



Discovering novel direct acting antiviral agents for HBV using *in silico* screening



Yoshiki Murakami^{a,*}, Michiyo Hayakawa^a, Yoshihiko Yano^b, Toshihito Tanahashi^c, Masaru Enomoto^a, Akihiro Tamori^a, Norifumi Kawada^a, Mitsuo Iwadate^d, Hideaki Umeyama^d

^a Department of Hepatology, Graduate School of Medicine, Osaka City University, Osaka 545-8585, Japan

^b Division of Gastroenterology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan

^c Department of Medical Pharmaceutics, Kobe Pharmaceutical University, Kobe 650-0017, Japan

^d Department of Biological Science, Chuo University, Tokyo 112-8551, Japan

ARTICLE INFO

Article history:

Received 21 October 2014

Available online 15 November 2014

Keywords:

Hepatitis B virus

In silico screening

Reverse transcriptase

ABSTRACT

The treatments for chronic hepatitis B (CHB) are interferon and nucleoside analogues reverse transcriptase (RT) inhibitors. Because both treatments are less than ideal, we conducted to identify novel anti-viral agents for HBV-reverse transcriptase (HBV-RT). We determined the ligand-binding site of the HBV-RT by conducting a homological search of the amino acid sequence and then we also determined not only structural arrangement of the target protein but the target protein-binding site of the ligand using known protein–ligand complexes in registered in the protein data bank (PDB). Finally we simulated binding between the ligand candidates and the HBV-RT and evaluated the degree of binding (*in silico* screening). PXB cells derived from human-mouse chimeric mouse liver, infected with HBV were administrated with the candidates, and HBVDNA in the culture medium was monitored by realtime qPCR. Among compounds from the AKosSamples database, twelve candidates that can inhibit RT were also identified, two of which seem to have the potential to control HBV replication *in vitro*.

© 2014 Elsevier Inc. All rights reserved.

1. Introduction

An estimated 350 million people worldwide are chronically infected with hepatitis B virus (HBV). Fifteen to forty percent of these patients will develop serious sequelae during their lifetime, and then progress to liver cirrhosis (LC) or hepatocellular carcinoma (HCC). Approximately 12–20% of infected patients will experience a 5-year progression from chronic hepatitis B (CHB) to LC. The 5-year cumulative risk of developing HCC is estimated to fall between 10% and 17% in patients with LC. These figures vary from country to country according to the disease endemicity and prevalence [1–3]. CHB is complex and meanders through different

immunological phases that may overlap. In its early phases, HBV infection is characterized by minimal liver damage, a high level of HBV replication and presence of HBe-antigen (HBeAg). Patients in this “immunotolerant phase” [3,4] are asymptomatic and have normal levels of serum alanine aminotransferase (ALT). The goal of CHB therapy is to suppress HBV replication thereby preventing progression to LC, decompensated LC, HCC, and death [5]. The ideal treatment outcome is the elimination of HBsAg, however currently available anti-HBV agents are not efficacious in this regard. As a result, the induction of sustained or maintained virological remission is the highest outcome that can be expected from current treatments.

Over the last 20 years, seven HBV life cycle inhibitors have been approved by the United States Food and Drug Administration. Two are interferon (IFN)-based therapies that participate in activating immunity response in infected hepatocytes or RNA degradation (IFN and pegylated interferon). The other five are oral nucleoside/nucleotide analogues: lamivudine (LMV), adefovir dipivoxil (ADV), entecavir, telbivudine, and tenofovir disoproxil fumarate (TDF). IFN related treatments deliver sub-par outcomes and nucleic acid analogs need to be administered for the long term. Therefore,

Abbreviations: RT, reverse transcriptase; PDB, protein data bank; FP, fingerprint; LMV, lamivudine; ADV, adefovir dipivoxil; TDF, tenofovir disoproxil fumarate.

* Corresponding author at: Department of Hepatology, Graduate School of Medicine, Osaka City University, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan.

E-mail address: m2079633@med.osaka-cu.ac.jp (Y. Murakami).

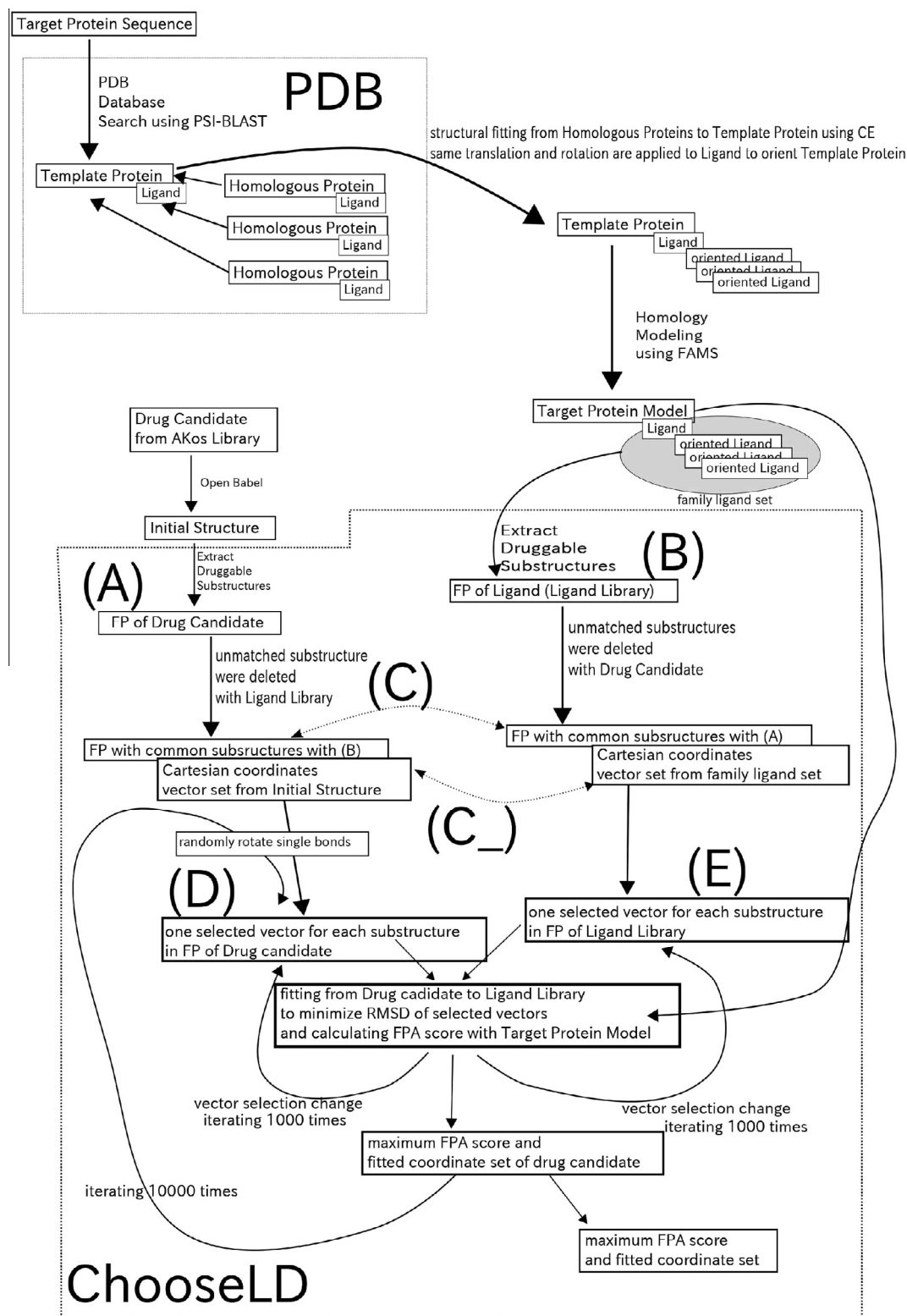
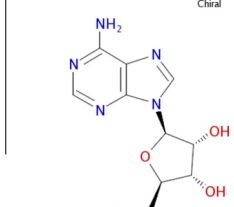
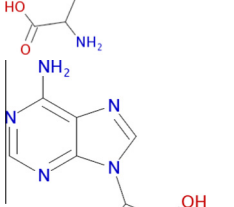
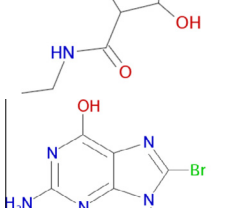
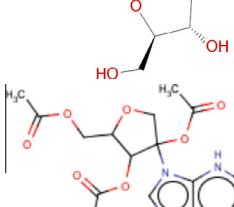


Fig. 1. Schematics of protein–ligand docking protocol. Two kinds of input data: “target protein sequence” and “drug candidate from AKos Library”. The target protein sequences were found in PDB and template protein binding with ligand was found. Using CE, homologous protein binding ligand was fit to template protein, then each ligand of each homologous protein oriented together. A structural model of the target protein was built using FAMS. The initial structure of the drug candidate compound was built using Open Babel, then druggable substructures were collected and named FP (A). Similarly ligand and oriented ligands were also collected and named FP (B). FP (A) and (B) were compared and matching substructures went to next step (C). In step (C_) Cartesian coordinates for each substructure were prepared in FP (A) and (B). In each substructure, one pair of Cartesian coordinates was selected, and used to calculate the FPA score. One thousand pairs were selected and the best FPA score was saved. Rotatable single bonds of drug candidate compound were randomly rotated, and the process repeated 1000 times. Drug candidates fitting the coordinates of the best FPA score were identified.

Table 1
List of anti-HBV candidates applied *in vitro*.

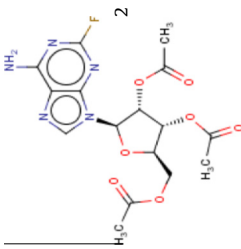
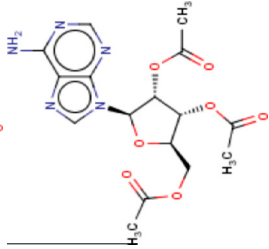
Code No./AKos accession No.	Chemical compound/molecular weight/rational formula	Solvent	FPAScore/ Bimolecular collision	Structural formula
Pyrimidine type M8/AKOS015896907	5'-Deoxy-5-fluorocytidine/245.2/C ₉ H ₁₂ F ₁ N ₃ O ₄	H ₂ O	1087.881/0,1,1	
M9/AKOS015853117	2'-Deoxy-5-fluorocytidine/245.2/C ₉ H ₁₂ F ₁ N ₃ O ₄	H ₂ O	1076.47/0,0,2	
M10/AKOS015896924	2'-Deoxy-5-methylcytidine/241.2/C ₁₀ H ₁₅ N ₃ O ₄	H ₂ O	1044.132/0,2,3	
M14/AKOS022143151	1-(3-O-Acetyl-2-deoxypentofuranosyl)-5-methyl-2,4(1H,3H)-pyrimidinedione/284.27/C ₁₂ H ₁₆ N ₂ O ₆	DMSO	1163.647/0,3,4	
M15/AKOS015966242	5'-Deoxy-2',3'-di-O-acetyl-5-fluorocytidine/ 329.281/C ₁₃ H ₁₆ F ₁ N ₃ O ₆	DMSO	1048.699/0,2,4	
M16/AKOS016315844	2',3'-Isopropylideneuridine/284.265/C ₁₂ H ₁₆ N ₂ O ₆	DMSO	1142.412/3,3,3	

Table 1 (continued)

Code No./AKos accession No.	Chemical compound/molecular weight/rational formula	Solvent	FPAScore/ Bimolecular collision	Structural formula
Purine type M11/AKOS022184395	S-Adenosylhomocysteine/384.411/C ₁₄ H ₂₀ N ₆ O ₅ S ₁	H ₂ O	1173.418/4,8,12	
M12/AKOS015951000	5'-N-Ethylcarboxamido-adenosine/308.293/C ₁₂ H ₁₆ N ₆ O ₄	DMSO	1010.174/2,3,4	
M13/AKOS015966900	8-Bromoguanosine/362.137/C ₁₀ H ₁₂ Br ₁ N ₅ O ₅	DMSO	1006.288/1,2,6	
M17/AKOS004910269	2,3,5-Tri-O-acetyl-1,4-anhydro-2-C-(2,6-dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)pentitol/410.336/C ₁₆ H ₁₈ N ₄ O ₉	DMSO	1077.439/3,4,5	

(continued on next page)

Table 1 (continued)

Code No./AKos accession No.	Chemical compound/molecular weight/rational formula	Solvent	FPAScore/ Bimolecular collision	Structural formula
M18/AKOS016009590	2',2',5'-Tri-O-acetyl-2-fluoroadenosine/411.342/C ₁₆ H ₁₈ F ₁ N ₅ O ₇	DM	1049.61/4.6,11	
M18/AKOS004903253	(2R,3R,4R,5R)-2-(6-amino-9-h-purin-9-yl)tetrahydrofuran-3,4-diyldiacetate/393.351/C ₁₆ H ₁₉ N ₅ O ₇	DMSO	1021.731/2.6,7	

despite the progress that has been made, there is still a pressing need for more effective treatments that target HBV.

Researchers have successfully discovered protein targets implicated in various diseases [6,7] resulting in fierce competition among pharmaceutical companies and other organizations to discover drug-candidate compounds that inhibit or activate these protein targets [8]. Experimental screening to determine the interaction of the experimental compound with the target protein is usually done using industrial robots. This type of screening is expensive and therefore, *in silico* screening is becoming increasingly popular to determine the efficacy of compounds against their targeted protein.

Our aim in this study was to discover HBV protein inhibitors using a novel method, ChooseLD (CHOOse biological information Semi-Empirically on the Ligand Docking), which uses simulated annealing based on bioinformatics for protein–ligand flexible docking. We believe this ChooseLD method can improve the search for new drug targeting proteins implicated in various diseases [9].

2. Material and methods

2.1. In silico screening

We selected one or more low molecular weight ligands. Fig. 1 shows the schematic diagram of our protein–ligand docking protocol; the target protein sequence is in the upper left corner. We did a query in the PDB [10] for the amino acid sequence of the target protein and restricted the results to those with a CE Z-score of 3.7 [11] that included ligand molecules, termed the family ligand set. Amino acid sequence alignment methods such as PSI-BLAST [12] were then applied.

A fingerprint (FP) of the chemical descriptor was defined based on the presence of particular substructures in the molecule. Each substructure is composed of two to four atoms connected with covalent bonds, has no ring and includes information about the atom-type based on SYBYL mol2 format (<http://tripos.com>). The drug candidate compounds were selected from the AKos (Germany) library. The initial 3D structures were written in mol2 format and were built using Open Babel software. Druggable substructures were extracted based on MDL comprehensive Medical Chemistry (CMD) Library. FP of drug candidate was built by collecting druggable substructures, (A) in Fig. 1.

The same process was applied to the family ligand set, (B). The FPs of (A) and (B) were compared and unmatched substructures were deleted to generate FP with common substructure as shown in (C). Cartesian coordinate vectors of FP (C) were subsequently used to calculate the FP alignment score (FPA score). (C₁) represents Cartesian coordinate's vectors in FP(C). Each substructure in the (C₁) band includes the assembly of several atom vectors, each with its own Cartesian coordinates. For example, the substructure of two aliphatic carbon atoms frequently appears in most organic compounds and other types of substructures are common in drug candidate molecules or family ligand set. If the same substructure was found in the FP(A) drug candidate molecule or in FP(B) in family ligand set, that substructure was considered redundant. However, such redundant substructures still had Cartesian coordinates that differed in (C₁). In the (D) or (E) step, substructures of the same type in the drug candidate or family ligand set, to compose a Cartesian coordinates vector, one vector set was selected from redundant substructure. One Cartesian coordinate with a redundant Cartesian coordinate vector in the (D) or (E) band was selected during the simulated annealing process. We performed the substructure selection process and changed the conformation one thousand times by random rotations about single

bonds. At each step of the simulated annealing, the maximum FPA score and Cartesian coordinates of the drug candidate were stored.

2.2. In vitro HBV infection

2.2.1. Human sample

Serum samples were obtained from a 54 year-old CHB patient with slightly elevated levels of serum alanine aminotransferase who was positive for HBs and HBe antigens and had high-level viremia with genotype C. No histological examination was performed for this patient. The exclusion criteria for the patient were: co-infection with human immunodeficiency virus (HIV) types 1 and 2, decompensated liver disease, organ transplantation, immune suppression, autoimmune disorders, consumption of >20 g/day alcohol, and past history of intravenous drug use. The patient provided written informed consent, and Osaka City University Graduate School and Faculty of Medicine's Ethics Committee approved this study in accordance with the Helsinki Declaration 2013.

2.3. Transfection procedure

4×10^5 PXB cells/well (for 24 well plate) (Phoenix bio, Hiroshima, Japan) was cultured in dHCGM medium [13]. 5 GEq of HBVDNA from patient's serum was mixed with 4% PEG8000 (MP Biomedicals, CA) and then inoculated into the PXB cells. dHCGM medium was changed 24 h, 48 h, and 168 h (5 days) after starting incubation. Thereafter, the medium was changed every five days.

The chemical compound was added each time the dHCGM medium was changed.

2.4. DNA extraction and quantification of HBVDNA

HBVDNA was extracted from 50 μ l of serum or 100 μ l of culture medium using SMI TEST EX R&D (Medical & Biological Laboratories CO.LTD Nagano, Japan) and was dissolved in H₂O at 20 μ l for serum or 50 μ l for medium. HBVDNA in medium was quantified by real time qPCR (Roche Diagnostic, Tokyo, Japan) by comparing serially diluted HBV/C1.24 (HBVDNA containing plasmid) obtained from Prof. Yasuhito Tanaka, Nagoya City University [14]. Primer sequences used were modified as follows: Forward primer 5'-CACA TCAGGATTCCTAGGACC-3', Reverse primer 5'-AGGTTGGTGAGTGA TTGGAG-3', Taqman probe 5'-FAM-CAGAGTCTAGACTCGTGGTGG ACTTC-TAMRA-3'.

2.5. Quantification of HBsAg in medium

HBsAg in the medium was measured by Mycell II HBsAg detection kit (Institute of Immunology Co., Ltd., Tokyo, Japan) according to the manufacturer's instruction.

2.6. Quantification of albumin

The human albumin content in the medium was detected using the Human albumin ELISA quantitation kit (Belthy, Montgomery, AL, USA) according to the manufacture instructions. Samples were analyzed in duplicate under each condition.

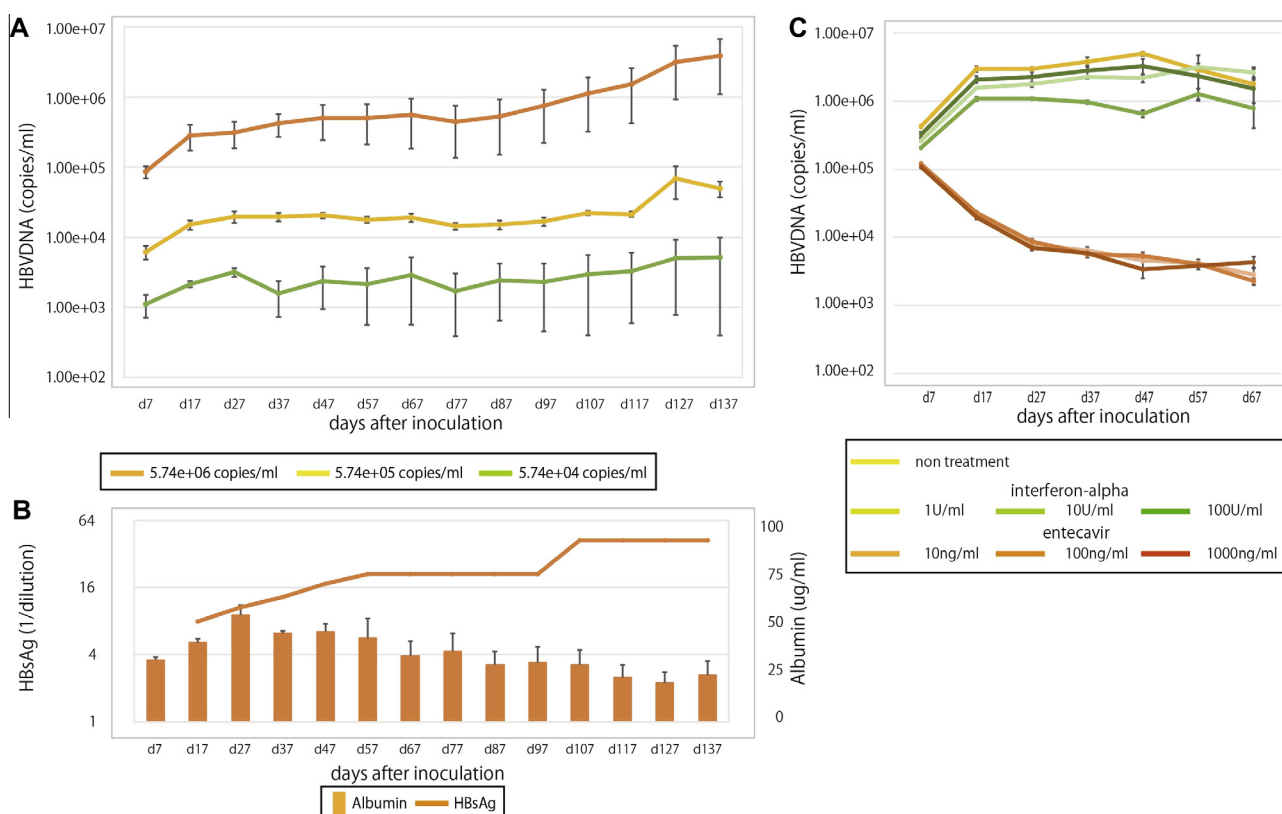


Fig. 2. In vitro HBV infection. (A) Time course of HBVDNA levels post HBV inoculation, three levels of HBVDNA from CHB patients serum were chosen as baselines; 5.74E+06 (high titer), 5.74E+05 (moderate titer), and 5.74E+04 (low titer). HBVDNA level was examined three times independently. (B) Time course of the level of HBsAg and amount of albumin after inoculation of HBV, left and vertical axis indicate the levels of HBVDNA and HBsAg. Left and vertical bars indicate HBsAg levels in the medium; semi-quantitative HBsAg assay was done with serial dilutions of serum samples. Right and vertical bars indicate the amount of albumin in the medium. These experiments were performed in duplicate. (C) Time course of the effect of interferon-alpha and entecavir, the three dosages of each drug were used and the antiviral effect was measured. The HBVDNA was quantified by real-time qPCR.

2.7. Chemical compound

Entecavir and IFN- α were purchased from Toronto Research Chemicals (Toronto, ON) and Sigma–Aldrich (St. Louis, MO), respectively. Chemical compounds for the *in vitro* study are listed in Table 1. Code No. M11, M12, M16, and M19 were purchased from Sigma–Aldrich. Code No. M8, M9, M10 and M13 were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). M14, M15, M17, M18 were purchased from AKOs.

2.8. Statistical analysis

Statistical analysis was performed using two-way repeated measures ANOVA and Dunnett's test (SPSS, IBM, Chicago, IL). *P* values of less than 0.05 were considered statistically significant.

3. Result

3.1. In silico screening for reverse transcriptase inhibitors

A model experiment on the HBV RT domain was performed using the reference domain for the HIV RT. Chemical compounds were selected from the 2,200,000 contained in the AKos databank

and then tested by *in silico* screening for their RT inhibiting activity. In order to test this *in silico* screening method, we evaluated several medicines that were used in prior HIV or HBV clinical studies. The FPAScore of medicines selected are: LMV 677.884, ADV 618.177, Entecavir 481.639, and TDF 698.330. Among compounds from the AKos library, sixty candidates for anti-viral agents for HBV-RT were identified using *in silico* analysis; thirty were pyrimidine-type nucleotides and thirty were purine-type nucleotides.

3.2. In vitro HBV transfection

HBVDNA in the PXB cell medium gradually increased after the cells were inoculated with HBV-positive serum (Fig. 2A). The amount of HBVDNA in the medium correlated with the amount used to infect the PXB cells (5.74×10^6 , 5.74×10^5 , and 5.74×10^4 copies/mL, respectively). The detection of HBsAg in the medium lead to the detection of HBVDNA (Fig. 2B).

3.3. Drug response in vitro transfection system

To test whether PXB cells infected with HBV was a suitable culture for screening the anti-HBV compounds, the culture was treated with either IFN- α (10–1000 U/mL) or entecavir (1–100 ng/mL),

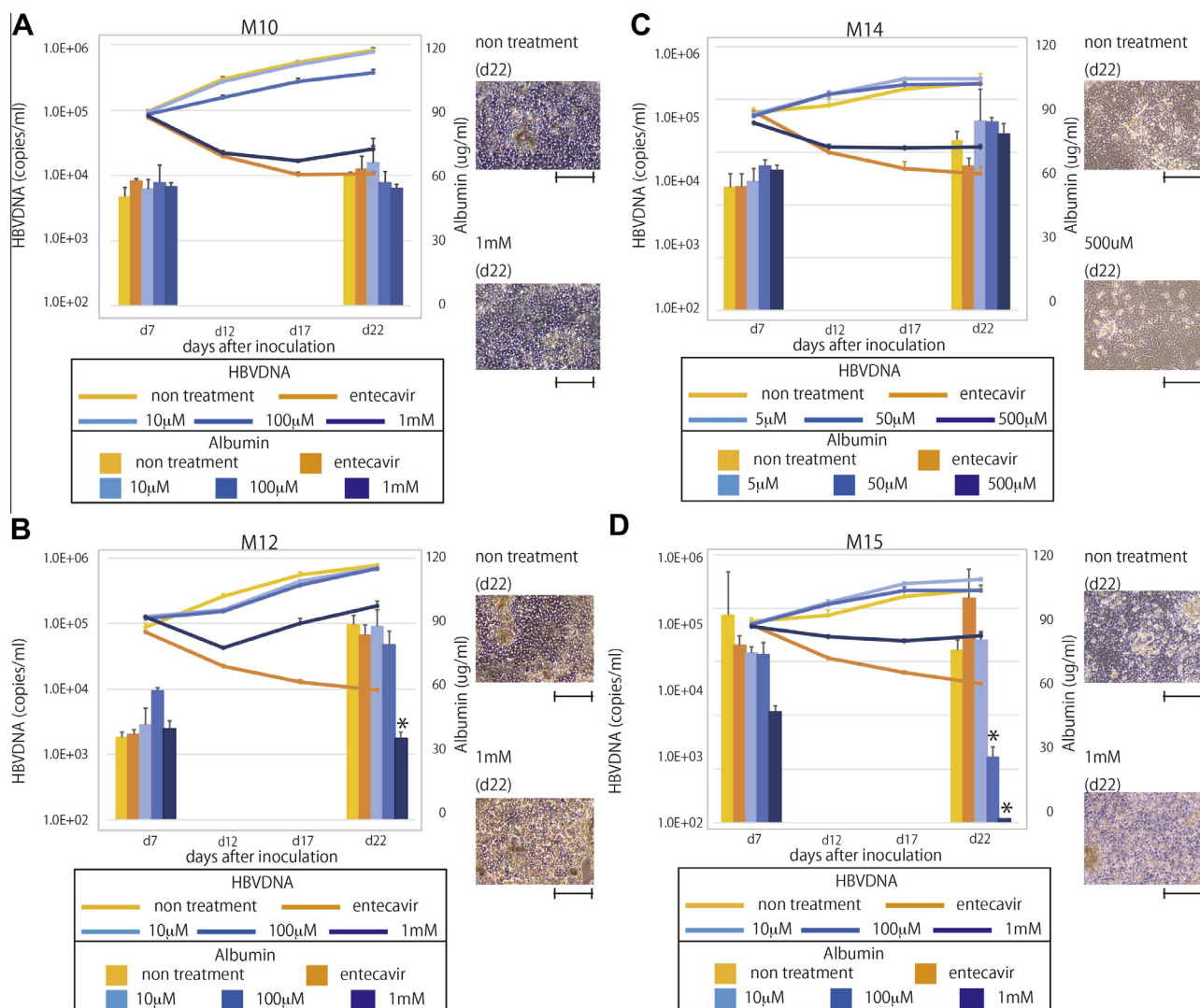


Fig. 3. Anti-viral effect by novel candidates. HBV infected PXB cells were treated with three amounts of M10 (A), M12 (B), M14 (C), and M15 (D). The line graph indicates HBVDNA levels in the medium while the bar graph depicts human albumin level in the medium. The data was taken from three independent experiments. Asterisks indicate a significant difference of *p* < 0.05. Cell morphology treated in 22 days after adding each candidate is shown in right part. Scale is 200 μ m.

established medicines that target CHB. The quantity of HBVDNA used for the infection was same as in high titer (Fig. 1A). As shown in Fig. 2C, 47 days after inoculation IFN treatment reduced HBVDNA in the +culture medium by half or two-thirds unlike the culture without IFN treatment. In the cell lines treated with entecavir, HBVDNA gradually decreased by 99% 47 days after inoculation (Fig. 2C). No obvious difference in anti-viral effect was observed even when the drug dosage was changed.

3.4. Novel candidate for anti-HBV drug

We were able to identify 60 candidates by *in silico* screening; from this twelve candidates were chosen using two exclusion conditions: prior *in vitro* or *in vivo* use in HIV or HBV treatment, and cell toxicity for example anti-cancerous drug.

Six of the twelve candidates were purine type chemical compounds: S-Adenosylhomocysteine (M11), 5'-N-ethylcarboxamido-adenosine (M12), 8-bromoguanosine (M13), 2,3,5-Tri-O-acetyl-1,4-anhydro-2-C-(2,6-dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl) pentitol (M17), 2',2',5'-Tri-O-acetyl-2-fluoroadenosine (M18), and (2R,3R,4R,5R)-2-(acetoxymethyl)-5-(6-amino-9H-purin-9-yl) tetrahydrofuran-3,4-diyl diacetate (M19). The remaining six were pyrimidine compounds: 5'-Deoxy-5-fluorocytidine (M8), 2'-Deoxy-5-fluorocytidine (M9), 2'-Deoxy-5-methylcytidine (M10), 1-(3-O-Acetyl-2-deoxypentofuranosyl)-5-methyl-2,4(1H,3H)-pyrimidinedione (M14), 5'-Deoxy-2',3'-di-O-acetyl-5-fluorocytidine (M15), and 2',3'-Isopropylideneuridine (M16) (Table 1). In PBX cell mediums treated with M10, M12, M14, and M15, HBVDNA significantly reduced compared to non-treated mediums (Fig. 3). Treatment did not affect cell viability morphologically and did not change the value of albumin in the medium even when high concentrations of M10 and M14 were used (Fig. 3A and C). However, at high concentrations of M12 and M15, cell viability was affected morphologically and the concentration of human albumin in the medium was significantly reduced (Fig. 3B and D).

4. Discussion

Standard clinical treatment of CHB uses nucleot(s)ide analogues that inhibit viral RT activity. Most nucleotide analogues result in side effects while entecavir and tenofovir are known to have viral resistance to HBV after several years. In addition, nucleotide analogue monotherapy cannot eliminate HBVDNA completely. Taking these current problems into account, our goal was to identify novel HBV protein-inhibiting agents.

In silico screening has been proven effective for identifying novel inhibitors for a variety of proteins. To date, several drug candidates have been found, for example, inhibitors for ALK, EGLN3, and NUA1, which are indicated in squamous cell carcinoma in the esophagus [15], lung small cell carcinoma (Umeyama et al., BMC Genomics, in press), and autoimmune disease such as systemic lupus erythematosus, rheumatoid arthritis, and diabetes mellitus [16].

We simulated Induced fit for RT using HIV RT domain and identified 30 purine-type and 30 pyrimidine-type inhibitor candidates from two million chemical compounds in the AkosSamples database. For each type, six candidates with no prior use as HIV and HBV medicines were chosen. Two of the 12 compounds had an antiviral effect *in vitro* using PBX hepatocytes. Large amounts of these compounds are used in *in vitro* experiment however this approach did not rely on newly synthesized compounds; therefore, these related costs and time could potentially be eliminated thereby allowing compounds to be screened more efficiently. The

primary aim of *in silico* screening is to identify compounds with anti-viral activity; the secondary aim is to increase the antiviral effect of compounds identified by chemical modification, however, this level of research will be done in the future.

Since the life cycle and replication course of HBV are complicated, it is difficult to eradicate HBV genes only by controlling the function of DNA polymerase. We created a composition of inhibitors for HBV related proteins in parallel with DNA polymerase and established a protein model of HBV surface and core antigens in order to adopt an induced fit configuration (unpublished results). Identifying these protein inhibitors would be done in the near future.

In summary, nucleos(t)ide analogues that target CHB result in potent antiviral response, however they also have a high proclivity for developing drug resistance HBV. Using reliable public drug databases, our *in silico* method can help to identify drug targets for HBV efficiently. Moreover, this algorithm can effectively identify candidates with the potential to suppress HBV replication *in vitro* HBV infected systems.

Competing interests

The authors have no competing interests to declare.

Acknowledgments

YM, MI, YY, TT, ME, AT, NK, and HU were supported by KOSEI-KAKENHI, (H25-B sou-Kan-en-general-018). YM, and MH performed the research. MI, YY, TT, ME, AT, HU, MI, NK, YT, and HU designed and performed the research. YM, MI, YT, and UH wrote the paper.

Reference

- [1] W.M. Lee, Hepatitis B virus infection, *N. Engl. J. Med.* 337 (1997) 1733–1745.
- [2] R.P. Beasley, Hepatitis B virus. The major etiology of hepatocellular carcinoma, *Cancer* 61 (1988) 1942–1956.
- [3] J. Shao, L. Wei, H. Wang, Y. Sun, L.F. Zhang, J. Li, J.Q. Dong, Relationship between hepatitis B virus DNA levels and liver histology in patients with chronic hepatitis B, *World J. Gastroenterol.* 13 (2007) 2104–2107.
- [4] G. Daniels, A century of human blood groups, *Wien. Klin. Wochenschr.* 113 (2001) 781–786.
- [5] L. European, Association for the study of the EASL clinical practice guidelines: management of chronic hepatitis B virus infection, *J. Hepatol.* 57 (2012) 167–185.
- [6] E.R. Wood, A.T. Truesdale, O.B. McDonald, D. Yuan, A. Hassell, S.H. Dickerson, B. Ellis, C. Pennisi, E. Horne, K. Lackey, K.J. Alligood, D.W. Rusnak, T.M. Gilmer, L. Shewchuk, A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells, *Cancer Res.* 64 (2004) 6652–6659.
- [7] J. Stamos, M.X. Sliwkowski, C. Eigenbrot, Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor, *J. Biol. Chem.* 277 (2002) 46265–46272.
- [8] K. Nakamura, E. Taguchi, T. Miura, A. Yamamoto, K. Takahashi, F. Bichat, N. Guilbaud, K. Hasegawa, K. Kubo, Y. Fujiwara, R. Suzuki, K. Kubo, M. Shibuya, T. Isae, KR951, a highly potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, has antitumor activities and affects functional vascular properties, *Cancer Res.* 66 (2006) 9134–9142.
- [9] D. Takaya, M. Takeda-Shitaka, G. Terashi, K. Kanou, M. Iwadate, H. Umeyama, Bioinformatics based Ligand-Docking and in-silico screening, *Chem. Pharm. Bull.* 56 (2008) 742–744.
- [10] J. Westbrook, Z. Feng, L. Chen, H. Yang, H.M. Berman, The Protein Data Bank and structural genomics, *Nucleic Acids Res.* 31 (2003) 489–491.
- [11] I.N. Shindyalov, P.E. Bourne, Protein structure alignment by incremental combinatorial extension (CE) of the optimal path, *Protein Eng.* 11 (1998) 739–747.
- [12] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res.* 25 (1997) 3389–3402.
- [13] C. Yamasaki, C. Tatenno, A. Aratani, C. Ohnishi, S. Katayama, T. Kohashi, H. Hino, H. Marusawa, T. Asahara, K. Yoshizato, Growth and differentiation of colony-forming human hepatocytes *in vitro*, *J. Hepatol.* 44 (2006) 749–757.

- [14] A. Abe, K. Inoue, T. Tanaka, J. Kato, N. Kajiyama, R. Kawaguchi, S. Tanaka, M. Yoshida, M. Kohara, Quantitation of hepatitis B virus genomic DNA by real-time detection PCR, *J. Clin. Microbiol.* 37 (1999) 2899–2903.
- [15] R. Kinoshita, M. Iwadate, H. Umeyama, Y.H. Taguchi, Genes associated with genotype-specific DNA methylation in squamous cell carcinoma as candidate drug targets, *BMC Syst. Biol.* 8 (Suppl. 1) (2014) S4.
- [16] S. Ishida, H. Umeyama, M. Iwadate, Y.H. Taguchi, Bioinformatic screening of autoimmune disease genes and protein structure prediction with FAMS for drug discovery, *Protein Pept. Lett.* 21 (2014) 828–839.