50} = 1.1 μM, SI > 90.0) was selected as a benchmark compound for further investigation.[29]

In 2010, Gong *et al.*[30] synthesized a series of novel 1-methyl-1*H*-benzimidazol-5-ol derivatives and evaluated their anti-HBV activity and cytotoxicity in the HepG2.2.15 cell line, using Lamivudine (IC_{50} = 240.0 ± 2.2 μM, SI = 9.1) as a control reference. Some of the analogues in this series exhibited an inhibitory activity superior to that of Lamivudine. Of them, compound **24** (IC_{50} = 7.8 ± 1.5 μM, SI = 13.0) was the most prominent.

As HBsAg plays an important role in the life cycle of HBV, Tang's group[31] used ELISA to detect HBsAg quantitatively in 2014. They undertook a high-throughput screening (HTS) of their molecular library and found a series of benzimidazole derivatives. Further study confirmed that benzimidazole derivatives can decrease the secretion of HBV virions and HBsAg. Compound **25** (BM601), was identified to inhibit the secretion of HBV virions and HBsAg, with EC_{50} values of 0.6 μM and 1.5 μM, as well as a CC_{50} of 24.5 μM. BM601 has no effect on transcription, protein production, nucleocapsid formation or intracellular HBV DNA synthesis. Furthermore, BM601 does not trigger a cellular stress response or affect HBeAg or host protein secretion.

This was the first time that benzimidazole derivatives were reported as virion and HBsAg secretion inhibitors. BM601 could be used as a model drug to investigate the secretion pathway of HBV and shed light on anti-HBV studies of benzimidazole derivatives.[31]

Phenylpropenamides

In a random screening, a series of phenylpropenamide derivatives (Fig. 4) displayed potent anti-HBV activity in the HepAD38 cellular line. In addition to high levels of potency, these compounds generally exhibited low cellular toxicity.[32,33] The antiviral activity of phenylpropenamide derivatives was

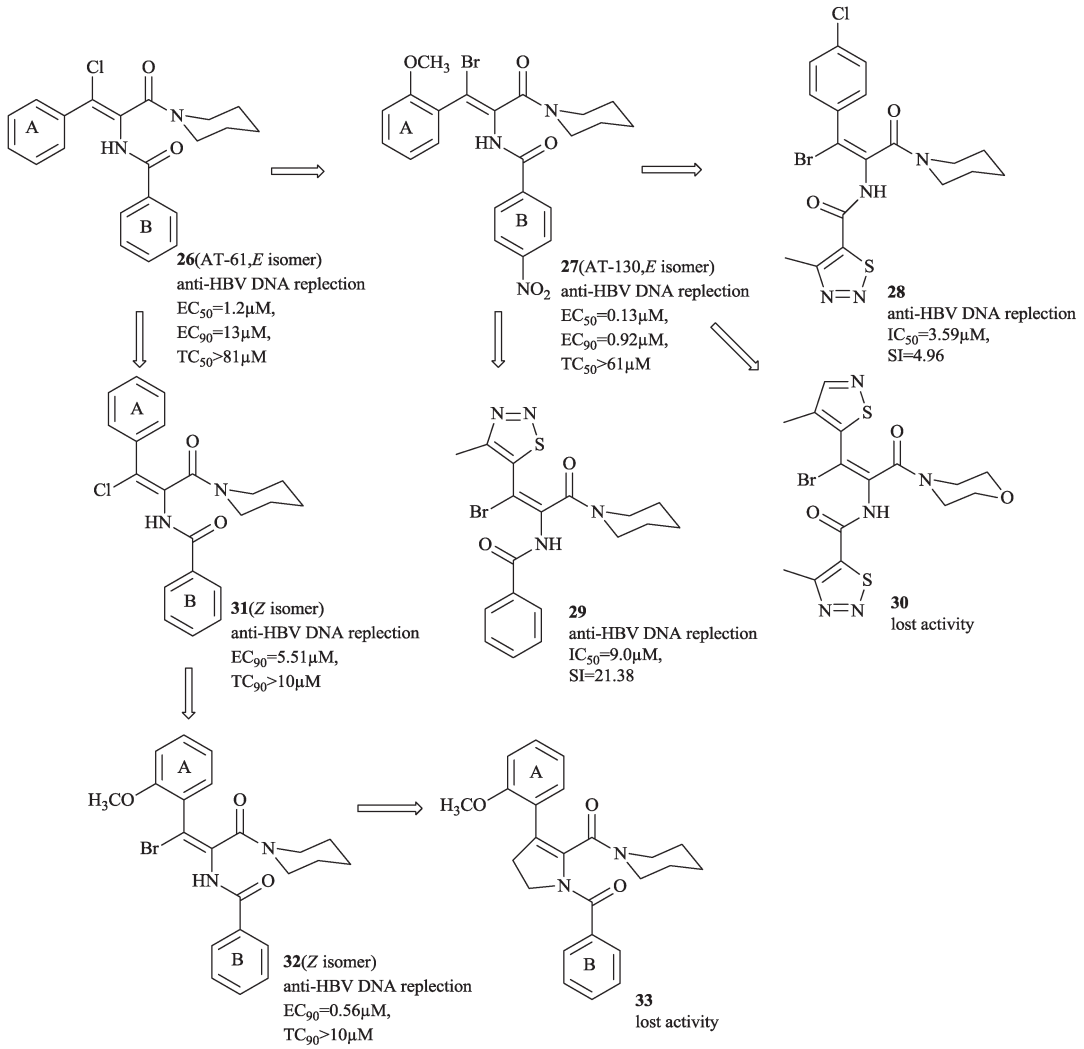

Fig. 4 Structures of phenylpropenamide derivatives.

specific for HBV replication and most likely occurs at one of the steps between the synthesis of viral RNA and the packaging of pregenomic RNA into immature core particles.[33] Initially, this mechanism was considered to interfere with the packaging of pregenomic RNA into the immature core particles, and not the HBV polymerase. Further research revealed an assembly effector mechanism underlying the apparent blocking of RNA packaging.[34,35]

In 2000, B. Perni *et al.*[32] synthesized a series of phenylpropenamides and evaluated their antiviral activity in the HepAD38 cellular assay. The SAR study on rings A and B of the initial hit compound **26** (AT-61, EC_{50} = 1.2 μM, EC_{90} = 13 μM, TC_{50} > 81 μM) was summarized. Uniformly, various secondary and tertiary amides were much less active than the corresponding piperidine amides. Generally, replacement of vinyl chlorides with vinyl bromides resulted in more favourable activity and less toxicity. In general, substituents at the 2-position of ring A or B had little effect on the activity. As a steric effect, substituents at the 4-position of ring A decreased the activity, while electron-withdrawing substituents at the 4-position of ring B improved the inhibitory activity. Most compounds displayed no toxic effects, even at the highest concentrations tested. SAR studies showed that having substituents on both rings will benefit the antiviral activity. The combination of a suitable 4-substituent (nitro or halo) on ring B with a 2-fluoro or 2-methoxy moiety on the ring A led to the most potent compounds in this series. Among them, compound **27** (AT-130, EC_{50} = 0.13 μM, EC_{90} = 0.92 μM, TC_{50} > 61 μM) showed the best activity, which is currently in preclinical trials.[32] Besides, AT-61 and AT-130 also displayed antiviral activity against the YMDD (tyrosine–methionine–aspartate–aspartate) motif of the Lamivudine resistant variant *in vitro*.[36] As shown in Fig. 5, AT-130 was bound to a hydrophobic pocket in the HBV capsid formed by Tyr118, Trp102 and Pro25 and formed multiple connections with the binding site. Two carbonyl groups formed hydrogen bonds with the side chains of Trp102 and Ser106, respectively. Besides these, the terminal nitro group also engaged in multiple hydrogen bonds with the backbone carbonyl of Asp29 and the side chain hydroxyl group of Thr33. Collectively, this HBV capsid/AT-130 complex provided critical insight into the molecular basis of the high affinity and gave valuable clues for further structure optimization.

Based on the bioisoteric replacement of the phenyl group in phenylpropenamide with 1,2,3-thiadiazolyl, Zhao *et al.*[37] designed and synthesized a series of new acrylamide derivatives containing 1,2,3-thiadiazole and assessed their anti-HBV activities and cytotoxicity *in vitro*, using Lamivudine (IC_{50} = 14.80 μM, SI = 64.97) as a positive reference. When replacing rings A or B of compound AT-130 with 4-methyl-1,2,3-thidiazole, compounds **28** (IC_{50} = 3.59 μM, SI = 4.96) and **29** (IC_{50} = 9.0 μM, SI = 21.38) showed potent anti-HBV DNA activity. However, when simultaneously replacing the A and B rings of AT-130 with 4-methyl-1,2,3-thidiazole (compound **30**), the anti-HBV activity was lost and the cytotoxicity was higher.

In 2011, J. Sofia and co-workers[38] prepared a series of substituted arylpropenamide derivatives and separated the *E* and *Z* geometrical isomers. Contrary to previous reports, the activity of this class of molecules was ascribed to the *Z* isomer. Further SAR studies on the active *Z* isomer (compounds **31** and **32**) identified back-up derivatives with potent antiviral activity against HBV. Consistent with previous SAR studies, suitable substituents and their position on both the A and B rings can improve the activity. Moreover, unsubstituted piperidine and pyrrolidine amides were preferred. To maintain the overall conformation of the molecule, novel rings were introduced. Unfortunately, ring constrained analogues (compound **33**) did not lead to active HBV inhibitors.

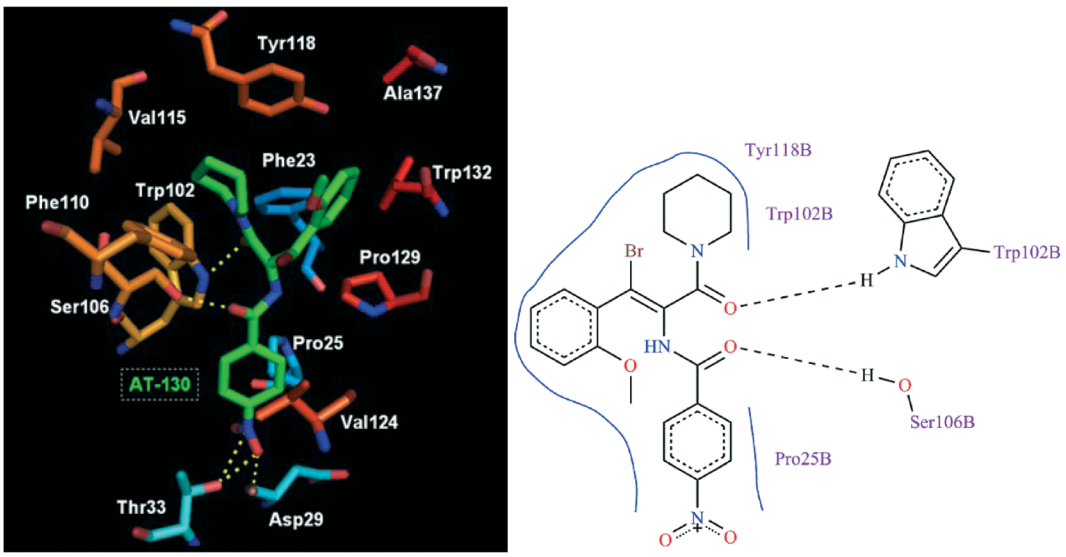

Fig. 5 A pictorial representation of the HBV capsid complexed with AT-130 (carbon: colored in green) (PDB code: 4G93). The figure was generated using PyMol (www.pymol.org).

Triazolo-pyrimidines

Triazolo-pyrimidine derivatives (Fig. 6) potentially target the HBV replication cycle of coating and secretion. In a HTS of a synthetic small-molecule library containing 80 288 compounds, compound **34** (HBF-0259) was identified as a specific and novel inhibitor of HBV.[39] HBF-0259 had no effect on HBV DNA synthesis, which was independent of viral genomic replication. Interestingly, HBF-0259 (EC_{50} = 1.5 μM, SI = 13.0) had a moderate antiviral activity, and it displayed no signs of toxicity through serum chemistry analysis and desirable pharmacokinetic profiles in male Sprague–Dawley rats.[40] HBF-0259 could inhibit the secretion of HBsAg, but had no effect on HBeAg secretion and HBV replication. It also exhibited slight accumulation of intracellular L and M, showing a different mechanism from BM601, which specifically inhibited HBsAg secretion without affecting the glycosylation of all three kinds of surface proteins or inducing any ER chaperone variation.[31,39] Thus, HBF-0259 was seen as a promising anti-HBV molecule aimed at potentiating the immune response by suppressing antigenemia.[40]

Due to its tetrahydro-tetrazolo-pyrimidine structure, HBF-0259 could undergo rearrangement *via* its open azide form, resulting in another isomer. To block this rearrangement process, Cuconati *et al.*[40] designed and synthesized the triazole analogue **35** by replacing *N*-2 with a methenyl group (on the A ring), and successfully addressed the potential stability issue. The potency of compound **35** (EC_{50} = 4.1 ± 2.1 μM, CC_{50} > 50 μM) was almost 3-fold more active than HBF-0259. No cytotoxicity was observed for either analogue up to the highest concentration tested (50 μM). Although there are two chiral centers present in the molecule, the triazolo-pyrimidine analogue **35** existed only as a pair of thermodynamically more stable *cis*-enantiomers. Neither of the enantiomers exhibited cytotoxicity up to 50 μM, and all the other compounds were tested as a *cis* racemic mixture in their study.

Cuconati *et al.*[40] synthesized a series of novel triazole analogues and evaluated their anti-HBV activities and cytotoxicities in the HepG2.2.15 cell line. The SAR study on the C ring indicated that the chloro/fluoro group at the *ortho* position was necessary for the inhibitory activity of HBsAg secretion. Introduction of an electron donating group instead of a halide on the C ring decreased the potency. Removal of the halide groups on the D ring significantly decreased the activity. However, it is noteworthy that biphenyl and 2-naphthyl analogues without halide motifs on the side chain exhibited remarkable potency.

Further structural modification of ring B in compound **35**, through the incorporation of double bonds, led to analogues **36** and **37**. Compound **36** (EC_{50} = 2.3 ± 2.1 μM, CC_{50} > 50 μM), with only one chiral center, had a modest, but consistently better activity compared with that for compound **35** in the cell culture assays. However, the nonchiral analogue **37** completely lost its activity.[40]

For this series, a SAR study indicated that 2,6-dihalide was the optimal substituent on the C ring, and on the D ring, *para* and *meta* chlorination equally improved the activity. Overall, the best compound was the difluoro analogue **38** (EC_{50} = 1.4 ± 0.4 μM, CC_{50} > 50 μM).[40]

Quinolin-2-ones (Quinolines)

As a part of the Cheng group's continuous search for active anti-HBV leads from natural sources and synthetic compounds, a rational screening suggested that compound **39** (4-aryl-6-chloro-quinolin-2-one) possessed moderate activity (IC_{50} = 0.458 mM, SI = 2.6) towards inhibiting the production of HBsAg in HBV-infected HepG2.2.15 cells.[41] A series of

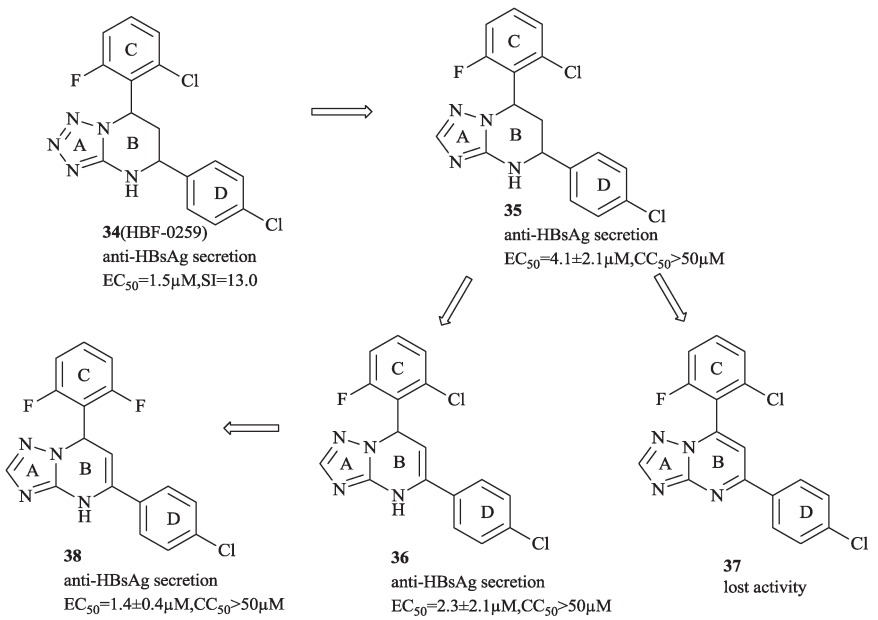

Fig. 6 Structures of triazolo-pyrimidine derivatives.

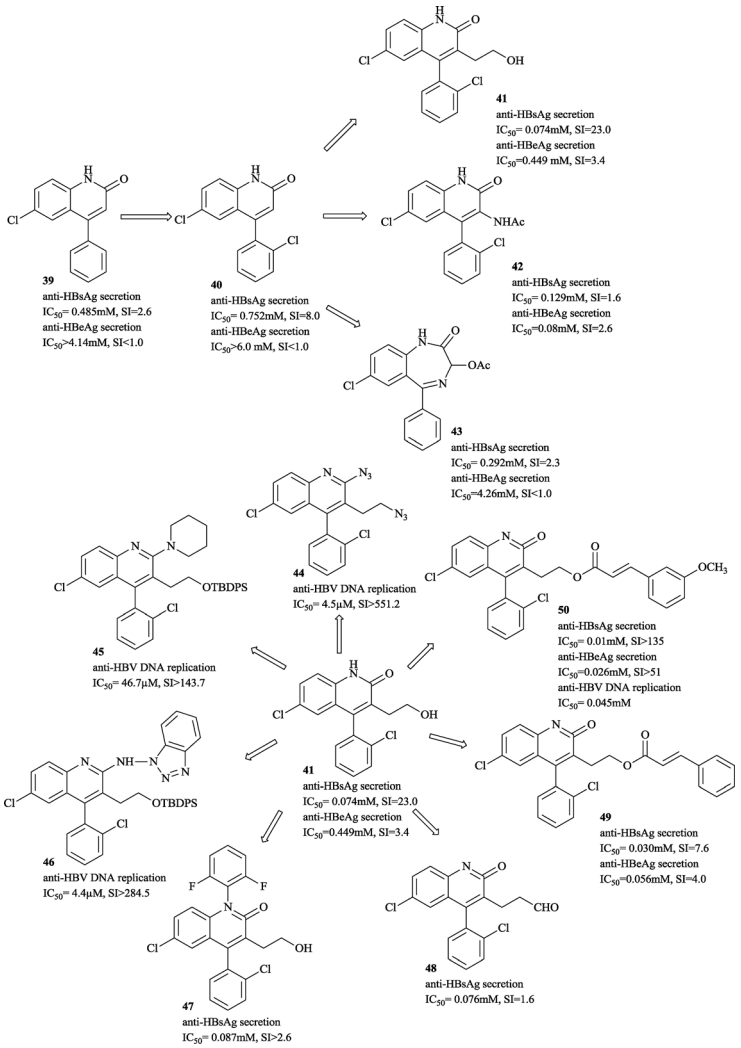

Fig. 7 Structures of quinolin-2-one and quinoline derivatives.

4-aryl-6-chloro quinolin-2-ones and 5-aryl-7-chloro-1,4-benzodiazepines (Fig. 7) were synthesized and evaluated for anti-HBV activities and cytotoxicities for the first time *in vitro*. Cheng *et al.*[41] mainly modified at *C*-3 of compound **39** to get 3-substituted-quinolin-2-ones, and at the quinoline ring *via* ring expansion to afford 5-aryl-7-chloro-1,4-benzodiazepines. The analogues bearing a halide (2-fluoro or 2-chloro) on the phenyl ring exhibited lower cytotoxicities, especially compound **40** (anti-HBsAg secretion IC_{50} = 0.752 mM, SI = 8.0). Compound **41**, derived from hydroxyl ethyl introduction onto to *C*-3 of compound **40**, showed increased suppression of the secretions of HBsAg (IC_{50} = 0.074 mM, SI = 23.0) and HBeAg (IC_{50} = 0.449 mM, SI = 3.4), which could be chosen as a benchmark for further optimization. Besides, introducing an amino group onto *C*-3 of compound **40** both inhibitory activities against HBsAg secretion and cytotoxicities increased, and only compound **42** showed an SI_{HBeAg} value of 2.6. Compared with the quinolin-2-one derivatives, 1,4-benzodiazepines demonstrated increased cytotoxicities, almost the same activities of inhibition for HBsAg secretion and reduced suppression of HBeAg secretion. Among this subseries, the best one is compound **43** (anti-HBsAg secretion IC_{50} = 0.292 mM, SI = 2.3, anti-HBeAg secretion $IC_{50} > 4.26$ mM, SI < 1.0).

In 2011, using compound **41** as the lead, Guo *et al.*[42] synthesized a series of 4-aryl-6-chloro-quinoline derivatives and evaluated their anti-HBV activity using Tenofovir (IC_{50} = 0.54 μM, SI > 3222) as a positive control. Most of them showed moderate inhibitory activity against the secretion of HBsAg and HBeAg. Nine compounds exhibited potent inhibition of HBV DNA replication. The modification primarily targeted the 2-position and *C*-3 of the hydroxyethyl moiety. Owing to their low cytotoxicities, compounds **44** (IC_{50} = 4.5 μM, SI > 551.2), **45** (IC_{50} = 6.7 μM, SI > 143.7) and **46** (IC_{50} = 4.4 μM, SI > 284.5) possessed promising inhibition of HBV DNA replication. For *C*-2 substituted quinoline derivatives, the anti-HBV activity largely depended on the size and character of the substituents, and this position could accommodate some substituents without decreasing the anti-HBV activity. TBDPS (*tert*-butyldiphenylsilyl) substitution at the 3-position was an important feature, conferring relatively low

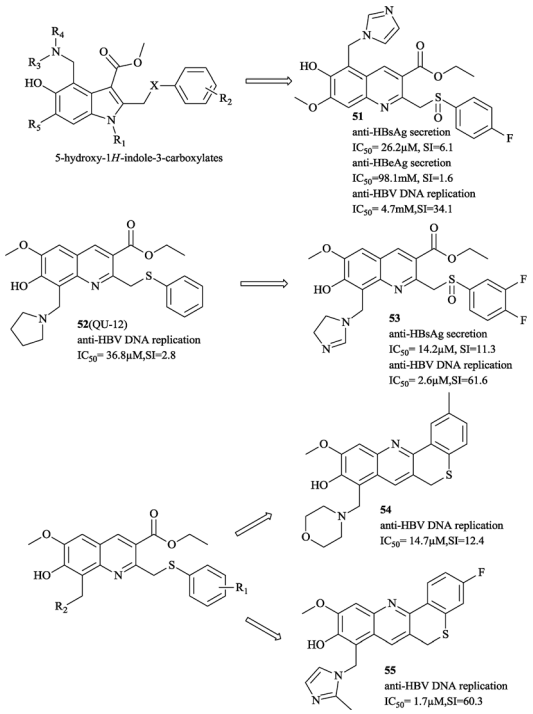

Fig. 7 *(continued)*

cytotoxicity to the compound. But for 2-*N*-substituted analogues, TBDPS substitution caused the compound to lose the ability to suppress the secretion of HBsAg and HBeAg. For 2-*O*-substituted analogues, etherification of the hydroxyl group at *C*-3 of 4-aryl-6-chloro-quinoline decreased the anti-HBV activity.

As part of their ongoing efforts, Guo and co-workers[43] synthesized a series of novel 6-chloro-4-(2-chlorophenyl)-3-(2-hydroxyethyl) quinolin-2(1*H*)-one derivatives *via* chemical modifications on *N*-1, *C*-2, and the hydroxyethyl moiety and evaluated their anti-HBV activities *in vitro* in 2011. Introducing different combinations of substituents (methyl, alkyl, benzyl, or 2,6-difluorobenzyl) to the *N*-1 position of compound **41** resulted in relatively low SI values. Only compound **47** (IC_{50} = 0.087 mM, SI > 2.6) retained potency against the secretion of HBsAg and had a low cytotoxicity (CC_{50} > 2.3 mM). Compound **41** was oxidised to its corresponding aldehyde **48** which retained the activity against secretion of HBsAg (IC_{50} = 0.076 mM, SI = 1.6) and lost the ability to suppress the secretion of HBeAg, whereas the carboxylic acid analogues showed decreased inhibition. A range of acyls with different lipophilic, electronic, and steric characters were tolerated by the hydroxyethyl moiety, and were potent suppressants of the secretion of HBsAg. Compound **49** exhibited 2.9-fold and 6.4-fold increased activities against the secretion of HBsAg (IC_{50} = 0.030 mM, SI = 7.6) and HBeAg (IC_{50} = 0.056 mM, SI = 4.0) in comparison with compound **41**. Different combinations of substitutions were introduced to the phenyl ring of the ester moiety, and the 3′-methoxyl substituted compound **50** with high potency against the secretion of HBsAg (IC_{50} = 0.010 mM), HBeAg (IC_{50} = 0.026 mM) and low cytotoxicity (CC_{50} > 1.3 mM), resulted in remarkable SI values (SI_{HBsAg} > 135, SI_{HBsAg} > 51). A preliminary mechanistic study suggested that compound **50** could mainly enhance the transcript activity of HBV enhancer I (ENI) and enhancer II (ENII). The presence of the phenyl ring on the ester moiety was tolerated, however, replacement of this phenyl moiety with an alkyl group or a pyridine ring resulted in a decrease in the antiviral activity of the resulting compounds in comparison with that for compound **49**. The length of alkyl linkers had little effect on the ability of a compound to suppress the secretion of HBsAg and HBeAg.

Another series of quinolones has been investigated in the Gong group since 2008. In their continuing work on seeking potent anti-HBV inhibitors, Liu *et al.*[44] designed and prepared some novel ethyl 6-hydroxyquinoline-3-carboxylate derivatives based on a series of 5-hydroxy-1*H*-indole-3-carboxylate derivatives studied previously.[45,46] Other changes mainly focused on the 2- and 5- positions on the quinoline ring. The presence of a fluorine atom at the 2-position on the phenyl ring could increase the HBV DNA replication inhibition ratio, while 2-pyridyl derivatives could eliminate anti-HBV activities completely. As for the Mannich groups pyrrolidinyl, piperidyl and imidazolyl, the presence of these at the 5-position on the ring was preferential, whilst for morpholinyl and 4-methyl piperazinyl groups it was not. Oxidation of the sulfide contained in the compounds to a sulfinyl group, had little influence on anti-HBV activity and cytotoxicity. Most of these compounds showed good antiviral activity, and compound **51** (IC_{50} = 4.7 μM, SI = 7.6) was the most potent, a highly specific inhibitor of HBV DNA replication in cell culture.[44] Meanwhile, taking compound **52** (QU-12, IC_{50} = 36.8 μM, SI = 2.8) as the lead compound, Liu and coworkers[47] synthesized several new ethyl 8-imidazolylmethyl-7-hydroxyquinoline-3-carboxylate derivatives

and evaluated their anti-HBV activity *in vitro*. Of them, compound **53** (IC_{50} = 2.6 μM, SI = 61.6) exhibited the best activity for inhibiting the replication of HBV DNA.

In 2009, a series of novel 6*H*-[1] benzothiopyrano [4,3-*b*] quinoline derivatives were prepared and evaluated for their anti-HBV activity and cytotoxicity in human hepatoblastoma-derived liver HepG2 cells by the Gong group[48] based on previous work from the literature.[44,47] The methyl group and the fluorine atom were tolerated at the 2-, 3- and 4-positions of the 6*H*-[1] benzothiopyrano [4,3-*b*] quinoline ring, and the methyl group at the R_1 position was preferential for potent anti-HBV activity. Mannich base functionalities significantly affected the anti-HBsAg activity. One member of this series, compound **54** (IC_{50} = 14.7 μM, SI = 12.4), was found to be more potent than Lamivudine on anti-HBV DNA activity.[48] In the meantime, another series of 9-methoxy-6*H*-[1] benzothiopyrano [4,3-*b*] quinolin-10-ols compounds with a Mannich side chain were synthesized and evaluated for their anti-HBV activity in HepG2.2.15 cells by Jia[49] and compound **55** (IC_{50} = 1.7 μM, SI = 60.3) was found to be the most effective.

The detailed mechanism of the quinolin-2-one's (quinolines) activity is still under on-going investigation.

Thiazolides

Compound **56** (Nitazoxanide, NTZ, Fig. 8), a drug with anti-infective activity against anaerobic bacteria, protozoa and viruses, is currently in phase II clinical development for treating chronic hepatitis C.[50–52] In 2008, Korba *et al.* had proved that NTZ (EC_{50} = 0.12 μM, SI > 121) was a potent HBV inhibitor demonstrating a synergistic effect in combination with either Lamivudine or Adefovir against the HBV *in vitro*.[51–53] NTZ and related agents represented a class of small molecules that modulate host antiviral pathways *via* protein kinase activation, thereby acting as interferon immune enhancers.[52] The complete elucidation of its mechanism of action is underway. Based on the previous work on NTZ and its analogues, Stachulski *et al.*[54] synthesized and evaluated a wide range of thiazolide analogues, particularly as selective antiviral agents. The modification mainly targeted the substituents on both of the rings and some related salicyloylanilides were developed. Compound **57** (TIZ, EC_{50} = 0.15 μM, SI > 172), the metabolite of NTZ, displayed potent activity. The SAR results showed that electron-withdrawing groups at C-5′ on the thiazole ring, especially 5′-nitro and 5′-halo, were favourable. The 5′-chloro analogue **58** (EC_{50} = 0.33 μM, SI = 43) could be selected for further development. Furthermore, The 5-methyl analogue **59** (EC_{50} = 0.33 μM, SI > 120) was slightly superior to its analogue **58**, but later proved to have an unfavorable metabolic/toxicity profile. Compound **60** (EC_{50} = 0.15 μM, SI > 166) exhibited potent anti-HBV activity. The Stachulski group also prepared and screened a set of salicyloyl anilides. Among this subseries, the mono- and bis-trifluoromethyl derivatives showed moderate activity and the trisubstituted **61** (EC_{50} = 0.28 μM, SI = 122) showed good activity.

2-Pyridones

In the process of screening an in-house library for anti-HBV activity, Li's group[55] identified compound **62** (IC_{50} = 20 μM, CC_{50} = 179 μM), a modest inhibitor of the HBV, as a candidate for further modification. Then, Sheng *et al.*[55] synthesized a series of novel 2-pyridone derivatives (Fig. 9) and evaluated their anti-HBV activity and cytotoxicity *in vitro*. Various substitutions (methoxy, chloro, iodo, nitro *etc.*) on the phenyl ring attached to the nitro atom of the 2-pyridones were important for the antiviral activity. Compounds **63** (IC_{50} = 0.206 μM, SI > 532) and **64** (IC_{50} = 0.8 μM, SI = 198.75) showed excellent antiviral activity against HBV DNA replication, which were more potent than the reference drug Adefovir (IC_{50} = 0.517 μM, SI = 1044.5). A nitro or acetyl group incorporated onto the 4-position of the phenyl ring led to a loss of anti-HBV activity. Alkyl groups, steric groups larger than the phenyl ring, would decrease the anti-HBV activity. Compound **65** (IC_{50} = 99.6 μM, SI = 0.15), with a

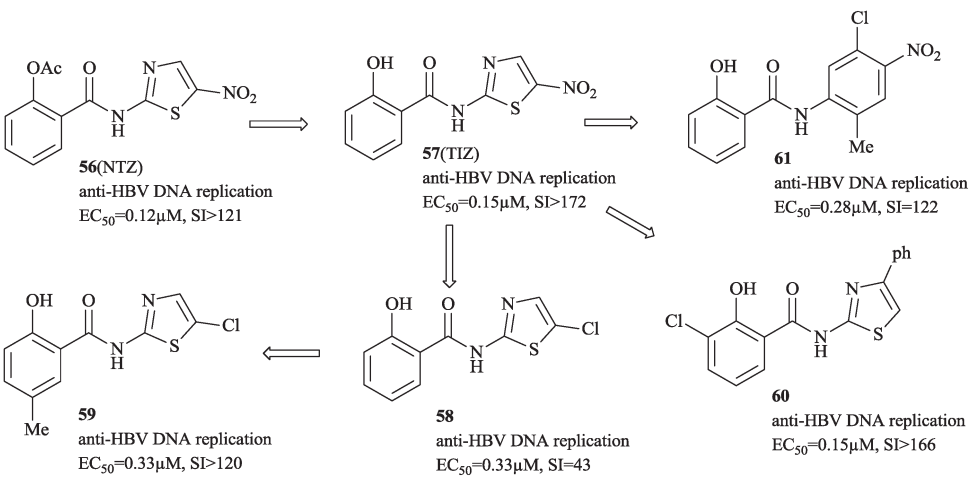

Fig. 8 Structures of thiazolide derivatives.

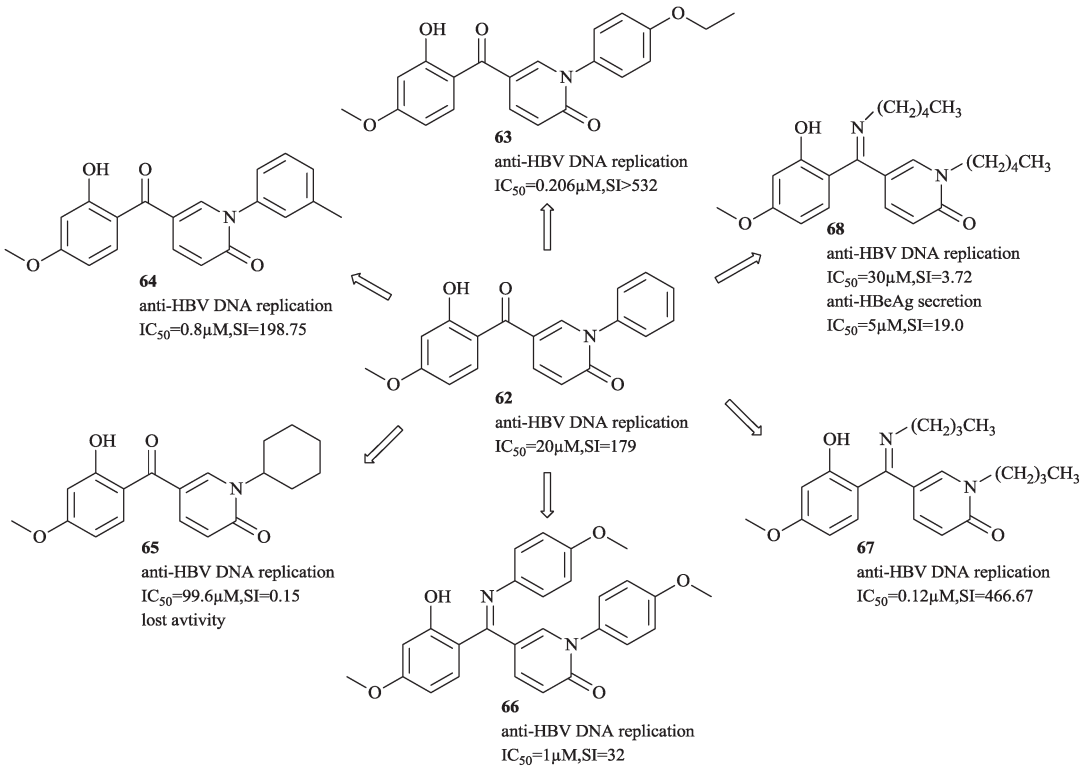

Fig. 9 Structures of 2-pyridone derivatives.

cyclohexyl group attached to the nitrogen atom of 2-pyridone, almost lost its activity against HBV DNA replication. The same substitutions on both the nitrogen atom of 2-pyridone and the imino group were introduced, and the dibenzyl or dialkyl substituted derivatives did not show improved anti-HBV activity. The results suggested that the imino groups were not indispensable for their anti-HBV activities. Compound **66** (IC_{50} = 1 μM, SI = 32), the most potent one in the dibenzyl subseries, showed a poor IC_{50} value against the HBV DNA replication, but it indicated that the methoxy group at the 4-position on the benzyl group was vital to the antiviral activity. It is interesting to note that compound **67** (IC_{50} = 0.12 μM, SI = 466.67), the dialkyl analogue, revealed the best antiviral activity for HBV DNA replication.

Meanwhile, Zhang and coworkers of the Li group synthesized four novel 5-substituted pyridine-2(1*H*)-one derivatives, which were highly efficient against HBV in cultured HepG2 2.2.15 cells. Especially, compound **68** (IC_{50} = 30 μM, SI = 3.72) which was the most potent, and showed preferable anti-HBeAg activity (IC_{50} = 5 μM, SI = 19.0).[56]

Considering this result, it is a pity that the mechanism of action is yet to be determined.

Sulfanilamides and benzenesulfonamides

In 2012, Guo *et al.*[57] screened an in-house small molecule library consisting of 85 000 drug-like compounds and confirmed compounds **69** (CCC-0975, EC_{50} = 4.55 μM, CC_{50} > 50 μM) and **70** (CCC-0346, EC_{50} = 0.35 μM, CC_{50} = 2.57 μM) as inhibitors of HBeAg production in HepDE19 cells (Fig. 10). Further mechanistic studies demonstrated that disubstituted sulfonamides interfered primarily with rcDNA conversion into cccDNA. It was the first attempt to identify small molecules that target cccDNA formation, and disubstituted sulfonamides could thus potentially serve as proof-of-concept drug candidates for development into therapeutics to eliminate cccDNA from chronic HBV infection.

In 2013, in a similar way, screening of a small molecules library led to the discovery of a series of sulfamoylbenzamides that significantly reduce the amount of cytoplasmic HBV DNA. Mechanistic analyses revealed that the compounds did dependably inhibit the formation of pgRNA-containing nucleocapsids of the HBV but not for other animal hepadnaviruses. SAR studies suggested that small cycloalkyl or open alkyl substitutions conferred to the most potent antiviral activity. Halide substitution, especially fluorine, onto both of the phenyl rings proved to be beneficial to the activity. Compound **71** (DVR-01) with a chlorine atom at the R_9 and a cycloheptyl ring at the R_x positions displayed moderate activity both in AML12HBV10 (EC_{50} = 1.7 μM, CC_{50} = 50 μM) and HepDES19 cells (EC_{50} = 1.6 μM, CC_{50} = 50 μM). Compounds **72** (DVR-23, in AML12HBV10 EC_{50} = 0.3 μM, CC_{50} > 50 μM, in HepDES19 cells EC_{50} = 0.1 μM, CC_{50} > 50 μM) and **73** (DVR-56 in AML12HBV10 EC_{50} = 0.39 μM, CC_{50} > 50 μM, in HepDES19 cells EC_{50} = 0.14 μM, CC_{50} > 50 μM) which shared common fluorine substituents, exhibited more potent activity. Further lead optimization efforts are being carried out in this group.[58] Besides this study, another group also chose sulfanilamides

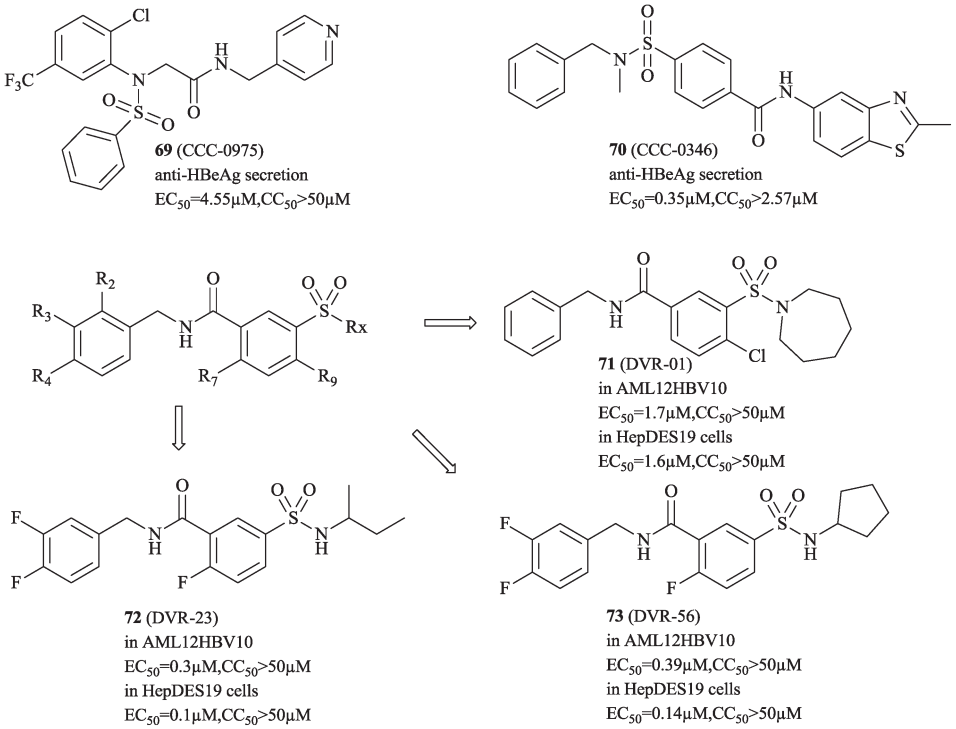

Fig. 10 Structures of sulfanilamides and benzenesulfonamide derivatives.

and benzenesulfonamides as molecular backbones. The resulting molecules were found to significantly change the protein conformation and reduce the assembly affinity of the core protein, leading to a decrease in the number of assembled capsids or virions.[59] Such a mechanism of action might provide a new therapeutic strategy to combat HBV infection.

Others

In a modified enzyme-linked immunosorbent assay, a chemical library consisting of 5600 compounds was screened for their interaction between the core protein and PreS region of the surface protein. KSG00011 (compound **74**, Fig. 11, EC_{50} = 5.4 μM, CC_{50} > 50 μM) and KKJ00626 (compound **75**, IC_{50} = 0.12 μM, CC_{50} > 50 μM) were identified as inhibitors from their inhibition of the production of HBV particles in Huh-7 cells transfected with HBV genomic DNA.[60]

NZ-4 (compound **76**, IC_{50} = 0.14 μM, CC_{50} > 50 μM), a derivative of bis-heterocycle tandem pairs from the natural product leucamide A, was active against the replication of various drug resistant HBV mutants. NZ-4 inhibits HBV replication by interfering with the interaction between pgRNA and

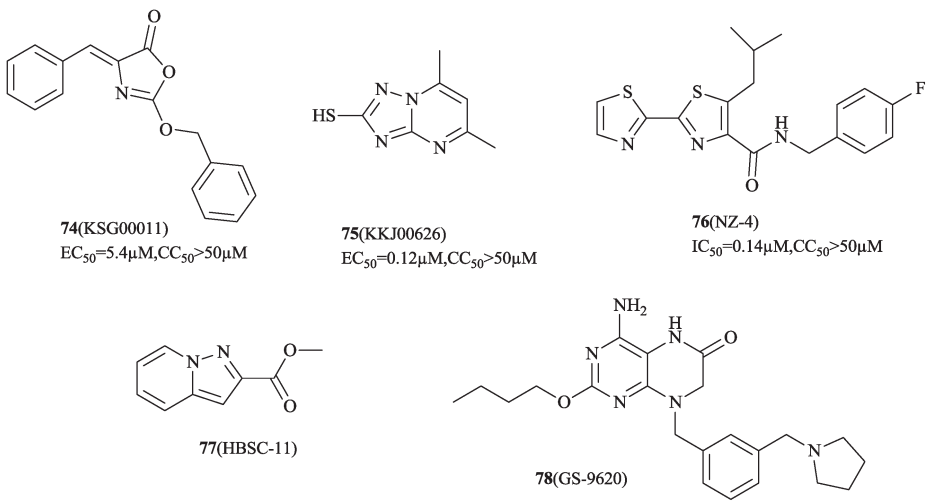

Fig. 11 Structures of other compounds.

HBcAg in the capsid assembly process, thus increasing the replication-deficient HBV capsids.[61–63]

The human La (hLa) protein, which forms a stable complex with HBV RNA ribonucleoprotein to promote HBV replication, is a promising target for molecular therapy. HBSC-11 (compound 77) was found to have an obvious inhibitory effect on hLa transcription and expression, and thus could be chosen as a benchmark to design novel inhibitors with a new mechanism.[64]

Pteridinone-based Toll-like receptor 7 (TLR7) agonists were identified as potent and selective alternatives to the previously reported adenine-based agonists, leading to the discovery of GS-9620 (compound **78**). GS-9620 is currently under clinical evaluation for the treatment of HBV infection.[65]

Conclusions

This review provided a fairly comprehensive overview of the structural modification, SARs and mechanisms of action for several classes of synthetic non-nucleoside molecules that are promising anti-HBV agents. Obviously, great achievements have been made in this field. However, currently, there are as yet no non-nucleoside compounds approved for the treatment of HBV infection. Therefore, there is still an urgent need to further develop novel, highly potent non-nucleoside anti-HBV agents with improved drug resistance profiles and favorable pharmacokinetic properties. Besides this, more efficient strategies that facilitate the drug discovery process would be extremely beneficial.

Nowadays, the progress of drug development for the treatment of HBV infection *via* a structure-based rational approach is being hindered by the deficiency of high-resolution crystallographic structures of key proteins in the HBV life cycle. Therefore, to date, advances in the medicinal chemistry of anti-HBV agents have mostly relied on a random screening approach and ligand-based modification. Structure modification *via* the bioisosteric replacement or scaffold hopping of the central core in bioactive molecules combined with the introduction of diverse substituents was regarded as a common practice in the current anti-HBV drug discovery field, in order to obtain intellectual property and novel drug candidates, as well as to improve synthetic accessibility.

Altogether, this review will provide valuable information for understanding the progress of the discovery of anti-HBV agents *via* ligand-based approaches.

We envisioned that with continued efforts in the development of computational methods and structural biology information on HBV proteins, structure-based approaches using a combination of medicinal chemistry strategies, structural biology, and computational chemistry will improve and help to facilitate anti-HBV drug discovery. Besides several other achievements including novel synthetic methodologies for privileged heterocycles, diversity-oriented synthesis (DOS) in combination with new HTS technologies (such as microarrays) can be effectively exploited to further revolutionize the lead discovery and optimization steps in anti-HBV drug research.

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