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TITLE:
Oligonucleotide aptamers: potential novel molecules against viral hepatitis

ABSTRACT:
Viral hepatitis, as an international public health concern, seriously affects communities and health system. In recent years, great strides have been taken for development of new potential tools against viral hepatitis. Among these efforts, a valuable strategy introduced new molecules called "aptamers". Aptamers as potential alternatives for antibodies could be directed against any protein in infected cells and any components of viral particles. In this review, we will focus on recent advances in the diagnosis and treatment of viral hepatitis based on aptamer technology. In recent years, various types of aptamers including RNA and DNA were introduced against viral hepatitis. Some of these aptamers can be utilized for early and precise diagnosis of hepatitis infections and other group selected as therapeutic tools against viral targets. Designing diagnostic and therapeutic platforms based on aptamer technology is a promising approach in viral infections. The obtained aptamers in the recent years showed obvious potential for use as diagnostic and therapeutic tools against viral hepatitis. Although some modifications to increase the biostability and half-life of aptamers are underway, it seems these molecules will be a favorable substitute for monoclonal antibody in near future.

2.1. Aptamers and aptamer-based biosensors for diagnosis of HBV ::: 2. Aptamer in the diagnosis of viral hepatitis ::: 1. INTRODUCTION:
The first reported aptamer for HBV diagnosis was introduced in 2010 by Liu, et al. The selected RNA aptamer is bound specifically to the infected hepatoma cell line which expresses hepatitis B surface antigen (HBsAg). Accordingly, this aptamer can find HBV infected cells, while in previous test only purified antigen was used as detection tool(25).
After finding this aptamer Liu, et al. exploited fluoresce in isothiocyanate (FITC) -conjugated RNA aptamer, HBs-A22, to detect HBsAg that is expressed on the surface of infected HepG2.2.15 cells in fluorescence microscopy. Replacing FITC with pharmaceutical agents in the structure of designed RNA aptamers was proposed by this group(25). Zhijiang Xi, et al. selected specific single strand DNA (ssDNA) aptamers by immobilizing HBsAg on the surface of carboxylated magnetic nanoparticles (MNPs) in a SELEX process after 13 rounds of selection. Three different aptamers with a same hairpin loop structure were successfully separated in vitro and a chemiluminescence aptasensor based on magnetic separation and immunoassay was constructed to indicate HBsAg in pure protein form or actual serum samples. As results, the H01 aptasen samples. As results, the H01 aptasensor worked well and indicated high specificity for contributing to better detection of hepatitis B virus infection. The detection limit of H01 aptasensor was 0.1 ng/mL, which is five times lower than ELISA as a routine method(26). While examinations proved this construct successfully bound to the S form of HBsAg, it seems that further investigations are required to identify the aptamer cross-reactivity with other forms of HBs Ag (M and L) and the kind of interaction with particle assembly.

2.2. Aptamers and aptamer-based biosensors for diagnosis of HCV ::: 2. Aptamer in the diagnosis of viral hepatitis ::: 1. INTRODUCTION:
Aptamers not only can fit into clefts on protein surfaces, particularly into the active site of enzymes to inhibit the catalytic activity like traditional antiviral small molecules, e.g., HCV protease inhibitors (boceprevir), but also they can bind to protruding parts of proteins. For this reason discovery of novel methods relying on aptamers for rapid and more cost-effective diagnosis of HCV is not unexpected.
In this regard, Lee, et al. obtained the construction of a biosensor utilizing the fluorescent dye (Cyanine3) RNA aptamer directed against HCV core antigen via Chip-based detection method(27), whereas Chen, et al. reported the ssDNA aptamer as molecular probes that bound viral glycoprotein E2(28). The aptamers against HCV glycoprotein E2 was selected by Park, et al. in 2013 which used in enzyme linked apto-sorbent assay (ELASA) an innovative detection assay-provided qualitative and quantitative analysis of virus particles in the tested samples at the same time. Additionally ELASA could be a proper method for evaluation of antiviral treatment(29).
These findings might be important for the early detection of hepatitis C while patients are in "window period", because serum antibodies have not been shown viral infection yet. Ordinarily,

the fluorescent dye-conjugated aptamers seem to be valuable in various types of diagnostic assays.

3.1. Aptamers for treatment of hepatitis B virus ::: 3. Aptamers in the treatment of viral hepatitis ::: 1. INTRODUCTION:

Some protective and symptomatic therapy such as interferon-α and nucleos(t)ide analogues (adefovir, lamivudine and entecavir), are usually prescribed for the treatment of chronic hepatitis B infection(46). Although, these medicines have therapeutic effects, their use are restricted by the severe side effects and development of drug-resistant strain(4748).

HBV genome contains a partially relaxed circular double-stranded DNA (rcDNA), meaning that consists of a complete (–) strand and the (+) strand DNA which is variable and shorter in length(49). During hepatitis progression, this unusual DNA forms covalently closed circular DNA in the nucleus to activate RNA transcription of HBV. Viral genome translates into the four overlapping open reading frames, including core proteins (C), viral reverse DNA polymerase (P), surface antigen (S) and X protein (HBx)(49). Several target proteins encoded by HBV have been suitably subjected to the selection of aptamers.

Interaction between the viral protein R (RNA-dependent RNA polymerase) with stem-loop structure of ε sequence located at the 5' side of the pregenomic RNA is a critical step for replication and assembly of HBV. Based on this fact, Feng, et al. developed S9 RNA aptamer as anti-HBV replication, which interacted with viral polymerase with high affinity and competed a binding site of viral genetic material. Inhibition of replication in HBV infected cell line, HepG2.2.15, which was determined after transfection with a plasmid vector encoding S9 RNA aptamer by southern blot analysis notably showed no signs of cell toxicity in S9 aptamer-transfected cells(50).

Zhang, et al. in 2014 found aptamers against the core protein of HBV (HBc) from a random DNA pool by SELEX procedure. This new aptamer, Apt.No.28, demonstrated a high affinity and inhibited the assembly of the nucleocapsid, reducing extracellular DNA, whose synthesis based on the formation of the nucleocapsid, confirmed its role in suppressing HBV replication. This aptamer was suggested as a new targeting molecule to facilitate the strategy for targeted therapy of HBV-related diseases. There was no documented report for estimation of Kd (dissociation constant) for selected aptamer in this research(51).

Aptamer against matrix binding domain (MBD) on the HBV capsid surface were designed by Orabi, et al. After thirteen rounds of SELEX using the wild type (WT) capsids for positive selection and the I126A mutant capsids for counter selection AO-01 aptamer with the lowest Kd was selected against WT capsids. Its Kd value against the I126A mutant capsids was at least 7 fold higher. Inhibition potential test in transiently cotransfected HuH-7 cell performed 47 % HBV inhibition via AO-01 and no inhibition with an aptamer with random sequence AO-N. These results showed that the selected aptamer is specific for the MBD(52).

3.2. Aptamers for treatment of hepatitis C virus ::: 3. Aptamers in the treatment of viral hepatitis ::: 1. INTRODUCTION:

Entry inhibitors are the most commonly studied therapeutic tools which prevent viral fusion with the target cell and penetration. Virus penetration occurs with specific surface proteins into the cells, which present as ligands.

As generally proven, many viruses such as HCV, HBV and HIV-1 have an obvious tropism for specific cell types(34).

HCV encodes two envelope glycoproteins, E1 and E2, which are released by host signal peptidase from the polyprotein precursor(53). In HCV infection, E2 glycoprotein was an important target for aptamer selection. This glycoprotein is a co-receptor of human CD81, displayed on hepatocytes and B lymphocytes(54). Chen, et al. obtained DNA aptamer, defined as ZE2, competitively blocking E2 in most HCV serotypes. Its efficiency was verified in Huh7.5.1, a well differentiated hepatocyte derived carcinoma cell line. The reduction in HCV RNA levels and E2 protein concentrations after treatment were demonstrated by qRT-PCR analysis and in the western blot assay respectively(28).

HCV non-structural protein 5B (NS5B) is an RNA-dependent RNA polymerase that is a desired target for aptamer therapeutics, because of its importance for the virus replication(55). Biroccio, et al. constructed RNA aptamer, assigned as B.2 specified by stem-loop structure, with a unique sequence UAUGGACCAGUGGC that identifies an important element -a GTP binding site of NS5B- responsible for its function. In vitro analysis of the polymerase activity was positively illustrated the correlation between aptamer and inhibition of polymerase activity in a concentration-dependent manner(56). Bellecave, et al. selected 27v as DNA aptamer against same enzyme that suppressed NS5B activity with competition the polymerase-binding site with the viral RNA template. The observation was significantly proved the viral copy number reduction in Huh7 cell line (differentiated hepatocyte derived carcinoma cell line infected with HCV JFH1 strain) by qRT-PCR which positively correlated with obtained aptamer. RNA level of virus have been declined after treatment with concentration of 5 μM, 1 μM, and 100 nM by 90%, 68%, and 19%, respectively comparing with non-aptamer treated cells (7.8 × 106 to 22 × 106 HCV RNA copies). The aptamer molecules were observed intracellularly using confocal microscopy, despite the absence of the transfection agent(41).

Among other enzymes, which indirectly play role in virus replication, nonstructural protein 3 (NS3) showed helicase and protease activity which is related to the C- and N-terminal domains of the enzyme, respectively. For the replication of the flaviviridae family, including HCV, both domains of NS3 are essential. Protease domain changes viral proteins necessary for its life cycle, while helicase unwinds DNA and RNA duplexes and then unpackaged genetic material replicates by polymerase. The helicase domain of HCV NS3 binds preferentially to the poly (U) sequence situated in the 3'-untranslated region of the viral genome (3'-UTR) under normal conditions(57). Umehara, et al. created a bivalent aptamer with sequences coupled with a poly (U) linker. In this study, they determined the optimum length of the linker, i.e., 41 and 50 nucleotides. Consequently, NEO-35-s41 and G925-S50 aptamers showed highest synchronous reduction in both helicase and protease activity of NS3. These results were confirmed using an in vitro enzymatic assay(58).

In another study, Fukuda, et al. introduced ΔNEO-III-14U RNA aptamer, which powered by a competitor sequence (poly U), as characterized during in vitro enzymatic and in vivo tests. Selected aptamer significantly inhibited the activity of both NS3 protease and helicase domains in a dose-dependent manner. They supposed that ΔNEO-III-14U aptamer can compete with HCV in the 3'-UTR regions of genome for the binding site of helicase domain(59).

Nonstructural protein 5A (NS5A) is a zinc-binding and proline-rich hydrophilic phosphoprotein that plays a critical role in RNA replication of HCV and its assembly. Yu, et al. developed NS5A-4 and NS5A-5 aptamers, demonstrated one-fold decrease in viral RNA level after anti-NS5A aptamers treatment in Huh7.5 cells compared with control, as estimated by real-time PCR. The results of focus forming assay on naive Huh7.5 cells indicated coupling of NS5A by the aptamer that could block the production of new infectious virion. It seems when interferon response to viral infection remains inactive, this viewpoint-specific is efficient (transcription of interferon genes was evaluated by qRT-PCR)(60). Gao, et al. with accordant results about the safety approach established NS2-1, NS2-2 and NS2-3 aptamers against NS2 protein (nonstructural protein 2) of the HCV, which can powerfully influence on viral replication(61).

As an opportunity, aptamers not only can recognize viral proteins, but also able to detect its nucleic acids. Designing aptamers for specified region of HCV genome, which interact with viral life cycle involve proteins appears to be an impressive therapeutic tool. The internal ribosome entry site (IRES) sequence, associated with viral translation, is well-conserved among HCV isolates because it is a potential attractive target for anti-HCV drugs. IRES consists of four domains (I-IV) present in the 5'-untranslated region (5'-UTR) of the HCV genome, which allow cap and end-independent mRNA translation in the host cell. The internal initiation of translation begins with the binding of IRES to the small ribosomal subunit (40S) in the host cell, and eukaryotic translation initiation factor 3 (eTIF3). In 2011 Konno, et al. rendered RNA aptamer AP30 that targeted domain I of IRES in 3' end of the genome antisense strand. AP30 showed approximately 50% replication suppression via in vitro analysis. Two consensus sequences 5'-UGGAUC-3' and 5'-GAGUAC-3', which completely bound to the SL-E1 and SL-D1 loops in the domain I performed attachment prevention of NS5B(6263). Kikuchi, et al. applied RNA aptamer containing loop structure including a consensus sequence 5'-UAUGGCU-3', complementary to the loop of the IRES domain II. About 20%–40% reduction in luciferase activity was established by in vitro translation test of IRES-luciferase mRNA due to the exposure of 1-17 and 2-02 aptamers(64). In another study, the same team of researchers isolated aptamer 3-07 capable of binding to the IIId domain of IRES. This aptamer has positively operated by viral in vitro IRES-dependent translation blockage and targeted the second domain of IRES. A bout 10% depletion in the luciferase activity compared to the control levels was detected. The potency and selectivity of this aptamer was shown in transfected HeLa cells with 0.5 pmol 3-07 aptamer which demonstrated inhibition in luciferase activity up to 45%(65). Especial attention was paid to synchronous inhibition of IRES domains II and III-IV; particularly, IIId and IIIe regions as essential elements in viral translation. Consequently, conjugated forms of two aptamers, named as 2-02 and 3-07, was produced and proved a 10 times decrease in binding affinity to the target sequence than the two parental aptamers; also the IC50 value showed same decrease in translational activity, 10-fold lower than components alone(66). In addition, Romero-Lopez, et al. generated HH363-24 molecule that was an engineered aptamer composed the activity of hammer head ribozyme (HH363) with properties typical for aptamers. This construct bound to the essential IIId domain of the IRES, cleaved the HCV genome in 3' side and inhibited viral translation and replication simultaneously. HH363-24 have proved to be highly potent inhibitory molecules in Huh7.5.1 cells, containing HCV-1b subgenomic RNA replicons. HH363-24 also was efficiently inhibited HCV RNA synthesis up to 70% in a subgenomic replicon system in the qRT-PCR assay. Mutational analysis exposed that combining aptamers for various target recognition sites improved the inhibition activity by increasing the domain binding competency(44).

4.1. Aptamer degradation under physiological conditions ::: 4. Challenges of commercialization ::: 1. INTRODUCTION:
One of the main challenges of aptamer commercialization is serum endo- or exonucleases protection. Nowadays, based on nucleases sensitivity of unmodified nucleotides various chemical modifications can be used into the nucleotide sugars or internucleotide phosphodiester linkages. These modifications decrease obviously aptamer degradation rate, which occur with in vivo nucleases. Furthermore, natural form of nucleic acids can be replaced with synthetic nucleic acids like Xeno nucleic acid (XNA). These unnatural polymers not only will mimic DNA and RNA function, but demonstrate significant enhancement of nuclease resistance and then display longer half-lives in vivo(6869).

4.2. Renal filtration ::: 4. Challenges of commercialization ::: 1. INTRODUCTION:
Although rapid clearance in aptamer approach would be helpful for in vivo diagnostic imaging, but it can influence destructively on other biomedical applications. The renal filtration threshold is thought to be 30-50 kD usually. Antibodies due to their large size (~150 kD) and special recycling system circulate in the bloodstream with extended half-life, while clearance of non-modified aptamer happen quickly from the blood, with a half-life of minutes to hours. However, some tricks such aptamer conjugation via high molecular weight polymers like polyethylene glycol (PEG) not only can significantly enhance its half-life under physiological conditions but also improve stability and reduce aptamer toxicity accumulation in nontarget organs(70).

4.3. Toxicity ::: 4. Challenges of commercialization ::: 1. INTRODUCTION:
Aptamers as therapeutic agents can reveal toxicity effects in both on-target and off-target situations. Although clinical trials of the limited number of aptamers are being conducted, but studies generally indicated moderate or low toxicity effects of aptamers.
Other investigations have represented some oligonucleotide conjugation partners such as PEG or other high-molecular weight compounds may stimulate the production of antibodies that neutralize aptamers in bloodstream and reduce toxicity(7172).

4.4. Intellectual property ::: 4. Challenges of commercialization ::: 1. INTRODUCTION:
The therapeutic potentials and applications of aptamer may be limited by intellectual property rights. Now aptamers and their selection technologies are widely supported by a single intellectual property portfolio and therefore investment in this area is depend on the collaboration between researchers and companies. In contrast, antibody technologies are currently applied broadly for lack of patent protection or expiration of intellectual property rights. Although these protection rights for the SELEX method will expire in the near future(68).

5. CONCLUSION:
As outlined in this review, recent advances in aptamer technology, specifically recognizing viruses disclosed significant potential in a range of diagnostic and therapeutic applications. These molecules as a convincing substitute for antibodies have provided an effective tool for viral infection management.
Designing diagnostic platforms based on aptamer technology is a promising approach in viral infections. Aptamers with special features such as detection of early or late viral markers, ability to discriminate infected cell from normal or active and inactive virus looks extremely hopeful. Despite the obstacles of in vivo studies, several aptamers are undergoing clinical trials. Therapeutic aptamers can be effectively designed and applied against viral hepatitis by various mechanisms including blockage of virion penetration, inhibition of viral replication, delivery of antiviral agents and activation of immune system. Accordingly, aptamers can operate diverse commercial

applications to combat viral hepatitis by applying a series of modifications in pharmacokinetic properties.