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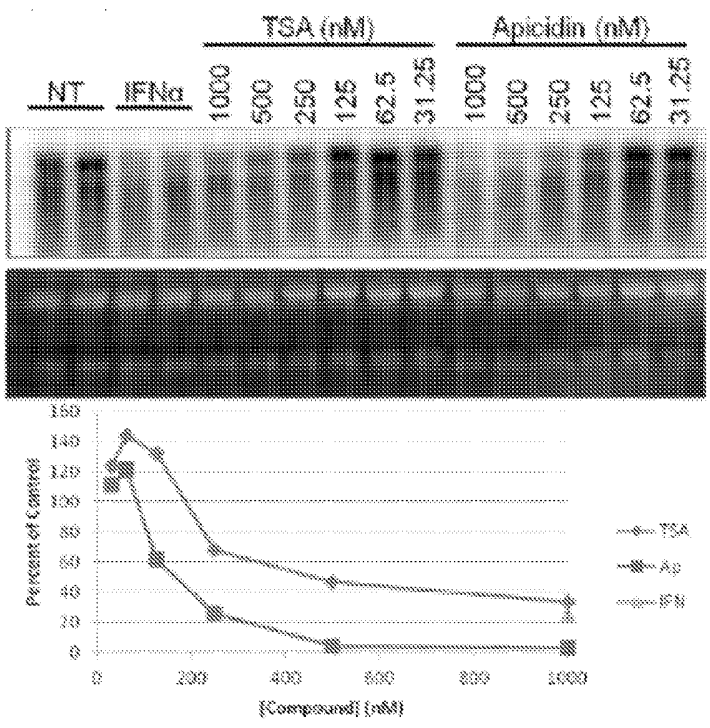
(54) Title: MODULATION OF HEPATITIS B VIRUS CCCDNA TRANSCRIPTION



(57) Abstract: The present disclosure pertains to the discovery of a new family of "first in class" small molecular inhibitors of hepatitis B virus (HBV) covalently closed circular (ccc) DNA for use as therapeutics in the management of chronic HBV. Provided are agents that provide epigenetic modification of the cccDNA, histone modifying agent, and inhibitors of histone deacetylace activity that in turn modulate HBV cccDNA, which has never been achieved with a pharmacological agent. Also provided are methods for modulating HBV cccDNA, for treating or preventing HBV in a subject, and for modulating cccDNA transcription of hepatitis B in a subject.

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MODULATION OF HEPATITIS B VIRUS CCCDNA TRANSCRIPTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority to U.S. Provisional App. No. 61/654,374, filed June 1, 2012, the entire contents of which are incorporated herein by reference.

TECHNICAL FIELD

[0002] The present disclosure pertains to the use of pharmacological agents, preferably with histone deacetylace activity, for modulating covalently closed circular DNA of hepatitis B virus, and for preventing or treating hepatitis B.

BACKGROUND

[0003] There are now seven medications approved by the United States Food & Drug Administration (FDA) for the management of chronic hepatitis B, which fall into one of two categories: the interferons (IFNs) and the polymerase inhibitors (*Lok, A. S., and B. J. McMahon. 2007. Chronic Hepatitis B. Hepatology 45:507-539*). These are recommended for use in approximately 50% or less of the infected population of more than 350 million. Although this is the highest risk population, those who fall outside the treatment guidelines may also benefit from intervention, since they are also at significantly elevated risk of liver diseases. The IFNs are limited by significant side effects. The pol inhibitors target the same viral life cycle step and thus combination therapy, the bulwark of HIV and curative HCV therapy, is of limited value. They require lifelong use, and are subject to eventual use limiting toxicities, as seen with HIV long term medication use, and the emergence of drug resistant mutants. Thus, alternatives and complements to the current portfolio of medications are needed.

[0004] There is a growing belief that a "cure", or at least sustained off-drug control of HBV, will require, or at least benefit from, drugs that control the viral nuclear genome, the covalently closed circular DNA (cccDNA). The 2006 NIDDK Liver Action Plan, reinforced by the 2010 Institute of Medicine report, all call for cccDNA inhibition as a priority for HBV drug development.

[0005] However, screening for HBV cccDNA inhibitors has been difficult, because of technical reasons: HBV cccDNA is made in amounts to low to be conveniently detected, and most viral gene products in conventionally transfected cells in culture are derived from transgenes of the viral genome, not cccDNA. The present inventors have created cell lines in

which HBV gene products such as the HBeAg are produced only from cccDNA, but not from integrated viral transgene and in amounts to be robustly detected, making screening realistic (*Cai, D., et al., 2012. Identification of the Disubstituted Sulfonamide Compounds as Specific Inhibitors of Hepatitis B Virus Covalently Closed Circular DNA Formation. Antimicrobial Agents and Chemotherapy: In Press; Zhou, T., et al., 2006. Hepatitis B virus e antigen production is dependent upon covalently closed circular (ccc) DNA in HepAD38 cell cultures and may serve as a cccDNA surrogate in antiviral screening assays. Antiviral Research 72:116-124*).

[0006] Given such challenges, it is unsurprising that there are no HBV therapeutics in use that target HBV cccDNA and, there have been few, if any, programs to screen and develop cccDNA inhibitors. This is largely due to technical difficulties (*see Block, T. M., et al. 2003. Molecular viral oncology of hepatocellular carcinoma. Oncogene 22:5093-5107; Locarnini, S. 2005. Therapies for hepatitis B: where to from here? Gastroenterology 128:789-792; Lok, A. S. 2011. Does antiviral therapy for hepatitis B and C prevent hepatocellular carcinoma? J Gastroenterol Hepatol 26:221-227*). In addition, the role of host functions in regulating HBV cccDNA transcription and stability is poorly understood further frustrating development of therapeutics. Thus, any work in this area would be innovative, and would address the outstanding and long-felt need for drugs that control the viral nuclear genome of hepatitis B and otherwise provide treatment for HBV infection.

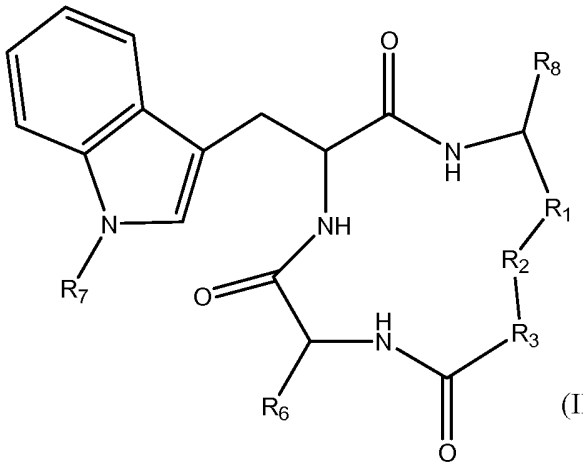
SUMMARY

[0007] Provided are methods of modulating cccDNA transcription of hepatitis B in a subject comprising administering to the subject an agent that provides epigenetic modification of the cccDNA, a histone modifying agent, or an inhibitor of histone deacetylace activity. For example, the epigenetic modifying agent, histone modifying agent, or inhibitor of histone deacetylace activity may be pharmacological, such as a small molecule.

[0008] Also provided are methods of treating hepatitis B in a subject comprising administering to the subject an inhibitor of histone deacetylace activity.

[0009] The present disclosure also pertains to method of modulating hepatitis B virus covalently closed circular DNA comprising contacting a hepatitis B virus with an inhibitor of histone deacetylace activity.

[0010] Also disclosed are compounds according to formula II:



(II)

wherein R_1-R_8 are defined as provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] **FIG. 1** provides data demonstrating that HBV cccDNA is efficiently formed and transcriptionally active in dstet5 cells.

[0012] **FIG. 2** relates to experiments demonstrating that cccDNA can be inhibited by IFN-a.

[0013] **FIG. 3** pertains to the present finding that Apicidin and TSA repress cccDNA transcription.

[0014] **FIG. 4** relates to the discovery that HDAC inhibitors dose-dependently stimulate DHBV pgRNA synthesis from transgene integrated in a host cellular chromosome.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0015] The present invention may be understood more readily by reference to the following detailed description taken in connection with the accompanying figures and examples, which form a part this disclosure. It is to be understood that this invention is not limited to the specific products, methods, conditions or parameters described and/or shown herein, and that the terminology used herein is for the purpose of describing particular embodiments by way of example only and is not intended to be limiting of the claimed invention.

[0016] The disclosures of each patent, patent application, and publication cited or described in this document are hereby incorporated herein by reference, in their entirety.

[0017] As employed above and throughout the disclosure, the following terms and abbreviations, unless otherwise indicated, shall be understood to have the following meanings.

[0018] In the present disclosure the singular forms "a," "an," and "the" include the plural reference, and reference to a particular numerical value includes at least that particular

value, unless the context clearly indicates otherwise. Thus, for example, a reference to "a compound" is a reference to one or more of such compounds and equivalents thereof known to those skilled in the art, and so forth. Furthermore, when indicating that a certain chemical moiety "may be" X, Y, or Z, it is not intended by such usage to exclude in all instances other choices for the moiety; for example, a statement to the effect that R_1 "may be alkyl, aryl, or amino" does not necessarily exclude other choices for R_1, such as halo, aralkyl, and the like.

[0019] When values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. As used herein, "about X" (where X is a numerical value) preferably refers to ±10% of the recited value, inclusive. For example, the phrase "about 8" refers to a value of 7.2 to 8.8, inclusive; as another example, the phrase " about 8%" refers to a value of 7.2% to 8.8%, inclusive. Where present, all ranges are inclusive and combinable. For example, when a range of "1 to 5" is recited, the recited range should be construed as including ranges "1 to 4", "1 to 3", "1-2", "1-2 & 4-5", "1-3 & 5", and the like. In addition, when a list of alternatives is positively provided, such listing can be interpreted to mean that any of the alternatives may be excluded, *e.g.*, by a negative limitation in the claims. For example, when a range of "1 to 5" is recited, the recited range may be construed as including situations whereby any of 1, 2, 3, 4, or 5 are negatively excluded; thus, a recitation of "1 to 5" may be construed as "1 and 3-5, but not 2", or simply "wherein 2 is not included." In another example, when a listing of possible substituents including "hydrogen, alkyl, and aryl" or ""hydrogen, alkyl, or aryl" is provided, the recited listing may be construed as including situations whereby any of "hydrogen, alkyl, and aryl" or "hydrogen, alkyl, or aryl" is negatively excluded; thus, a recitation of "hydrogen, alkyl, and aryl" or "hydrogen, alkyl, and aryl" may be construed as "hydrogen and/or aryl, but not alkyl", or simply "wherein the substituent is not alkyl".

[0020] As used herein, the terms "component," "composition of compounds," "compound," "drug," "pharmacologically active agent," "active agent," "therapeutic," "therapy," "treatment," or "medicament" are used interchangeably herein to refer to a compound or compounds or composition of matter which, when administered to a subject (human or animal) induces a desired pharmacological and/or physiologic effect by local and/or systemic action.

[0021] The abbreviations in the specification correspond to units of measure, techniques, properties, or compounds as follows: "min" means minute(s), "g" means gram(s), "mg" means milligram(s), "µg" means microgram(s), "eq" means equivalent(s), "h" means hour(s), "µL" means microliter(s), "mL" means milliliter(s), "mM" means millimolar, "M" means molar, "mmol" or "mmole" means millimole(s), "cm" means centimeters, "SEM" means

standard error of the mean, and "IU" means International Units. "IC_{50} value" or "IC_{50}" means dose of the compound which results in 50% alleviation or inhibition of the observed condition or effect.

[0022] "Apicidin" is a compound derived from a Fusarium species fungal metabolite. It has the structure cyclo(N-O- methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl).

[0023] "Natural analogs of Apicidin" refers to analogs of Apicidin that are produced in fermentations of Fusarium pallidoroseum species ATCC74322 and ATCC47289 (Apicidins A, B, C, D1, D2, D3, which are described in *JOC 67, 815 (2002)* and *Tet Lett, 37, 8077 (1996)*, and in WO 1996/9603428.

[0024] As used herein, "alkyl" refers to an optionally substituted, saturated straight, or branched, hydrocarbon radical having from about 1 to about 20 carbon atoms (and all combinations and subcombinations of ranges and specific numbers of carbon atoms therein). Where appropriate, "alkyl" can mean "alkylene"; for example, if X is $-R_1R_2$, and R_1 is said to be "alkyl", then "alkyl" may correctly be interpreted to mean "alkylene".

[0025] "Amino" refers to $-NH_2$ and may include one or more substituents that replace hydrogen. "Amino" is used interchangeably with amine and is also intended to include any pharmaceutically acceptable amine salts. For example, amino may refer to $-NH^+(X)(Y)Cl^-$, wherein X and Y are preferably and independently hydrogen or alkyl, wherein alkyl may include one or more halo substitutions.

[0026] As used herein, "aryl", "arene", and "aromatic" each refer to an optionally substituted, saturated or unsaturated, monocyclic, polycyclic, or other homo-, carbo- or heterocyclic aromatic ring system having from about 3 to about 50 ring members (and all combinations and subcombinations of ranges and specific numbers of carbon atoms therein), with from about 5 to about 10 ring atom members being preferred. Such moieties encompass (include) "heteroaryl" and "heteroarene" as defined *infra*. Where appropriate, "aryl" can mean "arene"; for example, if X is $-R_1R_2$, and R_1 is said to be "aryl", then "aryl" may correctly be interpreted to mean "arene".

[0027] As used herein, "alkenyl" refers to an alkyl radical having from about 2 to about 20 carbon atoms and one or more double bonds (and all combinations and subcombinations of ranges and specific numbers of carbon atoms therein), wherein alkyl is as previously defined. In some embodiments, it is preferred that the alkenyl groups have from about 2 to about 6 carbon atoms. Alkenyl groups may be optionally substituted.

[0028] As used herein, "aralkyl" refers to alkyl radicals bearing one or more aryl substituents and having from about 4 to about 50 carbon atoms (and all combinations and subcombinations of ranges and specific numbers of carbon atoms therein), wherein aryl and alkyl are as previously defined. In some preferred embodiments, the alkyl moieties of the aralkyl groups have from about 1 to about 4 carbon atoms. In other preferred embodiments, the alkyl moieties have from about 1 to about 3 carbon atoms. Aralkyl groups may be optionally substituted.

[0029] "Alkylamino" signifies alkyl-(NH)-, wherein alkyl is as previously described and NH is defined in accordance with the provided definition of amino. "Arylamino" represents aryl-(NH)-, wherein aryl is as defined herein and NH is defined in accordance with the provided definition of amino. Likewise, "aralkylamino" is used to denote aralkyl-(NH)-, wherein aralkyl is as previously defined and NH is defined in accordance with the provided definition of amino. "Alkylamido" refers to alkyl-CH(=O)NH-, wherein alkyl is as previously described. "Alkoxy" as used herein refers to the group R-O- where R is an alkyl group, and alkyl is as previously described. "Aralkoxy" stands for R-O-, wherein R is an aralkyl group as previously defined. "Alkylsulfonyl" means alkyl-SO_2-, wherein alkyl is as previously defined. "Aminooxy" as used herein refers to the group amino-(O)-, wherein amino is defined as above. "Aralkylaminooxy" as used herein is used to denote aryl-akyl-aminooxy-, wherein aryl, alkyl, and aminooxy are respectively defined as provided previously.

[0030] As used herein, "alkylene" refers to an optionally branched or substituted bivalent alkyl radical having the general formula -$(CH_2)_n$-, where n is 1 to 10. Non-limiting examples include methylene, trimethylene, pentamethylene, and hexamethylene.

[0031] "Alkyleneamino" refers to -$(CH_2)_n$-NH-, where n is 1 to 10 and wherein the bivalent alkyl radical may be optionally branched or substituted, and the amino group may include one or more substituents that replace hydrogen.

[0032] As used herein, "heteroaryl" or "heteroarene" refers to an aryl radical wherein in at least one of the rings, one or more of the carbon atom ring members is independently replaced by a heteroatom group selected from the group consisting of S, O, N, and NH, wherein aryl is as previously defined. Heteroaryl / heteroarene groups having a total of from about 3 to about 14 carbon atom ring members and heteroatom ring members are preferred. Likewise, a "heterocyclic ring" is an aryl radical wherein one or more of the carbon atom ring members may be (but are not necessarily) independently replaced by a heteroatom group selected from the group consisting of S, O, N, and NH. Heterocyclic rings having a total from about 3 to 14 ring

members and heteroatom ring members are preferred, but not necessarily present; for example, "heterocyclohexyl" may be a six-membered aryl radical with or without a heteroatom group.

[0033] "Halo" and "halogen" each refers to a fluoro, chloro, bromo, or iodo moiety, with fluoro, chloro, or bromo being preferred.

[0034] "Haloalkyl" signifies halo-alkyl- wherein alkyl and halo, respectively, are as previously described.

[0035] The phrase reading "[moiety] is absent" may mean that the substituents to which the moiety is attached are directly attached to each other.

[0036] Typically, substituted chemical moieties include one or more substituents that replace hydrogen. Exemplary substituents include, for example, halo (*e.g.*, F, Cl, Br, I), alkyl, cycloalkyl, alkylcycloalkyl, cycloalkylalkyl, alkenyl, alkynyl, aralkyl, aryl, heteroaryl, heteroaralkyl, spiroalkyl, heterocycloalkyl, hydroxyl (-OH), nitro ($-NO_2$), cyano (-CN), amino ($-NH_2$), -N-substituted amino (-NHR"), -N,N-disubstituted amino (-N(R")R"), oxo (=O), carboxy (-COOH), -O-C(=O)R", -C(=O)R", -OR", -C(=O)OR", -(alkylene)-C(=O)-OR", -NHC(=O)R", aminocarbonyl ($-C(=O)NH_2$), -N-substituted aminocarbonyl (-C(=O)NHR"), -N,N-disubstituted aminocarbonyl (-C(=O)N(R")R"), thiol, thiolato (-SR"), sulfonic acid ($-SO_3H$), phosphonic acid ($-PO_3H$), -P(=O)(OR")OR", -S(=O)R", $-S(=O)_2R$", $-S(=O)_2NH_2$, $-S(=O)_2$ NHR", $-S(=O)_2NR"R"$, $-NHS(=O)_2R"$, $-NR"S(=O)_2R"$, $-CF_3$, $-CF_2CF_3$, -NHC(=O)NHR", -NHC(=O)NR"R", -NR"C(=O)NHR", -NR"C(=O)NR"R", -NR"C(=O)R" and the like. In relation to the aforementioned substituents, each moiety R" can be, independently, any of H, alkyl, cycloalkyl, alkenyl, aryl, aralkyl, heteroaryl, or heterocycloalkyl, for example.

[0037] As used herein, the terms "treatment" or "therapy" (as well as different word forms thereof) includes preventative (*e.g.*, prophylactic), curative or palliative treatment.

[0038] As employed above and throughout the disclosure the term "effective amount" refers to an amount effective, at dosages, and for periods of time necessary, to achieve the desired result with respect to the treatment of the relevant disorder, condition, or side effect. It will be appreciated that the effective amount of components of the present invention will vary from patient to patient not only with the particular compound, component or composition selected, the route of administration, and the ability of the components to elicit a desired response in the individual, but also with factors such as the disease state or severity of the condition to be alleviated, hormone levels, age, sex, weight of the individual, the state of being of the patient, and the severity of the pathological condition being treated, concurrent medication or special diets then being followed by the particular patient, and other factors which those skilled in the art will recognize, with the appropriate dosage ultimately being at the discretion of

the attendant physician. Dosage regimens may be adjusted to provide the improved therapeutic response. An effective amount is also one in which any toxic or detrimental effects of the components are outweighed by the therapeutically beneficial effects. As an example, the compounds useful in the methods of the present invention are administered at a dosage and for a time such that the level of activation and adhesion activity of platelets is reduced as compared to the level of activity before the start of treatment.

[0039] "Pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem complications commensurate with a reasonable benefit/risk ratio.

[0040] Within the present invention, the disclosed compounds may be prepared in the form of pharmaceutically acceptable salts. "Pharmaceutically acceptable salts" refer to derivatives of the disclosed compounds wherein the parent compound is modified by making acid or base salts thereof. Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. The pharmaceutically acceptable salts include the conventional non-toxic salts or the quaternary ammonium salts of the parent compound formed, for example, from non-toxic inorganic or organic acids. For example, such conventional non-toxic salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric and the like; and the salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, pamoic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicylic, sulfanilic, 2-acetoxybenzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isethionic, and the like. These physiologically acceptable salts are prepared by methods known in the art, *e.g.*, by dissolving the free amine bases with an excess of the acid in aqueous alcohol, or neutralizing a free carboxylic acid with an alkali metal base such as a hydroxide, or with an amine.

[0041] Compounds described herein throughout, can be used or prepared in alternate forms. For example, many amino-containing compounds can be used or prepared as an acid addition salt. Often such salts improve isolation and handling properties of the compound. For example, depending on the reagents, reaction conditions and the like, compounds as described herein can be used or prepared, for example, as their hydrochloride or tosylate salts. Isomorphic

crystalline forms, all chiral and racemic forms, N-oxide, hydrates, solvates, and acid salt hydrates, are also contemplated to be within the scope of the present invention.

[0042] Certain acidic or basic compounds of the present invention may exist as zwitterions. All forms of the compounds, including free acid, free base and zwitterions, are contemplated to be within the scope of the present invention. It is well known in art that compounds containing both amino and carboxy groups often exist in equilibrium with their zwitterionic forms. Thus, any of the compounds described herein throughout that contain, for example, both amino and carboxy groups, also include reference to their corresponding zwitterions.

[0043] "Hydrate" refers to a compound of the present invention which is associated with water in the molecular form, i.e., in which the H-OH bond is not split, and may be represented, for example, by the formula $R \cdot H_2O$, where R is a compound of the invention. A given compound may form more than one hydrate including, for example, monohydrates ($R \cdot H_2O$) or polyhydrates ($R \cdot nH_2O$ wherein n is an integer > 1) including, for example, dihydrates ($R \cdot 2H_2O$), trihydrates ($R \cdot 3H_2O$), and the like, or hemihydrates, such as, for example, $R \cdot n/_2H_2O$, $R \cdot n/_3H_2O$, $R \cdot n/_4H_2O$ and the like wherein n is an integer.

[0044] "Solvate" refers to a compound of the present invention which is associated with solvent in the molecular form, i.e., in which the solvent is coordinatively bound, and may be represented, for example, by the formula R·(solvent), where R is a compound of the invention. A given compound may form more than one solvate including, for example, monosolvates (R·(solvent)) or polysolvates (R·n(solvent)) wherein n is an integer > 1) including, for example, disolvates (R·2(solvent)), trisolvates (R·3(solvent)), and the like, or hemisolvates, such as, for example, $R \cdot n/_2$(solvent), $R \cdot n/_3$(solvent), $R \cdot n/_4$(solvent) and the like wherein n is an integer. Solvents herein include mixed solvents, for example, methanol/water, and as such, the solvates may incorporate one or more solvents within the solvate.

[0045] "Acid hydrate" refers to a complex that may be formed through association of a compound having one or more base moieties with at least one compound having one or more acid moieties or through association of a compound having one or more acid moieties with at least one compound having one or more base moieties, said complex being further associated with water molecules so as to form a hydrate, wherein said hydrate is as previously defined and R represents the complex herein described above.

[0046] The term "stereoisomers" refers to compounds that have identical chemical constitution, but differ as regards the arrangement of the atoms or groups in space.

[0047] "Racemic" means having the capacity for resolution into forms of opposed optical activity.

[0048] As used herein, the term "partial stereoisomer" refers to stereoisomers having two or more chiral centers wherein at least one of the chiral centers has defined stereochemistry (i.e., *R* or *S*) and at least one has undefined stereochemistry (i.e., *R* or *S*). When the term "partial stereoisomers thereof" is used herein, it refers to any compound within the described genus whose configuration at chiral centers with defined stereochemistry centers is maintained and the configuration of each undefined chiral center is independently selected from *R* or *S*. For example, if a stereoisomer has three chiral centers and the stereochemical configuration of the first center is defined as having "*S*" stereochemistry, the term "or partial stereoisomer thereof" refers to stereoisomers having *SRR*, *SRS*, *SSR*, or *SSS* configurations at the three chiral centers, and mixtures thereof.

[0049] An "isotopically substituted analogue" is a compound of the present disclosure in which one or more atoms have been replaced with an isotope of that atom. For example, hydrogen (protium) may be substituted with deuterium or tritium. Other atoms that may be replaced with an isotope thereof in order to form an isotopically substituted analogue thereof include, for example, carbon (replaced with C^{13}), nitrogen (replaced with N^{15}), iodine (replaced with I^{131}), fluorine (replaced with F^{18}), or sulfur (replaced with S^{31}). Any available isotope may be used to form an isotopically substituted analogue thereof, and those of ordinary skill in the art will recognize available techniques for forming such analogues from a given compound.

[0050] "Prodrug" refers to compounds which are themselves inactive or minimally active for the activity desired, but through biotransformation can be converted into biologically active metabolites. For example, a prodrug of the present invention would include, *inter alia*, any compound which is convertible *in vivo* by metabolic means to a compound claimed or described in the present disclosure.

[0051] "N-oxide" refers to compounds wherein the basic nitrogen atom of either a heteroaromatic ring or tertiary amine is oxidized to give a quaternary nitrogen bearing a positive formal charge and an attached oxygen atom bearing a negative formal charge.

[0052] When any variable occurs more than one time in any constituent or in any formula, its definition in each occurrence is independent of its definition at every other occurrence. Combinations of substituents and/or variables are permissible only if such combinations result in stable compounds.

[0053] The term "administering" means either directly administering a compound or composition of the present invention, or administering a prodrug, derivative or analog which will form an equivalent amount of the active compound or substance within the body.

[0054] "Dosage unit" refers to physically discrete units suited as unitary dosages for the particular individual to be treated. Each unit may contain a predetermined quantity of active compound(s) calculated to produce the desired therapeutic effect(s) in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention may be dictated by (a) the unique characteristics of the active compound(s) and the particular therapeutic effect(s) to be achieved, and (b) the limitations inherent in the art of compounding such active compound(s).

[0055] "Subject" or "patient" refers to an embryonic, immature, or adult animal, including the human species, that is treatable with the compositions, and/or methods of the present invention.

[0056] It has presently been discovered that hepatitis B virus covalently closed circular DNA (cccDNA), existing and being expressed as an "episome" in the nucleus of an infected cell, is regulated differently than HBV DNA integrated in to the host chromosome, and that RNA expression from the HBV cccDNA can be pharmacologically suppressed, selectively, as compared to other genes (as described more fully herein). Indeed, the present inventors have identified numerous compounds that repress DHBV cccDNA transcription in a reproducible and robust manner, and that occurs at low concentrations and under conditions of no apparent toxicity. These results represent the first time that selective pharmacological suppression has been achieved, by design, with small molecules. The result that gene expression from HBV cccDNA is regulated differently that the same or similar DNA integrated in to the host chromosomes is surprising and a highly useful observation, in that it enables therapies that selectively repress cccDNA DNA (for example, as compared with integrated HBV DNA) without suppressing or otherwise affecting host chromosomal DNA. The present finding that HBV cccDNA can be suppressed pharmacologically was heretofore unknown, and offers the proof of useful concept of the prior statement, and demonstrates that such pharmacological suppression is possible.

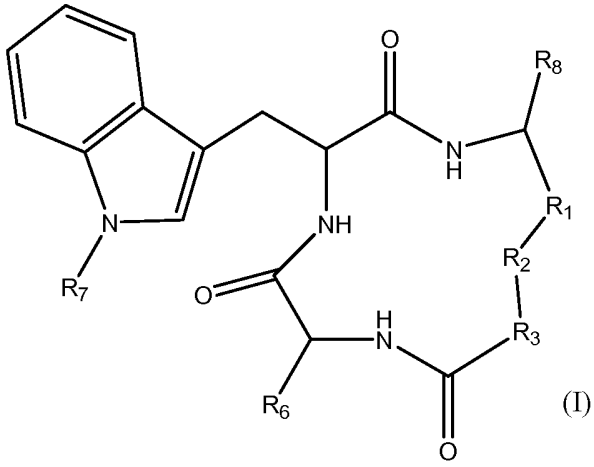
[0057] Accordingly, the present disclosure provides, *inter alia*, methods of modulating cccDNA transcription of hepatitis B in a subject comprising administering to the subject an agent that provides epigenetic modification of the cccDNA, a histone modifying agent, or an inhibitor of histone deacetylace activity. For example, the epigenetic modifying agent, histone modifying agent, or inhibitor of histone deacetylace activity may be pharmacological, such as a small

molecule. The epigenetic modifying agent, histone modifying agent, or inhibitor of histone deacetylace activity may be selective for the inhibition of cccDNA, as compared with integrated HBV DNA, *i.e.*, does not inhibit integrated HBV DNA, and/or as compared with cellular host DNA, *i.e.*, does not inhibit cellular host DNA. The inhibitor of histone deacetylace activity may be an inhibitor of multiple classes of histone deacetylase, or may be selective for a particular class of histone deacetylase. For example, the inhibitor may be an inhibitor of class I histone deacetylace activity, class II histone deacetylace activity, or both. Preferably, the inhibitor of histone deacetylace activity is an inhibitor of class I histone deacetylace activity. Numerous inhibitors of histone deacetylace activity are known, and any such HDAC inhibitor may be used pursuant to the present methods.

[0058] The present methods of modulating cccDNA transcription of hepatitis B may also include - in addition to the administration to the subject an agent that provides epigenetic modification of the cccDNA, a histone modifying agent, or an inhibitor of histone deacetylace activity - administering to the subject a therapeutically effective amount of a further agent that modulates hepatitis B virus. The further agent may be administered simultaneously with, or simply as a part of the same general therapy regimen as the agent that provides epigenetic modification of the cccDNA, histone modifying agent, or inhibitor of histone deacetylace activity. The further agent may be any substance that is presently used for modulation of HBV, of which numerous types are known among those skill in the art. For example, existing drugs for the modulation of HBV include interferons (*e.g.*, interferon alpha, pegylated interferon), nucleoside analogues (*e.g.,* lamivudine, adefovir dipivoxil, entecavir, telbivudine, tenofovir, clevudine, amdoxovir), non-nucleoside antivirals (e.g., BAM 205, ANA380, myrcludex B, HAP Compound Bay 41-4109, REP 9AC, nitazoxanide, dd-RNAi compound, ARC-520, NVR-1221), non-interferon immune enhancers (*e.g.,* thymosin alpha-1, interleukin-7, DV-601, HBV core antigen vaccine, GS-9620, GI13000), and post-exposure and/or post-liver transplant treatment drugs (*e.g.,* hyperHEP S/D, Nabi-GB, Hepa Gam B).

[0059] In particular, the further agent may be any other Direct Acting Antiviral anti hepatitis B agent (such as the polymerase inhibitors Barraclude, Tenofovir, lamivudine, telbivudine, and adefovir) and/or any other directing acting antiviral agents that work at a step in the virus life cycle other than suppression of cccDNA transcription, such as capsid inhibitors, secretion inhibitors, or entry inhibitors. The further agent may also be any other non-direct acting antiviral agent, such as an interferon or other immunomodulatory agent.

[0060] In accordance with the present methods of modulating cccDNA transcription of hepatitis B, the inhibitor of histone deacetylace activity may be, for example, Trichostatin A, suberoyl bis hydroxamic acid, dimethylamino hydroxy-benzamide, Apicidin, an Apicidin analog (for example, a natural analog of Apicidin or an analog that is synthesized de novo), or a compound according to formula (I)



(I)

wherein

R_1 is $-(CH_2)_n-$ or -C(=O)-;

R_2 is -C(=O)-, 3,5-triazolyl, or $-C(Z)N(R_4)-$;

R_4 is hydrogen, alkyl, aryl, aralkyl, dialkylaminoalkyl, or carboxyalkyl;

R_3 is $-CH(R_5)-$, or R_2 is nitrogen and R_3 is -CH- and R_2 and R_3 together form piperidinyl;

R_5 is hydrogen, $-CH_3$, or an alpha amino acid R group;

R_6 is $-(CH_2)_mC(X)Y$, $-(CH_2)_2CH_3$, or $-(CH_2)_q$-phenyl-$(CH_2)_mC(=O)NHOH$;

X is =O, H_2, $=N-NH_2$, or $=N-NH-C(=O)NH_2$;

Y is NHOH or $-CH_2CH_3$;

Z is H_2 or O;

R_7 is hydrogen or alkoxy;

R_8 is alkyl or carboxyalkyl;

n is 0-2;

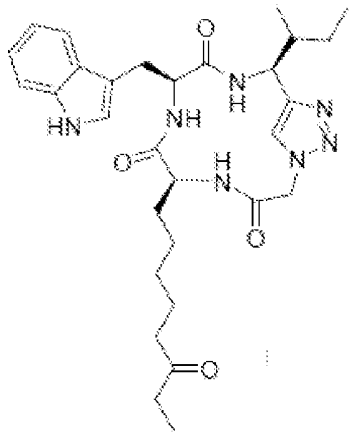
m is 0-6; and,

q is 0-3;

or a stereoisomer or pharmaceutically acceptable salt thereof.

[0061] As used herein, the phrase "alpha amino acid R group" refers to a side chain group from a a natural or unnatural amino acid.

[0062] In certain embodiments, the inhibitor of histone deacetylace activity is Apicidin,



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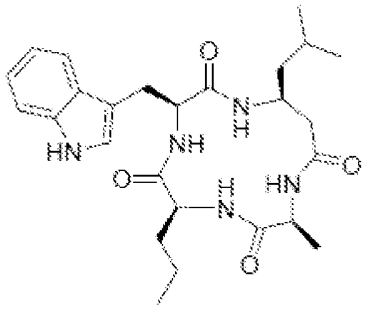

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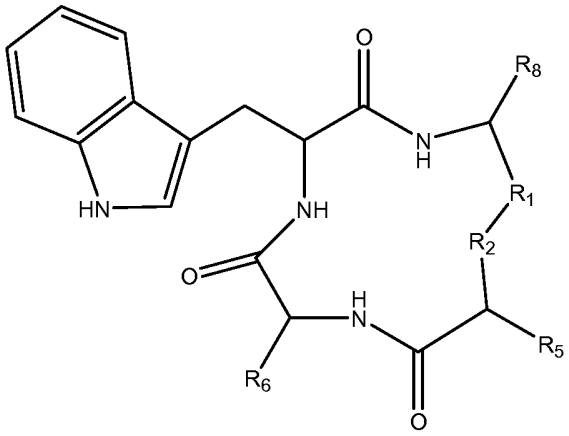


wherein

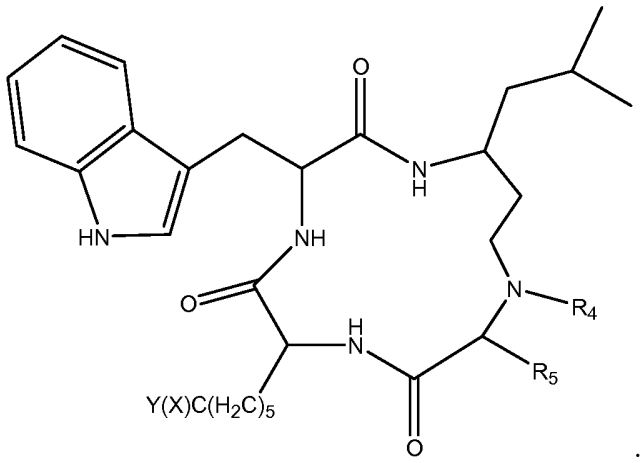
R_1 is $-(CH_2)-$,

and,

R_2 is $-C(Z)N(R_4)-$

or a stereoisomer or pharmaceutically acceptable salt thereof.

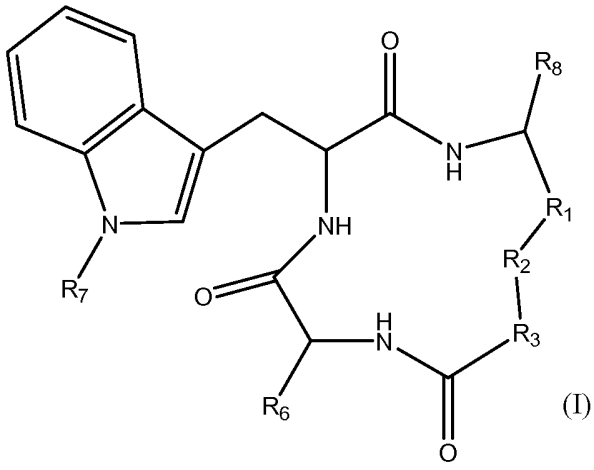
[0063] In other embodiments, the inhibitor of histone deacetylace activity is



or a stereoisomer or pharmaceutically acceptable salt thereof.

[0064] The present disclosure also pertains to methods of treating hepatitis B in a subject comprising administering to the subject an agent that provides epigenetic modification of the cccDNA, a histone modifying agent, or an inhibitor of histone deacetylace activity. For example, the epigenetic modifying agent, histone modifying agent, or inhibitor of histone deacetylace activity may be pharmacological, such as a small molecule. The epigenetic modifying agent, histone modifying agent, or inhibitor of histone deacetylace activity may be selective for the inhibition of cccDNA, as compared with integrated HBV DNA, *i.e.*, does not inhibit integrated HBV DNA, and/or as compared with cellular host DNA, *i.e.*, does not inhibit cellular host DNA. The inhibitor of histone deacetylace activity may be an inhibitor of multiple classes of histone deacetylase, or may be selective for a particular class of histone deacetylase. For example, the inhibitor may be an inhibitor of class I histone deacetylace activity, class II histone deacetylace activity, or both. Preferably, the inhibitor of histone deacetylace activity is an inhibitor of class I histone deacetylace activity. Numerous inhibitors of histone deacetylace activity are known, and any such HDAC inhibitor may be used pursuant to the present methods.

[0065] In accordance with the present methods of treating hepatitis B in a subject, the inhibitor of histone deacetylace activity may be, for example, Trichostatin A, suberoyl bis hydroxamic acid, dimethylamino hydroxy-benzamide, Apicidin, an Apicidin analog (for example, a natural analog of Apicidin or an analog that is synthesized de novo), or a compound according to formula (I)



(I)

wherein

R_1 is $-(CH_2)_n$- or -C(=O)-;

R_2 is -C(=O)-, 3,5-triazolyl, or -C(Z)N(R_4)-;

R_4 is hydrogen, alkyl, aryl, aralkyl, dialkylaminoalkyl, or carboxyalkyl;

R_3 is $-CH(R_5)$-, or R_2 is nitrogen and R_3 is -CH- and R_2 and R_3 together form piperidinyl;

R_5 is hydrogen, $-CH_3$, or an alpha amino acid R group;

R_6 is $-(CH_2)_mC(X)Y$, $-(CH_2)_2CH_3$, or $-(CH_2)_q$-phenyl-$(CH_2)_mC(=O)NHOH$;

X is =O, H_2, =N-NH_2, or =N-NH-C(=O)NH_2;

Y is NHOH or $-CH_2CH_3$;

Z is H_2 or O;

R_7 is hydrogen or alkoxy;

R_8 is alkyl or carboxyalkyl;

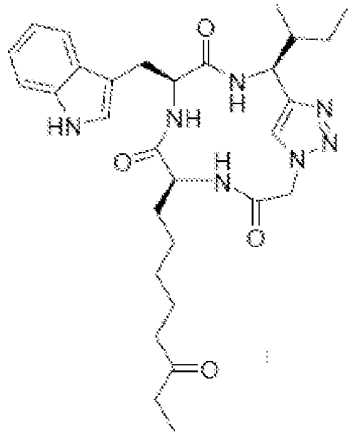
n is 0-2;

m is 0-6; and,

q is 0-3;

or a stereoisomer or pharmaceutically acceptable salt thereof.

[0066] In certain embodiments, the inhibitor of histone deacetylace activity is Apicidin,



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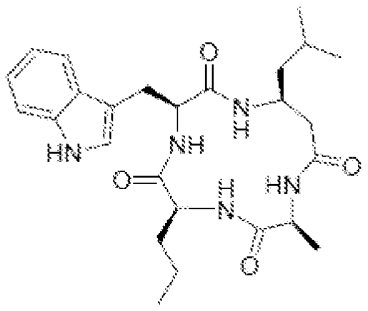

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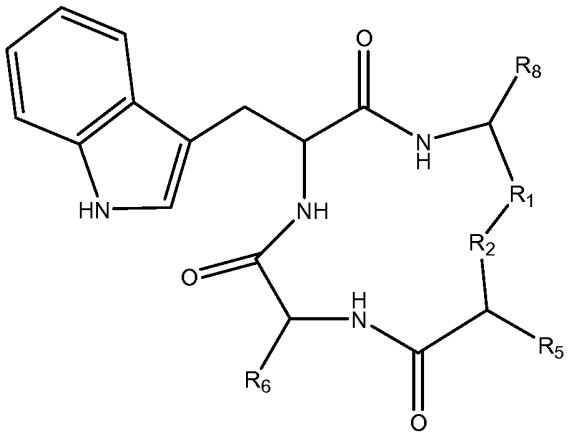


wherein

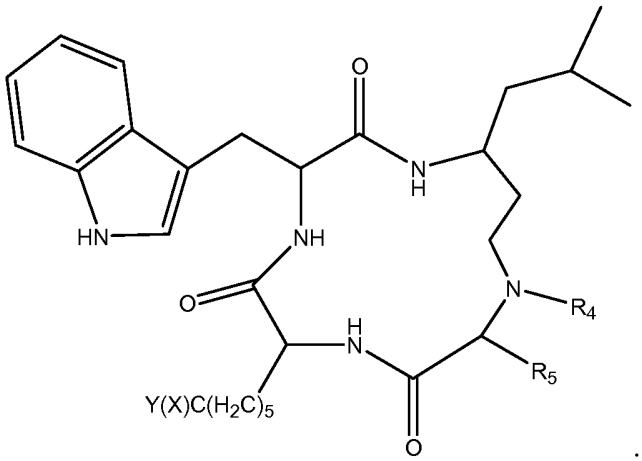
R_1 is $-(CH_2)-$,

and,

R_2 is $-C(Z)N(R_4)-$

or a stereoisomer or pharmaceutically acceptable salt thereof.

[0067] In other embodiments, the inhibitor of histone deacetylace activity is



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or a stereoisomer or pharmaceutically acceptable salt thereof.

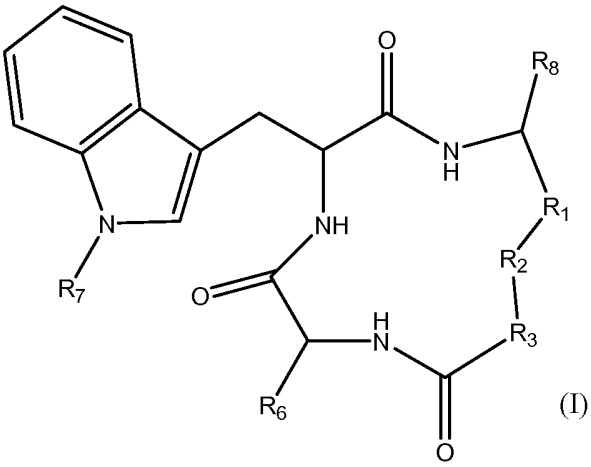
[0068] The present methods of treating hepatitis B in a subject may also include - in addition to the administration to the subject an agent that provides epigenetic modification of the cccDNA, a histone modifying agent, or an inhibitor of histone deacetylace activity - administering to the subject a therapeutically effective amount of a further agent that modulates hepatitis B virus. The further agent may be administered simultaneously with, or simply as a part of the same general therapy regimen as the agent that provides epigenetic modification of the cccDNA, histone modifying agent, or inhibitor of histone deacetylace activity. The further agent may be any substance that is presently used for modulation of HBV, of which numerous types are known among those skill in the art. For example, existing drugs for the modulation of HBV include interferons (*e.g.*, interferon alpha, pegylated interferon), nucleoside analogues (*e.g.*, lamivudine, adefovir dipivoxil, entecavir, telbivudine, tenofovir, clevudine, amdoxovir), non-nucleoside antivirals (e.g., BAM 205, ANA380, myrcludex B, HAP Compound Bay 41-4109, REP 9AC, nitazoxanide, dd-RNAi compound, ARC-520, NVR-1221), non-interferon immune enhancers (*e.g.*, thymosin alpha-1, interleukin-7, DV-601, HBV core antigen vaccine, GS-9620, GI13000), and post-exposure and/or post-liver transplant treatment drugs (*e.g.*, hyperHEP S/D, Nabi-GB, Hepa Gam B).

[0069] In particular, the further agent may be any other Direct Acting Antiviral anti hepatitis B agent (such as the polymerase inhibitors Barraclude, Tenofovir, lamivudine, telbivudine, and adefovir) and/or any other directing acting antiviral agents that work at a step in the virus life cycle other than suppression of cccDNA transcription, such as capsid inhibitors, secretion inhibitors, or entry inhibitors. The further agent may also be any other non-direct acting antiviral agent, such as an interferon or other immunomodulatory agent.

[0070] Also disclosed are methods of modulating hepatitis B virus covalently closed circular DNA comprising contacting a hepatitis B virus with an agent that provides epigenetic modification of the cccDNA, a histone modifying agent, or an inhibitor of histone deacetylace activity. For example, the epigenetic modifying agent, histone modifying agent, or inhibitor of histone deacetylace activity may be pharmacological, such as a small molecule. The epigenetic modifying agent, histone modifying agent, or inhibitor of histone deacetylace activity may be selective for the inhibition of cccDNA, as compared with integrated HBV DNA, *i.e.*, does not inhibit integrated HBV DNA, and/or as compared with cellular host DNA, *i.e.*, does not inhibit cellular host DNA. The inhibitor of histone deacetylace activity may be an inhibitor of multiple classes of histone deacetylase, or may be selective for a particular class of histone deacetylase. For example, the inhibitor may be an inhibitor of class I histone deacetylace activity, class II

histone deacetylace activity, or both. Preferably, the inhibitor of histone deacetylace activity is an inhibitor of class I histone deacetylace activity. Numerous inhibitors of histone deacetylace activity are known, and any such HDAC inhibitor may be used pursuant to the present methods.

[0071] In accordance with the present methods of modulating hepatitis B virus covalently closed circular DNA, the inhibitor of histone deacetylace activity may be, for example, Trichostatin A, suberoyl bis hydroxamic acid, dimethylamino hydroxy-benzamide, Apicidin, an Apicidin analog (for example, a natural analog of Apicidin or an analog that is synthesized de novo), or a compound according to formula (I)



(I)

wherein

R_1 is $-(CH_2)_n$- or -C(=O)-;

R_2 is -C(=O)-, 3,5-triazolyl, or -C(Z)N(R_4)-;

R_4 is hydrogen, alkyl, aryl, aralkyl, dialkylaminoalkyl, or carboxyalkyl;

R_3 is –CH(R_5)-, or R_2 is nitrogen and R_3 is -CH- and R_2 and R_3 together form piperidinyl;

R_5 is hydrogen, $-CH_3$, or an alpha amino acid R group;

R_6 is $-(CH_2)_mC(X)Y$, $-(CH_2)_2CH_3$, or $-(CH_2)_q$-phenyl-$(CH_2)_mC(=O)NHOH$;

X is =O, H_2, =N-NH_2, or =N-NH-C(=O)NH_2;

Y is NHOH or $-CH_2CH_3$;

Z is H_2 or O;

R_7 is hydrogen or alkoxy;

R_8 is alkyl or carboxyalkyl;

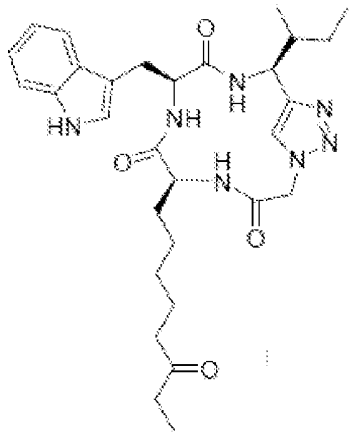
n is 0-2;

m is 0-6; and,

q is 0-3;

or a stereoisomer or pharmaceutically acceptable salt thereof.

[0072] In certain embodiments, the inhibitor of histone deacetylace activity is Apicidin,



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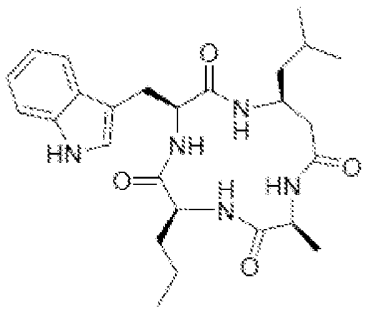

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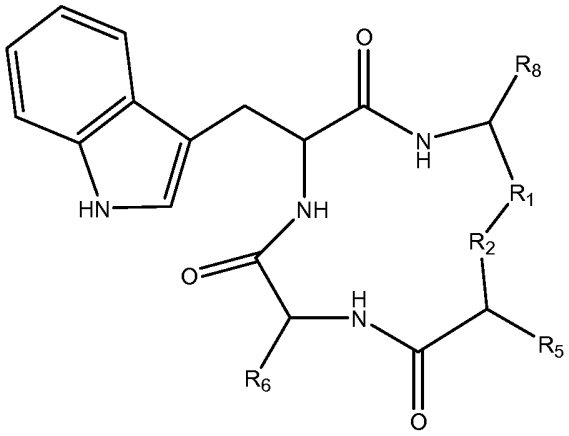


wherein

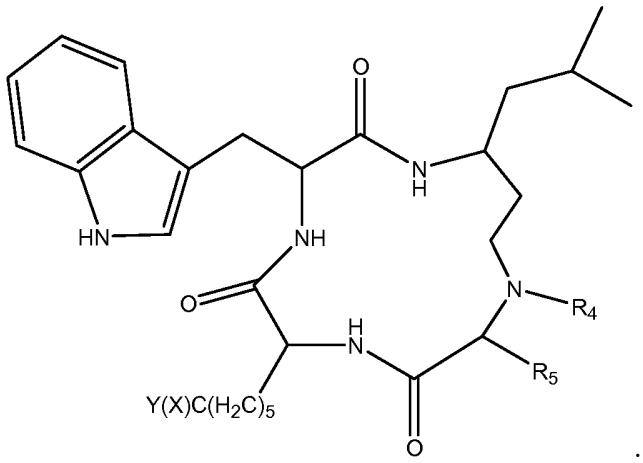
R_1 is $-(CH_2)-$,

and,

R_2 is $-C(Z)N(R_4)-$

or a stereoisomer or pharmaceutically acceptable salt thereof.

[0073] In other embodiments, the inhibitor of histone deacetylace activity is

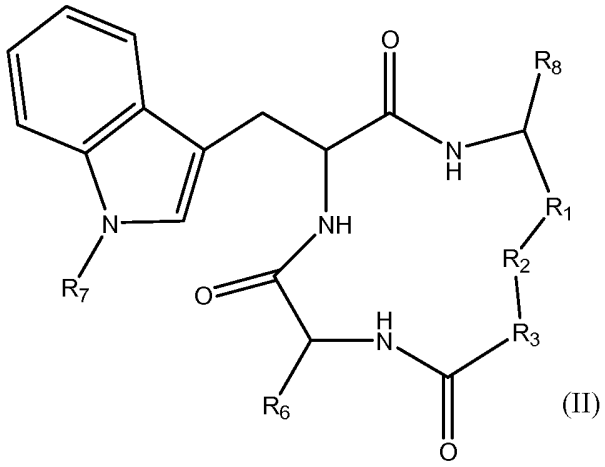


or a stereoisomer or pharmaceutically acceptable salt thereof.

[0074] The present methods of modulating hepatitis B virus covalently closed circular DNA may also include - in addition to the contacting of a hepatitis B virus with an agent that provides epigenetic modification of the cccDNA, a histone modifying agent, or an inhibitor of histone deacetylace activity - contacting the hepatitis B virus with a therapeutically effective amount of a further agent that modulates hepatitis B virus. The contacting of the further agent with the HBV may occur simultaneously with, or simply as a part of the same procedure that involves contacting the HBV with the agent that provides epigenetic modification of the cccDNA, histone modifying agent, or inhibitor of histone deacetylace activity. The further agent may be any substance that is presently used for modulation of HBV, of which numerous types are known among those skill in the art. For example, existing drugs for the modulation of HBV include interferons (*e.g.*, interferon alpha, pegylated interferon), nucleoside analogues (*e.g.*, lamivudine, adefovir dipivoxil, entecavir, telbivudine, tenofovir, clevudine, amdoxovir), non-nucleoside antivirals (e.g., BAM 205, ANA380, myrcludex B, HAP Compound Bay 41-4109, REP 9AC, nitazoxanide, dd-RNAi compound, ARC-520, NVR-1221), non-interferon immune enhancers (*e.g.*, thymosin alpha-1, interleukin-7, DV-601, HBV core antigen vaccine, GS-9620, GI13000), and post-exposure and/or post-liver transplant treatment drugs (*e.g.*, hyperHEP S/D, Nabi-GB, Hepa Gam B).

[0075] In particular, the further agent may be any other Direct Acting Antiviral anti hepatitis B agent (such as the polymerase inhibitors Barraclude, Tenofovir, lamivudine, telbivudine, and adefovir) and/or any other directing acting antiviral agents that work at a step in the virus life cycle other than suppression of cccDNA transcription, such as capsid inhibitors, secretion inhibitors, or entry inhibitors. The further agent may also be any other non-direct acting antiviral agent, such as an interferon or other immunomodulatory agent.

[0076] The present disclosure also pertains to compound according to formula II:



(II)

wherein

R_1 is $-(CH_2)_n-$ or -C(=O)-;

R_2 is -C(=O)- or $-C(Z)N(R_4)-$;

R_4 is hydrogen, alkyl, aryl, aralkyl, dialkylaminoalkyl, or carboxyalkyl;

R_3 is $-CH(R_5)-$;

R_5 is hydrogen, $-CH_3$, or an alpha amino acid R group;

R_6 is $-(CH_2)_mC(X)Y$, $-(CH_2)_2CH_3$, or $-(CH_2)_q$-phenyl-$(CH_2)_mC(=O)NHOH$;

X is =O, H_2, $=N-NH_2$, or $=N-NH-C(=O)NH_2$;

Y is NHOH or $-CH_2CH_3$;

Z is H_2 or O;

R_7 is hydrogen or alkoxy;

R_8 is alkyl or carboxyalkyl;

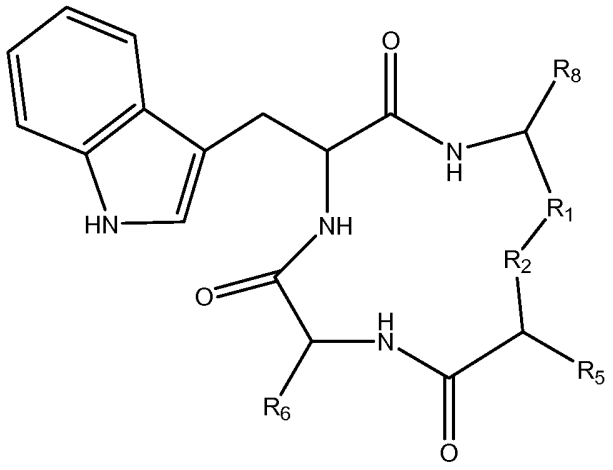
n is 0-2;

m is 0-6; and,

q is 0-3;

or a stereoisomer or pharmaceutically acceptable salt thereof,

[0077] For example, the compound may be



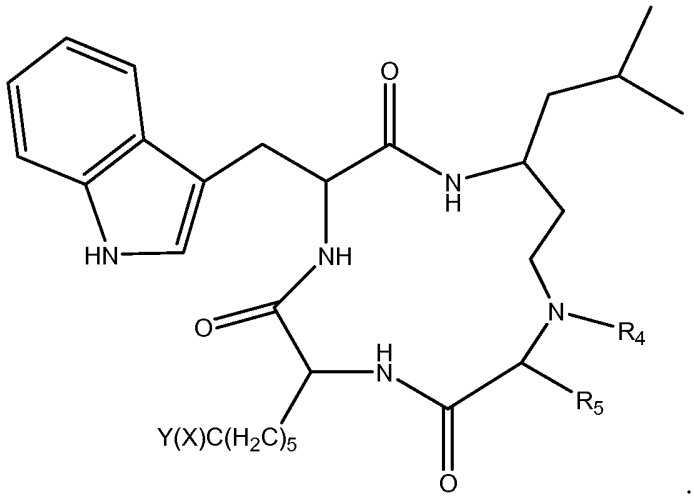
wherein

R_1 is -(CH_2)-,

and,

R_2 is -C(Z)N(R_4)-.

[0078] In other embodiments, the compound may be



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[0079] As will be readily understood, functional groups present may contain protecting groups during the course of synthesis. Protecting groups are known *per se* as chemical functional groups that can be selectively appended to and removed from functionalities, such as hydroxyl groups and carboxyl groups. These groups are present in a chemical compound to render such functionality in room temperature chemical reaction conditions to which the compound is exposed. Any of a variety of protecting groups may be employed with the present invention. Protecting groups that may be employed in accordance with the present invention may be described in *Greene, T.W. and Wuts, P.G.M., Protective Groups in Organic Synthesis 2d. Ed., Wiley & Sons, 1991.*

[0080] In a further aspect, the present disclosure relates to pharmaceutical compositions comprising a compound according to formula (I) or (II), or a pharmaceutically acceptable salt, isotopically substituted analogue, or stereoisomer thereof and a pharmaceutically acceptable carrier, diluent, or excipient. The applicable carrier, diluent, or excipient may be selected on the basis of the chosen route of administration and standard pharmaceutical practice as described, for example, in *Remington's Pharmaceutical Sciences (Mack Pub. Co., Easton, PA, 1985)*, the disclosure of which is hereby incorporated by reference in its entirety. The pharmaceutical compositions may further comprise a therapeutically effective amount of a further agent that modulates hepatitis B virus. For example, the further agent that modulates virus may be a known anti-viral agents. In certain embodiments, the present compositions comprise a therapeutically effective amount of a compound according to formula (I) or (II) which is administered in combination with immunizations or vaccines that are effective in preventing or lessening the symptoms of HBV. Examples include antibodies, immune suppressants, anti-inflammatory agents, and the like.

[0081] As used herein, the term "contacting" refers to the bringing together into physical or chemical communication of indicated moieties in an *in vitro* system or an *in vivo* system. For example, "contacting" an HBV virus with a compound in the invention may include the administration of a compound in the present invention to an individual or patient, such as a human, having an HBV infection, as well as, for example, introducing a compound of the invention into a sample containing a cellular or purified preparation containing cccDNA.

[0082] As used herein, the term "individual" or "patient," used interchangeably, refers to any animal, including mammals, such as mice, rats, other rodents, rabbits, dogs, cats, swine, cattle, sheep, horses, or primates, such as humans.

[0083] As used herein, the phrase "therapeutically effective amount" refers to the amount of active compound or pharmaceutical agent that elicits the biological or medicinal response that is being sought in a tissue, system, animal, individual or human by a researcher, veterinarian, medical doctor or other clinician, which includes one or more of the following:

(1) preventing the disease; for example, preventing a disease, condition or disorder in an individual who may be predisposed to the disease, condition or disorder but does not yet experience or display the pathology or symptomatology of the disease;

(2) inhibiting the disease; for example, inhibiting a disease, condition or disorder in an individual who is experiencing or displaying the pathology or symptomatology of the disease, condition or disorder (i.e., including arresting further development of the pathology and/or symptomatology); and

(3) ameliorating the disease; for example, ameliorating a disease, condition or disorder in an individual who is experiencing or displaying the pathology or symptomatology of the disease, condition or disorder (i.e., including reversing the pathology and/or symptomatology).

[0084] A subject or patient in whom administration of the therapeutic compound is an effective therapeutic regimen for a disease or disorder is preferably a human, but can be any animal, including a laboratory animal in the context of a clinical trial or screening or activity experiment. Thus, as can be readily appreciated by one of ordinary skill in the art, the methods, compounds and compositions of the present invention are particularly suited to administration to any animal, particularly a mammal, and including, but by no means limited to, humans, domestic animals, such as feline or canine subjects, farm animals, such as but not limited to bovine, equine, caprine, ovine, and porcine subjects, wild animals (whether in the wild or in a zoological garden), research animals, such as mice, rats, rabbits, goats, sheep, pigs, dogs, cats, and the like, avian species, such as chickens, turkeys, songbirds, and the like, *i.e.,* for veterinary medical use.

[0085] The compounds of this invention may be administered orally or parenterally, neat or in combination with conventional pharmaceutical carriers, diluents, or excipients, which may be liquid or solid. The applicable solid carrier, diluent, or excipient may function as, among other things, a binder, disintegrant, filler, lubricant, glidant, compression aid, processing aid, color, sweetener, preservative, suspensing/dispersing agent, tablet-disintegrating agent, encapsulating material, film former or coating, flavors, or printing ink. Of course, any material used in preparing any dosage unit form is preferably pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations. Parenteral administration in this respect includes administration by, *inter alia*, the following routes: intravenous, intramuscular, subcutaneous, intraocular, intrasynovial, transepithelial including transdermal, ophthalmic, sublingual and buccal; topically including ophthalmic, dermal, ocular, rectal and nasal inhalation via insufflation, aerosol, and rectal systemic.

[0086] In powders, the carrier, diluent, or excipient may be a finely divided solid that is in admixture with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier, diluent or excipient having the necessary compression properties in suitable proportions and compacted in the shape and size desired. For oral therapeutic administration, the active compound may be incorporated with the carrier, diluent, or excipient and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. The amount of active compound(s) in such therapeutically useful compositions is

preferably such that a suitable dosage will be obtained. The therapeutic compositions preferably contain up to about 99% of the active ingredient.

[0087] Liquid carriers, diluents, or excipients may be used in preparing solutions, suspensions, emulsions, syrups, elixirs, and the like. The active ingredient of this invention can be dissolved or suspended in a pharmaceutically acceptable liquid such as water, an organic solvent, a mixture of both, or pharmaceutically acceptable oils or fat. The liquid carrier, excipient, or diluent can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers, or osmo-regulators.

[0088] Suitable solid carriers, diluents, and excipients may include, for example, calcium phosphate, silicon dioxide, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, methyl cellulose, ethylcellulose, sodium carboxymethyl cellulose, microcrystalline cellulose, polyvinylpyrrolidine, low melting waxes, ion exchange resins, croscarmellose carbon, acacia, pregelatinized starch, crospovidone, HPMC, povidone, titanium dioxide, polycrystalline cellulose, aluminum methahydroxide, agar-agar, tragacanth, or mixtures thereof.

[0089] Suitable examples of liquid carriers, diluents and excipients for oral and parenteral administration include water (particularly containing additives as above, *e.g.* cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, *e.g.* glycols) and their derivatives, and oils (*e.g.* fractionated coconut oil and arachis oil), or mixtures thereof.

[0090] For parenteral administration, the carrier, diluent, or excipient can also be an oily ester such as ethyl oleate and isopropyl myristate. Also contemplated are sterile liquid carriers, diluents, or excipients, which are used in sterile liquid form compositions for parenteral administration. Solutions of the active compounds as free bases or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. A dispersion can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

[0091] The pharmaceutical forms suitable for injectable use include, for example, sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form is preferably sterile and fluid to provide easy syringability. It is preferably stable under the conditions of manufacture and storage and is preferably preserved against the contaminating action of microorganisms such as

bacteria and fungi. The carrier, diluent, or excipient may be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of a dispersion, and by the use of surfactants. The prevention of the action of microorganisms may be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions may be achieved by the use of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0092] Sterile injectable solutions may be prepared by incorporating the active compounds in the required amounts, in the appropriate solvent, with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions may be prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation may include vacuum drying and the freeze drying technique that yields a powder of the active ingredient or ingredients, plus any additional desired ingredient from the previously sterile-filtered solution thereof.

[0093] The compounds of the invention may be administered in an effective amount by any of the conventional techniques well-established in the medical field. The compounds employed in the methods of the present invention including the compounds of formulas (I) or (II) may be administered by any means that results in the contact of the active agents with the agents' site or sites of action in the body of a patient. The compounds may be administered by any conventional means available.

[0094] Preferably the pharmaceutical composition is in unit dosage form, *e.g.* as tablets, buccal tablets, troches, capsules, elixirs, powders, solutions, suspensions, emulsions, syrups, wafers, granules, suppositories, or the like. In such form, the composition is sub-divided in unit dose containing appropriate quantities of the active ingredient; the unit dosage forms can be packaged compositions, for example packeted powders, vials, ampoules, prefilled syringes or sachets containing liquids. The unit dosage form can be, for example, a capsule or tablet itself, or it can be the appropriate number of any such compositions in package form. In addition, dosage forms of the present invention can be in the form of capsules wherein one active

ingredient is compressed into a tablet or in the form of a plurality of microtablets, particles, granules or non-perils. These microtablets, particles, granules or non-perils are then placed into a capsule or compressed into a capsule, possibly along with a granulation of the another active ingredient.

[0095] The dosage of the compounds of the present invention that will be most suitable for prophylaxis or treatment will vary with the form of administration, the particular compound chosen and the physiological characteristics of the particular patient under treatment. Generally, small dosages may be used initially and, if necessary, increased by small increments until the desired effect under the circumstances is reached. Generally speaking, oral administration may require higher dosages.

[0096] The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, *e.g.*, into a number of discrete loosely spaced administrations. The dose may also be provided by controlled release of the compound, by techniques well known to those in the art.

[0097] Additional information regarding the preparation of the present compounds for administration and the formulation of compositions according to the present invention is provided *infra*.

[0098] The compounds useful in the methods of the present invention may be prepared in a number of ways well known to those skilled in the art. The compounds can be synthesized, for example, by the methods as described below, or variations thereon as appreciated by the skilled artisan. The reagents used in the preparation of the compounds of this invention can be either commercially obtained or can be prepared by standard procedures described in the literature. All processes disclosed in association with the present invention are contemplated to be practiced on any scale, including milligram, gram, multigram, kilogram, multikilogram or commercial industrial scale.

[0099] For compounds herein in which a variable appears more than once, each variable can be a different moiety selected from the Markush group defining the variable. For example, where a structure is described having two R groups that are simultaneously present on the same compound, the two R groups can represent different moieties selected from the Markush group defined for R.

[0100] It is further appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, can also be provided in combination in a single embodiment. Conversely, various features of the invention which are, for brevity,

described in the context of a single embodiment, can also be provided separately or in any suitable subcombination.

[0101] The present invention is further described in the following Examples. It should be understood that these examples, while indicating preferred embodiments of the invention, are given by way of illustration only, and should not be construed as limiting the appended claims. From the above discussion and these examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLES

Modulation of HBV cccDNA

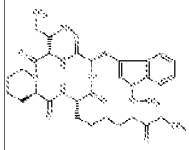
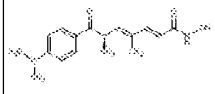
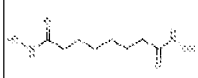
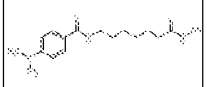
[0102] DHBV cccDNA in LMH derived dstet5 cells is efficiently produced and transcriptionally active. Most HBV producing cells lines produce HBV gene products from an HBV transgene integrated into the host chromosome, and thus cccDNA is not the major source of viral product. This makes screening for drugs that target cccDNA difficult. Cell lines were produced in which viral gene products are dependent upon cccDNA. It was established human Hep G2 and chicken hepatoma (LMH)- stable cell lines for this purpose with tetracycline (tet) regulated HBV/DHBV. As shown in FIG. 1, after culture in the absence of tet and presence of 2 mM of foscarnet (PFA) to block viral reverse transcription, DHBV RNAs accumulate, but DHBV replication is arrested at the stage of pgRNA-containing nucleocapsids (lane 0). Upon addition of tet back to media to block transgene transcription, and removal of PFA to allow the viral DNA synthesis in the pgRNA-containing capsid to proceed, there is a rapid decline of viral RNA (day 1 and 2), with an eventual increase to a higher level when cccDNA is made after day 3.

[0103] These results imply that cccDNA is efficiently formed and transcriptionally functional in dstet5 cells. These results are more thoroughly demonstrated in FIG. 2, where, under the conditions specified in which the transgene transcription is blocked with tet, appearance of new HBV RNA is closely associated with appearance of cccDNA (FIG. 2 Group II, core DNA shown), whereas, viral transcripts are rapidly degraded (1/2 life ~3 hrs) in cells in which both cccDNA synthesis and new transgene transcription is blocked (FIG. 2, Group I)

[0104] Identification of compounds that potently repress DHBV cccDNA transcription. With a system and conditions under which viral transcripts are produced in a cccDNA dependent manner (FIGS. 1, 2B), approximately 100 compounds were screened, including those from the inventors' in-house small compound library, those present in the inventors' Natural Products

collection, and selected compounds including inhibitors of cellular epigenetic modification enzymes, including HDACs, HATs, Sirtuins, histone methyltransferases, histone demethylases and DNA methyltransferases. Numerous compounds, including the four compounds shown in Table 1, significantly reduced the amounts of cccDNA-derived DHBV pgRNA. All possess HDAC class I inhibitory activity.

Table 1: Compounds that repress HBV cccDNA function and their activity against HDACs[1]

Hit	Apicidin	Trichostatin A (TSA)	Suberoyl bis hydroxamic acid (SBHA)	Dimethylamino-hydroxy-benzamide (M344)[5]
Structure				
HBVcccDNA (EC50, uM)	0.183	0.480	2.50	6.25
Toxicity (CC50,um)[2]	>20.00	>20.00	>40.00	>100.00
Selectivity Index (SI)[3]	>100	>40	>16	>16
HDAC-I inhibitor?	YES[4,5]	YES[5,6,9]	YES[4,6,7,10]	YES[4,7]
HDAC-II inhibitor?	NO[3,7]	YES[4,5,6,9]	YES & HDAC III[11]	YES & HDAC III,[7,11]

[1] Compounds found to suppress HBV cccDNA function in the dstet5 system, described in Prelim Evid, as illustrated in Fig. 2.
[2] Toxicity from our assays on dstet5 cells, as in text; Selectivity Index (SI) is the toxicity CC50 divided by the Effectiveness EC50, see text.
[3] Selectivity Index (SI) is concentration that reduces 50% of cell viability (CC_{50}) divided by the concentration reduces 50% of the HBV specific signal (RNA and/or HBeAg) (EC_{50}).
[4] (7); [5] (15)(26); [6] (45); [7] Reaction Biology Monograph; [8] (33); [9] (13); [10] (14); [11] (38)

As shown in FIG. 3, since Apicidin potently inhibited cccDNA (EC50 ~ 180 nM), with no toxicity at up to 20 uM for five days, and has nanomolar activity against class I but not class II HDACs, it appears that HDAC II inhibition is not necessary to suppress HBV in this system.

[0105] Apicidin and TSA repress HBV cccDNA transcription. FIG. 3 shows that Apicidin and TSA repress cccDNA transcription in Dstet5 cells. Evidence was also obtained demonstrating that these compounds also repress HBV cccDNA transcription in the HepG2 cells. In marked contrast, it was observed that Apicidin and TSA dose-dependently stimulate DHBV pgRNA transcription from transgene integrated in host cellular chromosome (FIG. 4). This is more typical of cellular gene responses to HDAC inhibitions and suggests that unlike chromosomal DNA, transcription from cccDNA "minichromosomes" are regulated differently. Moreover, there is even evidence that cccDNA levels were reduced, indicating, as seen in the Duck system, that transcriptional repression is followed by destabilization.

Effect of compounds upon HBV cccDNA transcription in human hepatoma cells.

[0106] HepDE19 cells are seeded into 6-well plates, cultured in the presence of tetracycline until confluence. Tet is removed from the culture media to allow pgRNA transcription, DNA synthesis and cccDNA formation to occur. Tet is added back to culture media to shut off transgene transcription. After day 3, the cells, in different wells, are left untreated or treated varying concentrations (*i.e.*, 0.1 to 10.0 uM) of each of the "Test" compounds (four "hits" from Table 1 and ~20 analogues) for 2 days. Intracellular HBV cccDNA, viral RNA and core DNA are quantified by Southern/Northern blot hybridization assays as described above and in known procedures. Intracellular full-length HBeAg precursor and secreted HBeAg are quantified with Western blot and ELISA assays respectively. HepG2.2.15 cells are used as a control, because all HBV expression is primarily from the HBV transgene in these cells. Interferon alpha, which has been shown to inhibit cccDNA transcription, and disubstituted-sulfonamides (DSS) CCC-0975, which inhibits cccDNA formation (from our screen, *Guo 2012*) will be included as positive drug controls. In some experiments, cultures are maintained for varying times (days) after removal of "Test" drug from the culture medium, to determine the durability of any drug induced repression of HBV cccDNA. In order to determine the selectivity of the testing compounds on cccDNA transcription, effects of the testing compounds on the expression of a panel of cellular genes, including, but not limited to, alpha1 antitrypsin, albumin, are also measured by quantitative RT-PCR or Northern blot hybridization. The cytotoxicity of the compounds are determined by MTT assay in parallel cultures.

[0107] The amount (0-100%) of reduction of HBeAg and HBV transcripts is taken as a measure of HBV cccDNA transcriptional repression. The amount (0-100%) of HBV cccDNA reduction is taken as a measure of destabilization and degradation of HBV cccDNA. The amount (0-100%) of repression of A1AT and/or albumin mRNA reduction is taken as a measure of cellular function inhibition in specificity determination. The amount of MTT (0-100%) activity is taken as a measure of cell viability and the basis of cell cytoxicity (CC). The Selectivity Index (SI) is as in the Table 1 legend.

[0108] Effect of compounds upon WHV cccDNA transcription in primary woodchuck hepatocytes. Woodchuck Hepatitis Virus (WHV) has been a useful model for evaluating therapeutics for HBV. Therefore, it is useful to know, for planning, if the lead compounds are active against WHV. Primary woodchuck hepatocytes cultures (PWHCs) are prepared by plating collagenase treated tissues, derived from small section biopsies obtained from chronically infected woodchucks, under conditions where 50-90% of the hepatocytes harbor WHV, and the

cultures (90% or greater) are hepatocytes and can be maintained for at least two months in culture, as done previously (*see Fletcher SP, et al. 2012. Transcriptomic analysis of the woodchuck model of chronic hepatitis B. Hepatology: In press*). Within 7 days of seeding, cultures are incubated in the absence or presence of test compounds, and the amount of WHV gene product in the culture medium (WHV virion associated DNA; WHs) and intracellularly (WHV DNA, WHV RNA transcripts) are determined, using the similar methods as previously used and published (*Guo, H., et al. 2010. Production and function of the cytoplasmic deproteinized relaxed circular DNA of hepadnaviruses. J Virol 84:387-396; Guo, et al. 2011. Alkylated porphyrins have broad antiviral activity against hepadnaviruses, flaviviruses, filoviruses, and arenaviruses. Antimicrob Agents Chemother 55:478-486*)

[0109] Where WHV is sensitive (as suspected) to Apicidin and other candidate cccDNA inhibitors, at SI's similar to that in the avian and human systems, then chronically infected woodchucks are used for an *in vivo* proof of efficacy study.

[0110] The inhibitors are ranked (i) by their selectivity index (SI), with the most selective in inhibiting HBV cccDNA transcription versus cellular viability, and then cellular function, being the most attractive; (ii) by their potency of inhibiting cccDNA transcription (lowest EC50) and finally, (iii) by "critical chemistry" (scalability type/formulation) issues. The compounds with the lowest values of EC_{50} (concentration that inhibits 50% of the cccDNA-transcribed RNA) and greatest SIs, are the most attractive.

Identification of HDAC Isoform

[0111] Each of the compounds identified in the primary screen share the property of having HDAC inhibitory activity (see, e.g., Table 1). It is likely that HDAC inhibition is either part of, or central to, the mechanism of the HBV antiviral action of these compounds. Although it is not necessary to precisely know the compound's mechanism, this information would be helpful in selecting or designing modified compounds, as well as in forecasting and reducing possible in vivo toxicities, and designing clinical studies. Also, since crystal structures are available for many HDACs, future drug design can be assisted. Taken together, the growing experience with HDAC inhibitors in research and in people, can could provide direction for the present clinical designs and future plans.

[0112] HDACs deacetylate polypeptides (i.e., histones) and are classified into four categories, based on function and DNA sequence homology. Class I and II HDACs are inhibited by trichostatin A (TSA). Apicidin efficiently inhibits class I, but not class II HDACs. Class III HDACs, called sirtuins, are a family of NAD+-dependent proteins, not affected by TSA. Class IV is considered an atypical category, based on DNA sequence. Because Apicidin and TSA

potently inhibited cccDNA transcription, HDAC isozymes in classes I and II are most relevant. However, since Apicidin inhibits only Class I, the initial focus is only on this class.

[0113] Experimental Details: Silencing of HDAC isozyme transcripts with shRNA and the affect upon HBV cccDNA function. Short hairpin RNAs (shRNAs) expressed from lentivirus transduction vectors are now standard tools to repress translation of the transcripts to which the shRNA is homologous. A focus is placed on the class I HDAC isozymes. Therefore, confluent monolayers of HepDE19 cells expressing HBV gene products in a cccDNA dependent manner (as described *supra*, after tet repression) are transduced with 100ul of lentivirus (~5 x 10^7/ml) in transduction mixture, expressing shRNA selective for class I-1,2,3 or 8 isozymes under conditions where at least 95% of the cells receive and express the shRNA. This is determined by expression of reporter from the retroviral transgene. The shRNA lentiviral expression vectors are provided by the vendor as transduction ready, and each vector targets different HDACs of sub-class I. They are purchased from Vendors (*e.g.,* Santa Cruz Bio, OpenBio), and contain and express short hairpins with 19-25 nts homologous to each HDAC isozyme transcript to be targeted. For example, one HDAC 1 specific shRNA contains 5′-GAT CCC CGC AGA … ATC TGC TTT TTG GAA A-3′, and others are similarly designed but specific for the other shRNAs, as provided by vendor and from previous work. There is 4- and 5-fold coverage for each HDAC isozyme. Control vectors contain scrambled sequences, and are used as negative controls. After 5 days of shRNA lentiviral transduction, repression of the specific HDAC is quantified by RNA analysis and western blot (with HDAC specific probes and monoclonal antibodies provided by vendor), and the amount of HBV cccDNA and cccDNA dependent transcript, and HBeAg, is measured as was performed in preceding description. Where transient transduction approaches are unsatisfactory, although a bit more involved, stable transductions are used, since shRNA constructs, with selectable markers are used. The amount of cellular gene expression (A1AT and albumin mRNA) are also quantified, as described in above, as a specificity control. Positive controls include incubation of the HepDE19 cells with Apicidin at 1000 nM, a concentration which represses HBV cccDNA (and will have been validated on HepDE19). Each of the different HDACs has been associated with specific cellular functions (i.e. 1, up regulation of p53, 2 & 3 p21, 8, deacetylation of H4) which are quantified as evidence of successful HDAC sub class inhibition, should that be desired.

[0114] Given the potency of Apicidin, it is expected that silencing at least one of the Class I HDACs will result in significant repression of HBV cccDNA function. It is recognized that the HDAC inhibitors, which act on multiple HDACs, may have a greater effect than can be achieved by a single HDAC transcript knock down. However, we knock down experiments are

also performed using lentivirus combinations covering all class I enzymes, since this should repress HBV cccDNA if class I enzymes are involved (as Apicidin suggests), but multiple enzymes must be repressed to detect the HBV cccDNA inhibition.

[0115] Where silencing a specific or group of HDAC transcripts results in repression of HBV cccDNA function, this is validated as a target for HBV antiviral action, and corroborated the finding that a mechanism of anti-HBV cccDNA action of the identified compounds involves HDAC inhibition. The compounds could, of course, use other mechanisms for HBV cccDNA suppression, but it will at least be known that HDAC inhibition does repress HBV cccDNA, and the door is now open for this new class of HBV therapeutic strategies.

[0116] Determining which of the identified compounds have the greatest inhibitory effect upon the HDACs isozymes responsible for HBV cccDNA repression. Having identified specific HDAC isozymes that are responsible for regulating HBV cccDNA, it is useful to identify the compounds that have the greatest selectivity for inhibiting the HBV cccDNA regulating isozyme. This allows for advancing the compounds with the greatest selectivity and help avoid off target effects resulting from needlessly inhibiting HDAC isozymes that are not involved in regulating HBV cccDNA. We note that of the four compounds denoted above in Table 1, Apicidin has the greatest selectivity index, and is also the one with the narrowest HDAC inhibitory profile (selective for HDAC class I). Therefore, it is possible to achieve even greater selectivity by avoiding broad HDAC inhibitors and zooming in on the specific HDAC sub-isozyme that is sufficient to repress HBV cccDNA.

[0117] Enzyme assays for each of the HDAC class I (1,2,3,8) isozymes are available as commercial kits, with positive and negative competitive inhibitor controls. Kits are purchased corresponding to the relevant isozyme as identified above, from BioTeK, BPS Bioscience, or other available sources. Briefly, with the BioTek system, sub class specific purified HDAC enzyme (recombinant, at ~10-50 ng/vessel) is provided, with a fluorogenic substrate, detected following deacetylation, with developer in a premixed reaction. The enzymes that were shown by silencing to be involved in HBV cccDNA repression are purchased. Varying amounts of control or each of the experimental compounds are incubated with the enzyme reaction mix

[0118] The assay read-out is optimized for linearity both as a function of time and enzyme concentration. Kits from the concentrations of the testing compounds required to inhibit 50% of the deacetylase activity of an HDAC isoform (*i.e.* IC50) are calculated by regression analysis using SigmaPlot software (Systat Software, Inc., San Jose, CA).

[0119] Ideally, and most logically, compounds found to be active according to the procedures described above are active against HDACs found to be most involved in HBV

cccDNA regulation, and these represent the favored compounds. Compounds that are active but broadly inhibit HDACs, some of which are found to be irrelevant to HBV cccDNA regulation, are somewhat less favored, since they may bring unnecessary side effects. Where, on the other hand, there is a disconnect, and the compounds active in the preceding assays do not inhibit the HDACs found to be most important to HBV cccDNA regulation, the compounds are advanced based on HBV cccDNA suppressive activity, and not HDAC inhibitory ranking.

Evaluation of lead compounds for their in vitro Absorption, Distribution, Metabolism and Toxicity (ADMET) properties HBV producing cells and non producing cells

[0120] Introduction and Rationale In vivo experiments are expensive and ethically constrained. Before testing in animals, it is therefore prudent to initially profile compounds for potential toxicity and other cell-serum - interactive properties that are, to the extent possible, predictive of in vivo performance. These studies have become standards in the field. Toxicity in *replicating* cells has also been found to be a good way to rank compounds with respect to toxicity. Finally, differing formulations are also usually necessary, before moving on to in vivo work, because solvents used in the tissue culture setting are not always compatible with in vivo administration. These are used, as below. An innovation in *in vitro* "ADMET" is presently proposed, in which the profiling is carried out with HBV producing cells in the presence of a currently approved antiviral therapies, in addition to the routine ADMET.

[0121] It is likely that new anti-HBV drugs, will be used in combination with the other HBV antiviral drugs, in current use. Combination therapy is standard for HIV and HCV and other infectious diseases. It is important to know if a new drug to treat HBV has toxicities or other altered profiles in the presence of the current standards of care, since there is evidence that many otherwise well tolerated medications have selective toxicities in chronically infected individuals. HBV producing cells may be more sensitive to some emdications than are non producing cells (Block, in progress). Therefore, the toxicity experiments, below, are carried out in the absence as well as the presence of HBV polymerase inhibitors and, in some cases, interferon alpha (IFNa).

[0122] Some of the present lead compounds may have already been used in animals (by others), there may be considerable information available. On the other hand, some of the leads may be new compounds for which there is no animal data. Compound profiles are also examined in the context of HBV infection, for the reason stated above.

[0123] Finally, compounds that suppress wild type HBV cccDNA function and are well tolerated in vitro are tested for their ability to suppress cccDNA from HBV that is resistant to

HBV polymerase inhibitors. Depending on the results of the preceding studies, human and/or duck HBV transfection (and for the duck, infection) systems are used.

[0124] For every experiment described below, controls with known toxicity, metabolism, protein permeability, membrane transport and defined formulation properties are included. For example, Barraclude and FIAU are included as controls for compounds that have no detectable toxicity in HBV producing cells, and those that do, respectively, and have reported PK and TK properties for which comparisons can be made.

[0125] Experimental Detail: In vitro "administration, distribution, metabolism, "elimination" and toxicity" (ADMET) studies. Some of these experiments are carried out under contract by a Vendor (i.e. Absorption Systems) and others, particularly where HBV producing cells and material are used, are carried out by the present inventors, as indicated, below.

[0126] *Standard cytoxicity assays:* Human hepatoma (HepG2, Huh7, HepRG) and HepG2-derived cell lines supporting constitutive (HepG2.2.15) and tetracycline-inducible HBV replication (HepDE19 and HepDES19) are seeded into 96-well plates at a density of 2×10^4 cells per well. Cells are treated with a serial dilution of testing compounds. The culture media is changed every other day. MTT assays are performed at day 2, 4, 6, 8 and 10 day since treatment.

[0127] *Toxicity to multiplying cells:* Varying concentrations of lead compound(s) are incubated with HepRG cells seeded at low density (100 cells per well of 32 mm dish) under HBV producing and non producing conditions, and cultured for 10 days, with media changes every 3 days.

[0128] *Metabolic stability in human and mouse liver microsomes:* The compounds are incubated with human and mouse liver microsomes from HBV producing and non producing cells (tissue culture source as above) in the presence of NADPH. In addition, the stability of compounds are evaluated in the presence of human simulated gastric fluid and simulated intestinal fluid. The purpose of this set of experiments is also to determine if the compounds are metabolized by the digestive enzymes. Since orally available compounds are pursued, it is important to find out what metabolites, if any, might be produced in the GI tract.

[0129] The toxicity and metabolic stability studies are carried out in the absence and presence of concentrations of lamivudine, barraclude, telbivudine, tenofovir and/or adefovir that are equal to and multiples (~0.1 ug/ml, for barraclude, – 10 ug/ml for lamivudine) or interferon alpha (IFNa) of the serum levels typically achieved in people. The cccDNA suppressive test compounds are used at 10 times their IC50, as determined in assays described above. Control compounds (with established toxicities and established metabolic profiles) are also included with each panel of tests (i.e. FIAU, statins, etc).

[0130] *Plasma protein binding*: Equilibrium dialysis is used in this assay to determine the percentage of compound that binds to human plasma proteins (by Vendor).

[0131] *Bidirectional permeability*: This assay is used to determine the permeability of compounds through Caco-2 cell monolayers in the apical-to-basolateral and basolateral-to-apical direction. (Contractor)

[0132] *Antiviral activity of lead compounds in the presence of interferons (IFNs).* The experiments above explore the in vitro ADMET of the lead compounds when used in combination with polymerase inhibitors or interferons in uninfected cells. It is also important to determine if the lead compounds have an impact upon an established antiviral agent's antiviral properties. Compared with pol inhibitors, IFN alpha (a) is less frequently used to manage HBV. When used, it is only for a period of months, unlike pol inhibitors, which are used for years and more likely to be co-administered with a cccDNA inhibitor. However, given the fact that IFNa mechanisms of antiviral action and toxicities may involve HDACs, it does make sense to evaluate the presently disclosed cccDNA inhibitors for their interaction profiles with IFNa, to the extent this can be evaluated in vitro. Therefore, the dSTET cells and AD38 cells programmed to produce transcripts from HBV cccDNA (as in prelim evidence and Cai 2012) seeded at cloning densities (for growth studies) and semi confluence (for antiviral/ cccDNA transcription studies) are incubated in the absence and presence of varying concentrations of candidate cccDNA inhibitor and the absence and presence of amounts of either avian IFN or human IFNa known to suppress HBV in vitro. Cell viability and the amount of HBV cccDNA derived gene products (transcripts) produced are determined as in previously described procedures and those known in the literature.

[0133] The compounds are also tested for in vitro activity in the presence of the currently used polymerase inhibitors. The emergence of mutant viruses resistant to the nucleoside/tide inhibitors of the HBV polymerase is a problem in the management of chronic infection, although the problem varies with the polymerase inhibitor used. Thus, compounds that suppress wild type HBV cccDNA function are tested for their ability to suppress cccDNA from HBV that is resistant to HBV polymerase inhibitors. All of the mutant viruses (DHBV and WHV) needed are available. Human and / or Duck HBV transfection (and for the Duck, infection) systems are used. Given the distinct mechanism of action, the present compounds retain antiviral activity.

[0134] *Formulation optimization:* For selected compounds, dosing vehicle development suitable for oral gavage are evaluated. The test vehicles include 1) pH manipulation, 2) co-solvents (such as glycin, polyethylene glycol propylene glycol, ethanol etc),

3) surfactants (such as polysorbates, polozamer, polyoxyl castor oil, glyceryl and PEG esters), 4) Non-aqueous systems (such as sesame oil, medium chain triglycerides, soybean oil, oleic acid), 5) complexing agents (such as cyclodextrins).

[0135] From an ADMET perspective, preferred are compounds that have properties similar to Barraclude, with respect to tolerability. Also preferred are compounds that have the same toxicity and metabolic stability profiles in the absence of HBV polymerase inhibitors (lamivudine, barraclude, interferon etc) as in their presence. Compounds with selective toxicity to HBV producing cells are disfavored, disqualified, or advanced with extra caution. Compounds that have enhanced, or enhance, the toxicity of current HBV antivirals, or antagonize the antiviral, activity those compounds, are still advanced, but with caution and tested in in vivo experiments for the possibility of enhanced toxicity in combination. It is possible to propose that the cccDNA active compounds not be used (or only used cautiously) in combination.

Lead compounds with favorable in vitro properties are scaled up and tested for in vivo Toxicity, Pharmaco kinetics (PK) and efficacy

[0136] Pharmacokinetic, Toxico-Kinetic (TK), and dose range finding studies. Prior to conducting in vivo efficacy studies, which are expensive, ethically constrained, and consume great amounts of compound, it is necessary to determine the maximum tolerated doses (MTDs) and pharmacokinetic properties (PK) of the candidate drugs, in vivo, in uninfected animals. This permits the identification of compounds worthy of advancement and establish proper dosing and routes of administration. Compounds are tested for efficacy in either (or both) duck and/or woodchuck models of chronic hepadnavirus infection, since these are the established and predictive animal models. The rationale for duck versus woodchuck is described below. Regarding Apicidin itself, a great deal will already be known about its PK/TK in animals, since it has already been used in mice. However, even for Apicidin, and certainly for any other of the present compounds, new PK, TK for the Duck and woodchuck study are needed. Therefore, a series of murine and rat PK and TK studies are conducted as follows.

[0137] Experimental Detail - *Single Dose Pharmacokinetic Study in mice, ducks and, if indicated, woodchucks.* The objective of this study is to obtain volume of distribution, systemic clearance, half-life (T1/2), maximal plasma concentration (Cmax) and bioavailability. These parameters are used to evaluate the clearance and bioavailability of each imino sugars so that the compounds can be ranked by their ability to maintain plasma concentration. In general, greater than 50% bioavailability is preferred for compounds to be advanced.

[0138] As described above, candidates are administered via i.v. injection (5mg/kg) or given orally (25mg/kg) to mice (6 week old Balb/c; 6 mice/group); Peking Ducks (6 week old) or

woodchucks (3 per group). Clinical observations are recorded at several intervals after dosing. Blood and urine samples for pharmacokinetics are collected predose, and at 5, 15, and 30 min, 1, 2, 4, 6, 8, 16 and 24 h post-dose. Samples are analyzed for the presence and amounts of administered drug (drug or prodrug) and in the case of administered prodrug, for the presence and amount of "drug" metabolite" as well. The samples are analyzed by Absorption Systems, who has established mouse plasma assays for our other compounds.

[0139] *Tissue distribution (murine).* Tissue is taken from mice (3 per dose group) receiving a single oral or iv administration of compound at various times after administration. Knowledge of the tissue distribution of a compound can significantly aid in evaluating potential as successful drug candidate. Although other in vitro parameters, such as plasma protein binding and volume of distribution have prediction values for rate and extent of distribution to extravascular tissues, the liver tissue concentration of drug is probably most relevant to efficacy. A focus is maintained on liver, in comparison to serum, kidney and abdominal fat tissue/ lymph nodes, for tissue concentration of candidates, using endpoint samples, following the single administration of the compound by an i.p. and oral route in mice. One point of interest is if active compound builds up in key tissue, which provides insights regarding its effective half life, in tissue. That is, although the serum half life of a drug might be ~2 hours, it could have a tissue half life in liver several fold times that, explaining a greater than expected efficacy (for a given dosing regimen), or greater than expected toxicity.

[0140] *Dose-finding Maximum Tolerated Dose (MTD) study.* Since the compounds are evaluated for antiviral activity in murine models, it is important to know the tolerability of the compounds in mice. Balb/c mice (6 week old, 6 per group); Ducks (6 week old, 3 per group) will be dosed by oral gavage (since we are pursuing orally available compounds) either "vehicle" alone, or vehicle in which compound has been dissolved. From previous experience, the range of compound administered is likely between 100 mg/kg to 500 mg/kg, 5 mice per dose group. Animals will be observed for up to 14 days, with daily readings of weight and an endpoint of survivability. Routine histology and clinical chemistry studies are be performed. The highest dose of compound that does not result in any mortality/toxicity is considered to be the MTD. Woodchucks can not be used for this MTD study; extrapolations from the murine study, combined with the PK woodchuck study will be necessary.

[0141] The compounds are ranked for their oral bioavialibility, tolerability, and half lives. The ideal compound is able to reach and sustain serum or liver tissue levels at least 10 times the tissue culture IC50 concentration, with soluble, oral, single day dosing, and have

MTDs more than 100 times that of the tissue culture IC50. Compounds are ranked with respect to these qualities, and the best and second best will be advanced.

[0142] *Is the lead compound efficacious in chronically infected animal models, in vivo?* Having demonstrated in vitro efficacy, and determined safe and rationale dosing for in vivo work, it is be important to know if the lead compounds can control viral levels in validated animal models of chronic HBV. This represents the first time a small molecule drug that targets cccDNA will have been tested in animals. Outcomes consistent with a safe, selective and cccDNA targeting agent are of interest. Efficacy end points include: rapid and coordinated reduction in viremia, antigenemia as well as amount of intra-liver cccDNA and replicative forms which would be indicative of cccDNA suppression. These goals dictate the animal models that are used, and length of treatment that is studied.

[0143] Several animal models of chronic HBV infection exist, and each has virtues as well as disadvantages. Ducks and woodchucks can be experimentally chronically infected with duck and woodchuck hepadnavirus, respectively. There are now several murine models, but since transgenic mice bearing HBV transgenes do not produce HBV from cccDNA templates, to test a cccDNA targeting compound, a chrimeric mouse with human hepatocytes would be necessary, such as the uPA mice. Practical considerations require making a choice. Experiments are designed for evaluation in the Duck model of chronic HBV, since the compounds are active against the Duck virus in avian cells in culture are already known. Studies in the chronically infected woodchuck are also prepared, since this is an established model for testing HBV therapeutics and is a natural infection. The uPA mice are very expensive but will are if woodchucks are not sensitive to the drugs, but human HBV is.

[0144] Therefore, preferred compounds are scaled up to the amount necessary and tested for efficacy, as defined below, in the following Duck, and if appropriate, woodchucks.

[0145] *Experimental Detail - Scale up production of preferred compounds.* Apicidins are produced in fermentations by Fusarium (i.e. sp. ATCC 74322). The strain is inoculated into a nutrient medium called MED5, shaken at 220 rpm, for 12-16 days in a controlled humidity atmosphere. At harvest, whole broth is extracted with methylethylketone and the extract is fractionated by gel filtration on Sephadex followed by final purification by RP-HPLC. Yields are on the order of 250 mg/L so scale up to gram amounts are routine.

[0146] *Duck hepadnavirus efficacy study.* Since it is known that Apicidin is highly active against the DHBV, in culture, it is tested in a chronically infected duck. The goal of this study is to determine the antiviral potential of preferred compounds. Serology and histology are secondary.

[0147] Six-week-old Peking Ducks, chronically infected with DHBV type 16 (Alberta Strain), are used. At 6 weeks, viremia and liver mass in ducks tends to have stabilized. Ducks are given, by either i.m. or oral gavage (depending on Aim 4 PK/TK results), test compound (3 dose groups, with dosing amount and frequency to depend on PK results, but aiming to achieve stable serum levels of at least 10 times the tissue culture IC50). There are three dose groups with 5-6 animals per dose group. Control dose groups (6 animals each group) include placebo treated animals and animals treated with either barraclude (1 mg/kg) or lamivudine (40 mg/kg) per day. At least three animals from all dose groups contribute at least one pre treatment and one post treatment liver biopsy. Treatment is for 10 weeks, since this exceeds the time for lamivudine to suppress viremia to beneath detectable levels and the reported ½ life of cccDNA in the duck. Ducks are followed with weekly serum collections for an additional 4 weeks after withdrawal of drug. Serum will be collected weekly.

[0148] Weekly serum is tested for standard "lab values" (hematology, albumin, AST, ALTs, The amount of DHBV viral DNA, sAg, sAb in the circulation is determined. Liver tissue derived from biopsies (some pre treatment and end of treatment from the same animals) is examined for DHBV DNA (cccDNA, replicative forms) and DHBV core (immunostained).

[0149] *WHV-infected woodchuck study.* The study uses 10 groups, with 5 animals per group, with drug treatment for 10 weeks followed by 10 weeks off drug (to test durability of affect). Due to variability in the levels of viremia and antigenemia, animals are stratified to groups by WHV viremia and antigenemia levels as determined seven days prior to study start, so that the average levels of both viral markers are evenly distributed among all groups of animals. Animals with abnormally low WHVsAg levels are not used in this study. Compound is administered daily, by a route and frequency to be finalized after bioavailability studies in rodents. The first day of dosing on the study is Study Day 1. Study Day 1 dose levels are calculated on a pretest body weight, and body weights are taken weekly for dose administration. Dosing range is as for the mouse study over four doses, with Group 10 treated with Barraclude as a reference compound (Tennant).

[0150] The primary endpoint is a dose dependent reduction in viremia and antigenemia on and off drug achieving durable off drug reductions.

[0151] *Viability and animal health.* Clinical observations are performed and recorded once daily for morbidity and mortality. Further toxicology is addressed via hematology, serum chemistry, and histology examination. It is also important to consider all biochemical and immunological endpoints in the context of general animal health to insure that decreases in viremia or antigenemia or other putative beneficial outcomes are not a secondary consequence

protocol (compound) toxicity. Gross physical characteristics (weight, stool and urine output and characterization, are determined on a weekly basis. In addition, liver function tests (performed on samples collected monthly), hematology and chemistry (performed on pre, mid and end of treatment samples (as described in the table) and, for selected animals (at pre-dose, mid dose and end of treatment times), histology on punch biopsy derived liver sections are also performed for assessment of toxicity as well as efficacy

[0152] Liver function test are determined by commercial service in the monthly samples as a marker of liver viability

[0153] *Evidence of humoral responsiveness.* The presence of antibodies that recognize WHsAg are determined by an ELISA. This assay is such that even WHs Abs complexed with antigen are detected.

[0154] *Toxicology.* Careful toxicology is carried out via hematology and serum chemistry as described for the mouse studies. In addition, histological examination of the punch biopsies of the livers is undertaken, including inflammation, bile duct proliferation, and portal and lobular hepatitis.

[0155] *WHV virus levels in the serum.* An assessment is performed on weekly (as slot blot hybridization and PCR or bi-monthly (southern blot).

[0156] *Biopsies.* Liver biopsies are collected before the start, middle, end of treatment, and end of study and used for histology and intracellular WHV DNA examination. Levels of replicative form and intrahepatic covalently-closed circular WHV DNA (WHV cccDNA) are quantitatively determined based on Hirt extraction. For immunostaining, separate tissue is used and accumulation of core and WHsAg in treated versus untreated animals will be determined.

[0157] For both the Duck and woodchuck studies, no technical difficulties are expected, since these studies are fairly routine, with all methods and reagents needed for evaluation being in hand. One possible problem with Ducks is the variations in viremia/ antigenemia that occur without drug. This is mitigated by using Ducks after 6 weeks of age, in which virology as usually stabilized.

[0158] The benchmark of positive activity is LFMAU treated animals. These animals are expected to have uniformly lost HBV viremia and even antigenemia, by 3 and 10 weeks of treatment, in the Duck and Woodchuck, respectively, with numbers of HBV infected hepatocytes greatly reduced, relative to pretreatment and untreated groups.

[0159] Inhibition of cccDNA transcription (and stability) should reduce the intracellular and extracellular amounts of all viral gene products (at a rate influenced by their serum half lives), even before there are reductions in the numbers of HBV infected cells (and possibly, out

of proportion to the number of HBV infected cell loss). Realistically, the clearest evidence of efficacy of our new compounds is time and dose dependent statically significant reductions in HBV DNA viremia and sAg antigenemia. Given the efficacy of the present compounds, in vitro, an at least a ten-fold reduction of serum surface antigen in either or both models is expected.

[0160] DHBsAgWHsAb levels are also measured. Control, chronically infected animals are expected to have no detectable (or very little detectable) Ag. There is a growing body of evidence that chronically infected people (and woodchucks) are capable, and do make, sAb, but it is suppressed or bound with circulating sAg. It is therefore possible that if and as Ag declines, sAb will declare itself.

[0161] Biopsy analysis is performed on immunostained for HBV core, sAg , using mounted liver tissue, and with extracts to examine the amounts of HBV nucleic acid, before and after treatment. Ideally, the numbers of infected cells will decline as a function of drug treatment. Useful information includes whether this occurs in a setting of increased hepatitis (cell infiltration).

[0162] Serum from animals for 10 & 4 weeks (Woodchuck and Duck, respectively) is also evaluated after drug treatment has been stopped. Stable, off drug, repression of antigenemia, viremia, with appearance of sAbs is considered the obtaining all major objectives. On drug suppression of viremia and antigenemia by amounts exceeding placebo, in the absence of any adverse reactions or events, is considered proof of a drug specific affect.

[0163] The animal studies outlined above permit definitive conclusions as to whether the compounds are effective at reducing antigenemia in an *in vivo* context.

[0164] Where inhibition of an HDAC is determined to repress HBV cccDNA transcription, the results are as surprising as they are useful, since HDAC inhibition has generally been associated with gene activation, including HBV DNA integrated into host chromosomes. The results may represent an example of how different is the regulation of HBV cccDNA from most cellular genes and, even if the inhibitors identified herein are not ultimately used in human systems, it is demonstrated that it is possible to non-catalytically inhibit cccDNA with small, pharmacologically, active compounds.

[0165] Taken together, this work delivers two very critical answers. First, it indicates the selective suppression of HBV cccDNA function in human and woodchuck cultures. Second, it determines which HDAC (the target of Apicidin) regulate HBV cccDNA. We understand that HDAC inhibition in HBV infected people must proceed with caution, and this work represents direction regarding how to go proceed with a revolutionary new therapeutic strategy.

General Synthesis

[0166] The compounds of this invention can be prepared from readily available starting materials using the following general methods and procedures. It will be appreciated that where typical or suitable process conditions (i.e., reaction temperatures, times, mole ratios of reactants, solvents, pressures, etc.) are given, other process conditions can also be used unless otherwise stated. Optimum reaction conditions may vary with the particular reactants or solvent used, but such conditions can be determined by one skilled in the art by routine optimization procedures.

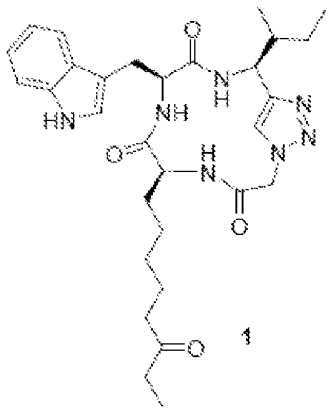
[0167] The processes described herein can be monitored according to any suitable method known in the art. For example, product formation can be monitored by spectroscopic means, such as nuclear magnetic resonance spectroscopy (e.g., ^{1}H or ^{13}C NMR), infrared spectroscopy (IR), spectrophotometry (*e.g.*, UV-visible), or mass spectrometry, or by chromatography such as high performance liquid chromatography (HPLC) or thin layer chromatography.

[0168] Preparation of compounds can involve the protection and deprotection of various chemical groups. The need for protection and deprotection, and the selection of appropriate protecting groups can be readily determined by one skilled in the art. The chemistry of protecting groups can be found, for example, in P. G. M. Wuts and T. Greene, *Greene's Protective Groups in Organic Synthesis*, 4th. Ed., Wiley & Sons, 2006, which is incorporated herein by reference in its entirety.

[0169] The reactions of the processes described herein can be carried out in suitable solvents which can be readily selected by one of skill in the art of organic synthesis. Suitable solvents can be substantially nonreactive with the starting materials (reactants), the intermediates, or products at the temperatures at which the reactions are carried out, *i.e.,* temperatures which can range from the solvent's freezing temperature to the solvent's boiling temperature. A given reaction can be carried out in one solvent or a mixture of more than one solvent. Depending on the particular reaction step, suitable solvents for a particular reaction step can be selected. The compounds of the invention can be prepared, for example, using the reaction pathways and techniques as described below.

Compound Synthesis

[0170] Apicidins have been derivitized and recent analogs **1** and **2**



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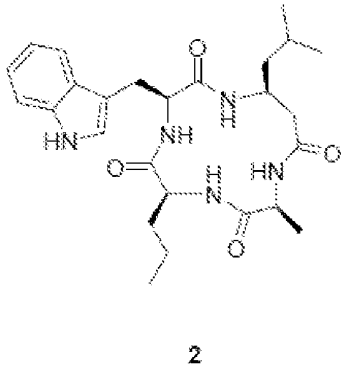

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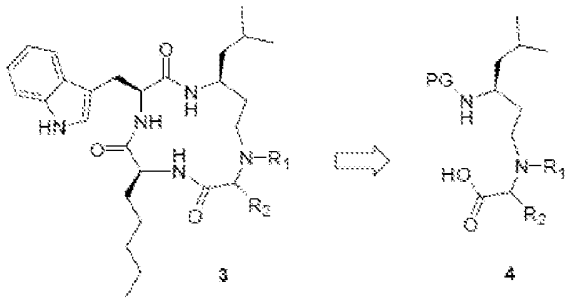
(*see Horne, W. S., C. A. Olsen, J. M. Beierle, A. Montero, and M. R. Ghadiri. 2009. Probing the bioactive conformation of an archetypal natural product HDAC inhibitor with conformationally homogeneous triazole-modified cyclic tetrapeptides. Angew Chem Int Ed Engl 48:4718-4724; Vickers, C. J., C. A. Olsen, L. J. Leman, and M. R. Ghadiri. 2012. Discovery of HDAC Inhibitors That Lack an Active Site Zn2+-Binding Functional Group. ACS Medicinal Chemistry Letters*) demonstrate that the Apicidin structure can be modified without loss of anti HDAC potency.

[0171] Further analogs were prepared with a focus on improving pharmaceutical properties relative to Apicidin, which has very poor aqueous solubility, oral bioavailability, and half life in vivo. Apicidin derivatives were prepared, *inter alia*, by standard solid and solution phase methods. In certain embodiments, the reduced beta-isoleucine amino acid derived fragments **4**



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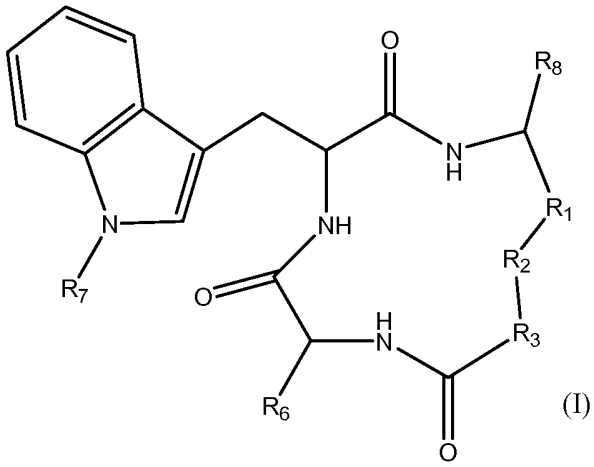
were prepared in a suitably protected form (PG = suitable protecting group, such as Fmoc or Boc) by solution phase methods and introduced into the amino acid sequence by solution or solid phase means, followed by cyclization using established methods.

What is Claimed:

1. A method of modulating cccDNA transcription of hepatitis B in a subject comprising administering to said subject an inhibitor of histone deacetylace activity.

2. The method according to claim 1 wherein said inhibitor of histone deacetylace activity is an inhibitor of class I histone deacetylace activity.

3. The method according to claim 1 wherein said inhibitor of histone deacetylace activity is Trichostatin A, suberoyl bis hydroxamic acid, dimethylamino hydroxy-benzamide, Apicidin or an analog thereof, or a compound according to formula (I)



(I)

wherein

R_1 is $-(CH_2)_n-$ or -C(=O)-;

R_2 is -C(=O)-, 3,5-triazolyl, or $-C(Z)N(R_4)-$;

R_4 is hydrogen, alkyl, aryl, aralkyl, dialkylaminoalkyl, or carboxyalkyl;

R_3 is $-CH(R_5)-$, or R_2 is nitrogen and R_3 is -CH- and R_2 and R_3 together form piperidinyl;

R_5 is hydrogen, $-CH_3$, or an alpha amino acid R group;

R_6 is $-(CH_2)_mC(X)Y$, $-(CH_2)_2CH_3$, or $-(CH_2)_q$-phenyl-$(CH_2)_mC(=O)NHOH$;

X is =O, H_2, $=N-NH_2$, or $=N-NH-C(=O)NH_2$;

Y is NHOH or $-CH_2CH_3$;

Z is H_2 or O;

R_7 is hydrogen or alkoxy;

R_8 is alkyl or carboxyalkyl;

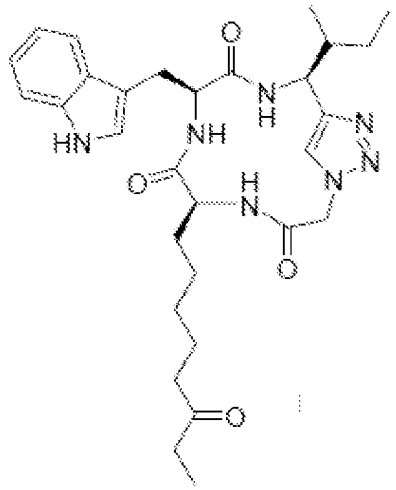
n is 0-2;

m is 0-6; and,

q is 0-3;

or a stereoisomer or pharmaceutically acceptable salt thereof.

4. The method according to claim 1 wherein said inhibitor of histone deacetylace activity is Apicidin,



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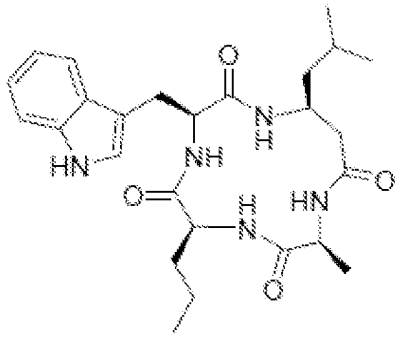

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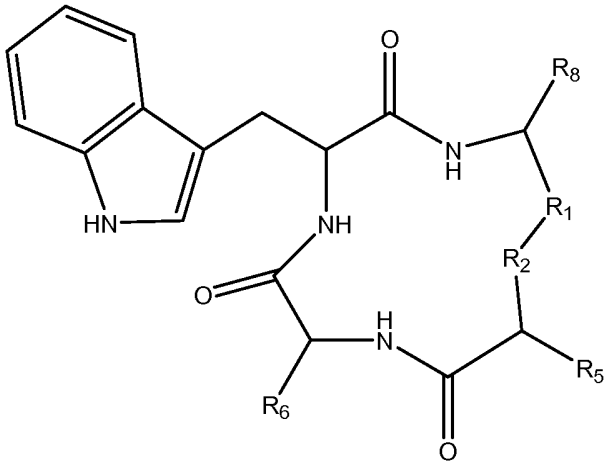


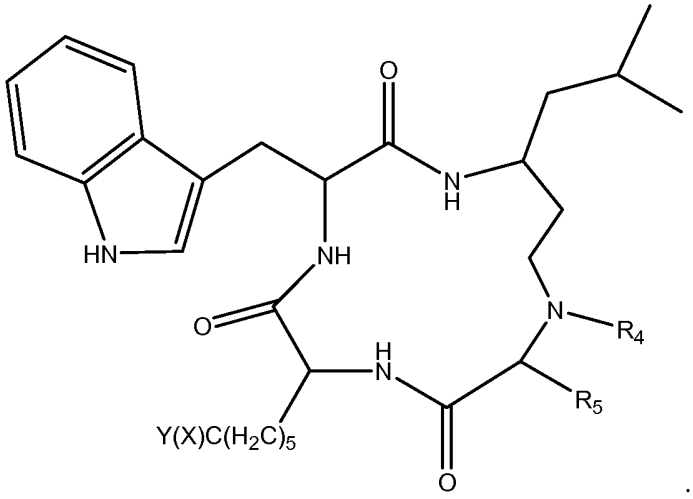
wherein

R_1 is -(CH_2)-,

and,

R_2 is -C(Z)N(R_4)-.

5. The method according to claim 3 wherein said inhibitor of histone deacetylace activity is

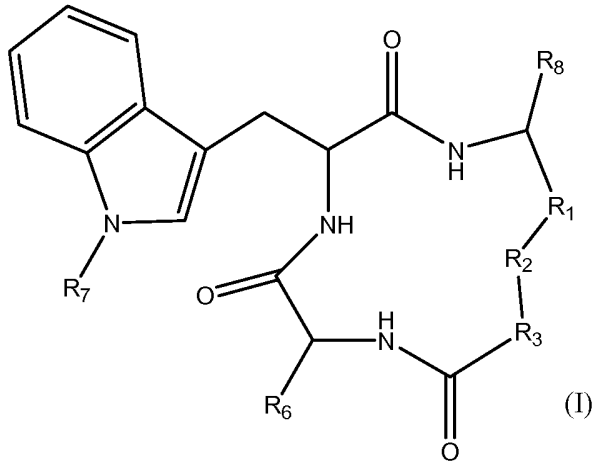


6. The method according to claim 1 further comprising administering to said subject a therapeutically effective amount of a further agent that modulates hepatitis B virus.

7. A method of treating hepatitis B in a subject comprising administering to said subject an inhibitor of histone deacetylace activity.

8. The method according to claim 7 wherein said inhibitor of histone deacetylace activity is an inhibitor of class I histone deacetylace activity.

9. The method according to claim 7 wherein said inhibitor of histone deacetylace activity is Trichostatin A, suberoyl bis hydroxamic acid, dimethylamino hydroxy-benzamide, Apicidin or an analog thereof, or a compound according to formula (I)



(I)

wherein

R_1 is $–(CH_2)_n$- or -C(=O)-;

R_2 is -C(=O)-, 3,5-triazolyl, or $–C(Z)N(R_4)$-;

R_4 is hydrogen, alkyl, aryl, aralkyl, dialkylaminoalkyl, or carboxyalkyl;

R_3 is $–CH(R_5)$-, or R_2 is nitrogen and R_3 is -CH- and R_2 and R_3 together form piperidinyl;

R_5 is hydrogen, $-CH_3$, or an alpha amino acid R group;

R_6 is $-(CH_2)_mC(X)Y$, $-(CH_2)_2CH_3$, or $-(CH_2)_q$-phenyl-$(CH_2)_mC(=O)NHOH$;

X is =O, H_2, $=N-NH_2$, or $=N-NH-C(=O)NH_2$;

Y is NHOH or $-CH_2CH_3$;

Z is H_2 or O;

R_7 is hydrogen or alkoxy;

R_8 is alkyl or carboxyalkyl;

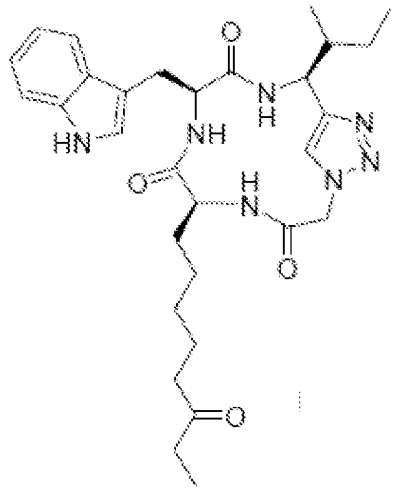
n is 0-2;

m is 0-6; and,

q is 0-3;

or a stereoisomer or pharmaceutically acceptable salt thereof.

10. The method according to claim 7 wherein said inhibitor of histone deacetylace activity is Apicidin,



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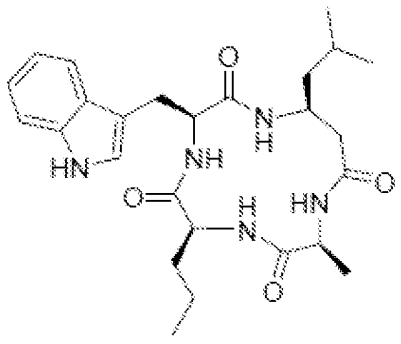

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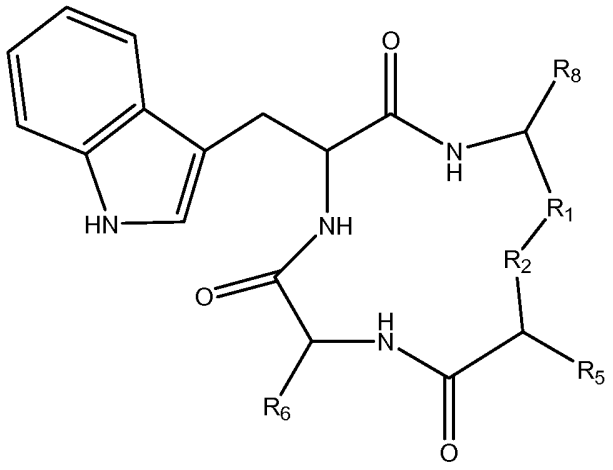


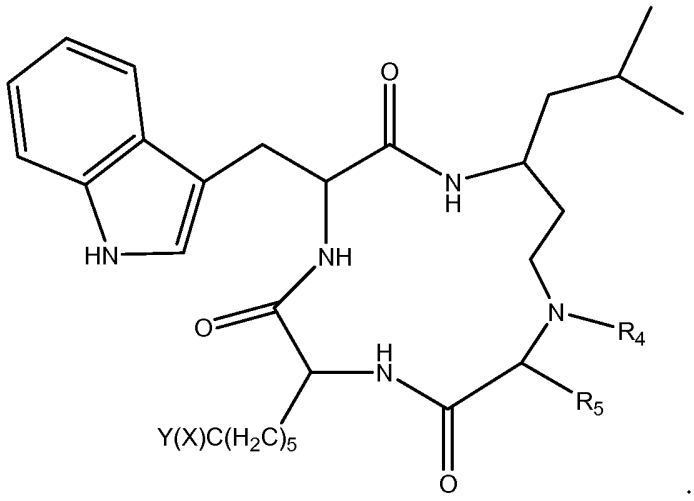
wherein

R_1 is $-(CH_2)-$,

and,

R_2 is $-C(Z)N(R_4)-$.

11. The method according to claim 10 wherein said inhibitor of histone deacetylace activity is



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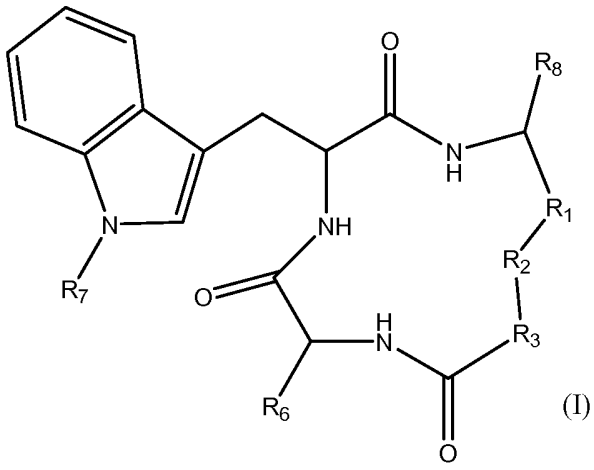
12. The method according to claim 7 further comprising administering to said subject a therapeutically effective amount of a further agent that modulates hepatitis B virus.

13. A method of modulating hepatitis B virus covalently closed circular DNA comprising contacting a hepatitis B virus with an inhibitor of histone deacetylace activity.

14. The method according to claim 13 wherein said inhibitor of histone deacetylace activity is an inhibitor of class I histone deacetylace activity.

15. The method according to claim 13 wherein said inhibitor of histone deacetylace activity is Trichostatin A, suberoyl bis hydroxamic acid, dimethylamino hydroxy-benzamide, Apicidin or an analog thereof, or a compound according to formula (I)



(I)

wherein

R_1 is $-(CH_2)_n$- or -C(=O)-;

R_2 is -C(=O)-, 3,5-triazolyl, or $-C(Z)N(R_4)$-;

R_4 is hydrogen, alkyl, aryl, aralkyl, dialkylaminoalkyl, or carboxyalkyl;

R_3 is $-CH(R_5)$-, or R_2 is nitrogen and R_3 is -CH- and R_2 and R_3 together form piperidinyl;

R_5 is hydrogen, $-CH_3$, or an alpha amino acid R group;

R_6 is $-(CH_2)_mC(X)Y$, $-(CH_2)_2CH_3$, or $-(CH_2)_q$-phenyl-$(CH_2)_mC(=O)NHOH$;

X is =O, H_2, =N-NH_2, or =N-NH-C(=O)NH_2;

Y is NHOH or $-CH_2CH_3$;

Z is H_2 or O;

R_7 is hydrogen or alkoxy;

R_8 is alkyl or carboxyalkyl;

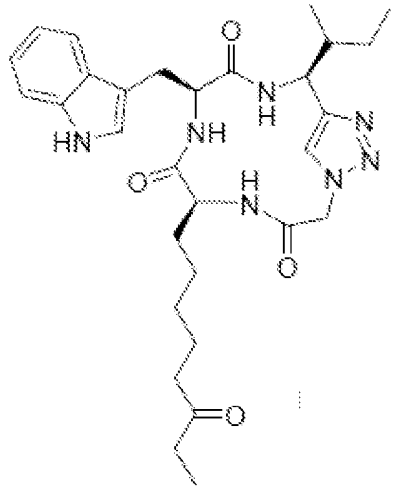
n is 0-2;

m is 0-6; and,

q is 0-3;

or a stereoisomer or pharmaceutically acceptable salt thereof.

16. The method according to claim 13 wherein said inhibitor of histone deacetylace activity is Apicidin,



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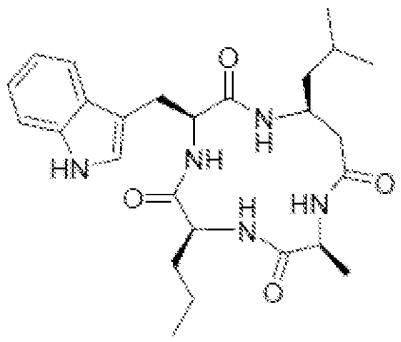
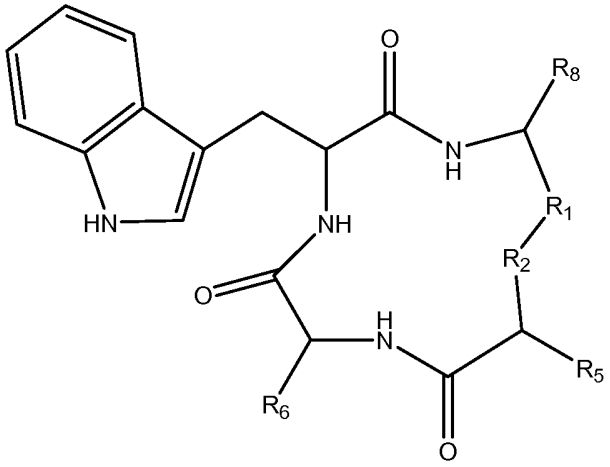


, or



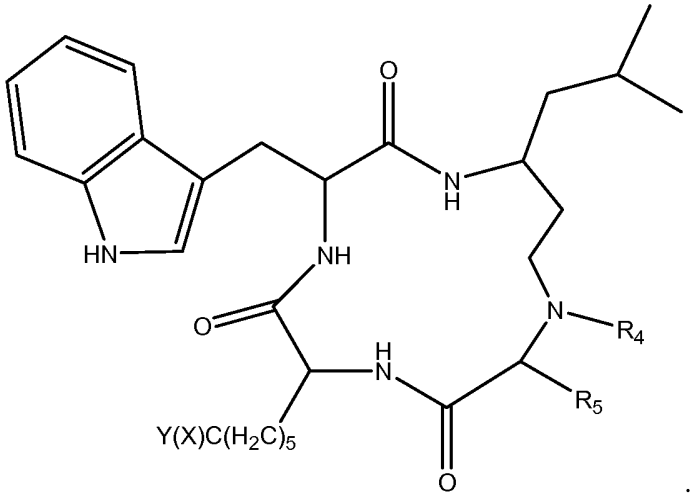
wherein

R_1 is -(CH_2)-,

and,

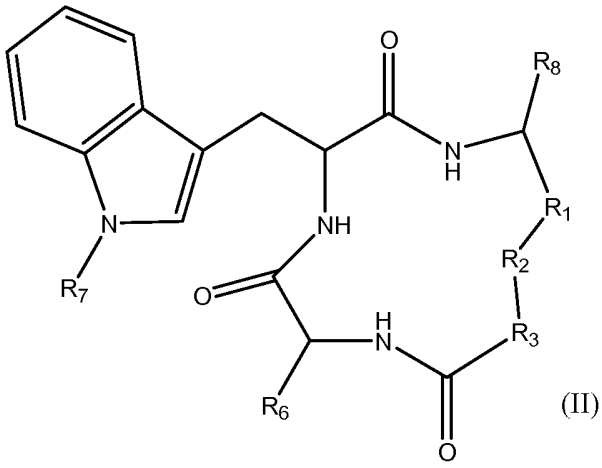
R_2 is -C(Z)N(R_4)-.

17. The method according to claim 13 wherein said inhibitor of histone deacetylace activity is



18. The method according to claim 13 further comprising contacting the hepatitis B virus with a further agent that modulates hepatitis B virus.

19. A compound according to formula II:



(II)

wherein

R_1 is $-(CH_2)_n-$ or -C(=O)-;

R_2 is -C(=O)- or $-C(Z)N(R_4)-$;

R_4 is hydrogen, alkyl, aryl, aralkyl, dialkylaminoalkyl, or carboxyalkyl;

R_3 is $-CH(R_5)-$;

R_5 is hydrogen, $-CH_3$, or an alpha amino acid R group;

R_6 is $-(CH_2)_mC(X)Y$, $-(CH_2)_2CH_3$, or $-(CH_2)_q$-phenyl-$(CH_2)_mC(=O)NHOH$;

X is =O, H_2, $=N-NH_2$, or $=N-NH-C(=O)NH_2$;

Y is NHOH or $-CH_2CH_3$;

Z is H_2 or O;

R_7 is hydrogen or alkoxy;

R_8 is alkyl or carboxyalkyl;

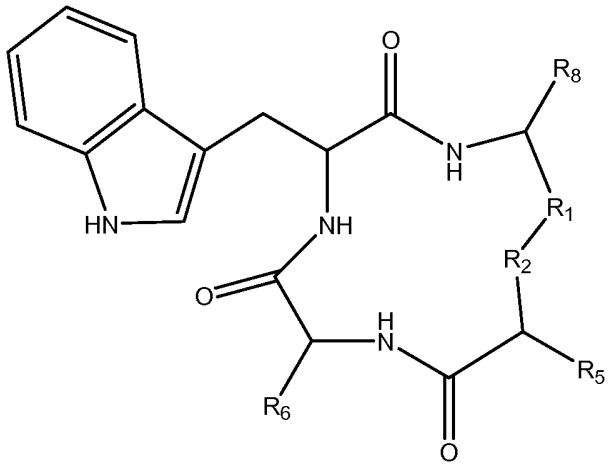
n is 0-2;

m is 0-6; and,

q is 0-3;

or a stereoisomer or pharmaceutically acceptable salt thereof,

20. The compound according to claim 19 wherein said compound is



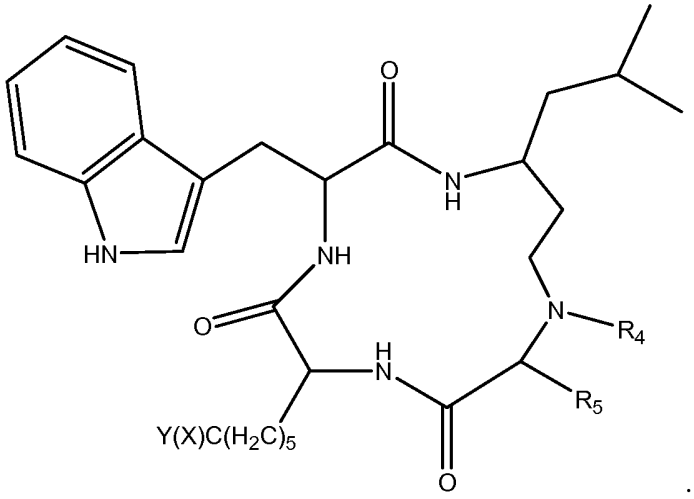
wherein

R_1 is -(CH_2)-,

and,

R_2 is -C(Z)N(R_4)-.

21. The compound according to claim 19 wherein said compound is


O
N
H
HN
NH
O
N
R4
H
N
R5
Y(X)C(H2C)5
O

.

FIG. 1

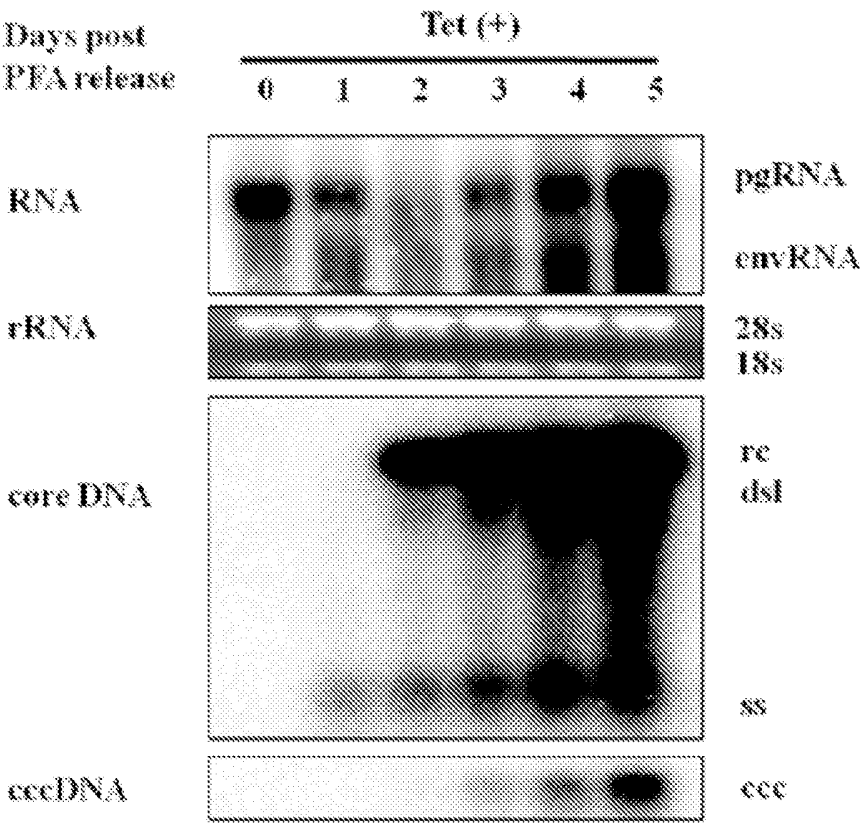

Days post PFA release
Tet (+)
0
1
2
3
4
5
RNA
pgRNA
envRNA
rRNA
28s
18s
core DNA
rc
dsl
ss
cccDNA
ccc

FIG. 2

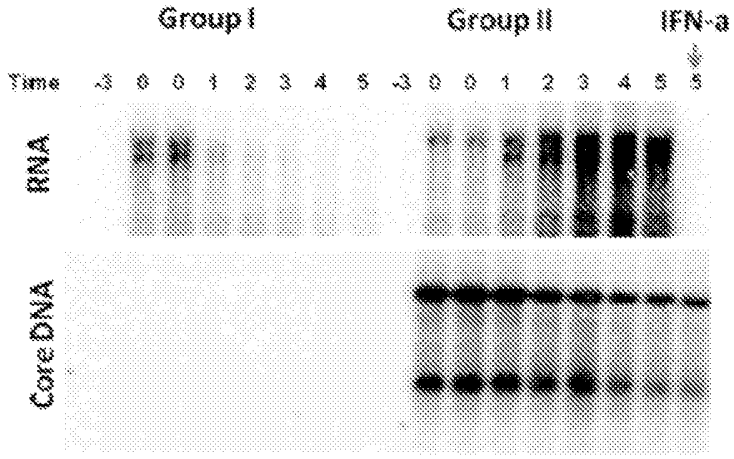

Group I
Group II
IFN-a
Time
-3
0
0
1
2
3
4
5
-3
0
0
1
2
3
4
5
5
RNA
Core DNA

FIG. 3

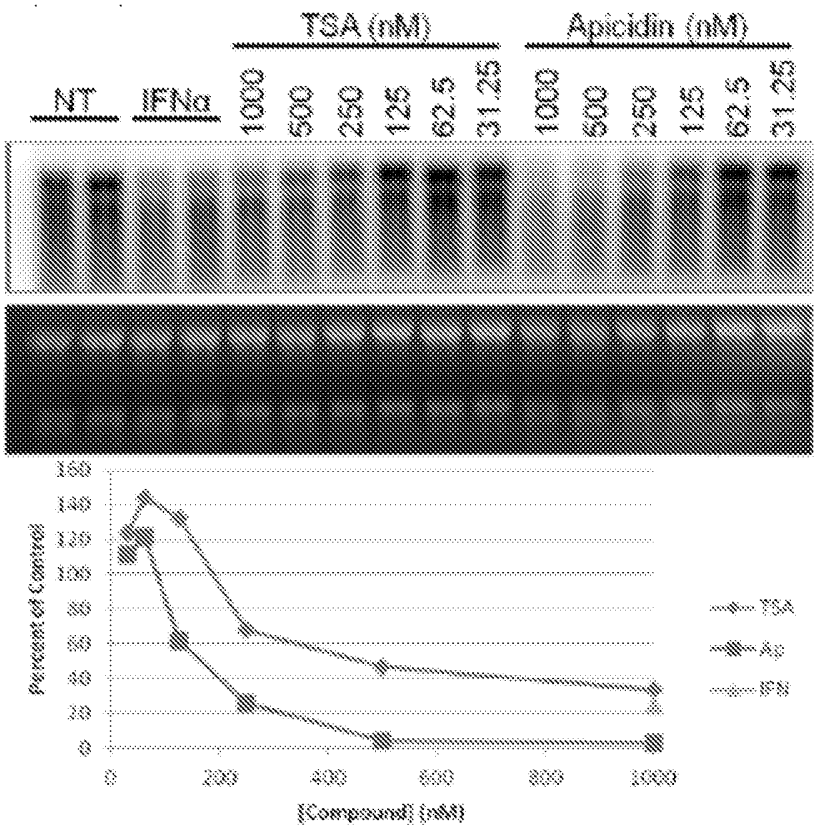
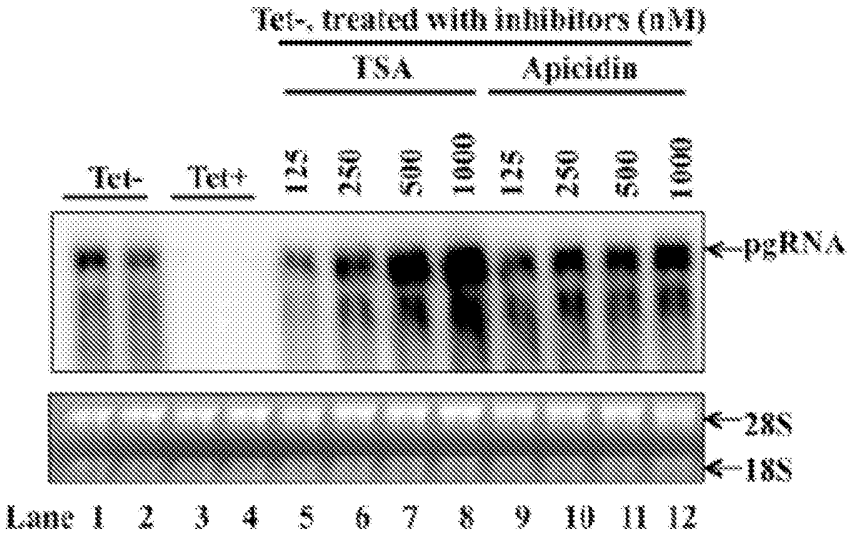

TSA (nM)
Apicidin (nM)
NT
IFNα
1000
500
250
125
62.5
31.25
Percent of Control
[Compound] (nM)
TSA
Ap
IFN

FIG. 4


Tet-, treated with inhibitors (nM)
TSA
Apicidin
Tet-
Tet+
125
250
500
1000
pgRNA
28S
18S
Lane 1 2 3 4 5 6 7 8 9 10 11 12

(19)中华人民共和国国家知识产权局

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代理人 刘慧 杨青
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C07K 5/00(2006.01)
A01N 47/34(2006.01)
C07D 245/00(2006.01)

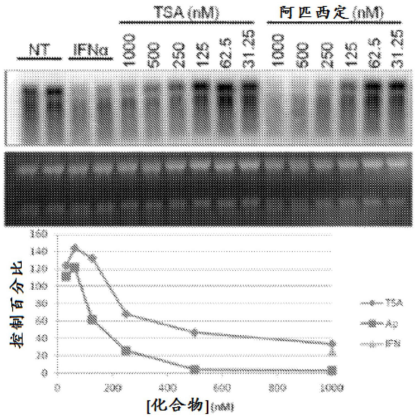
权利要求书8页 说明书30页 附图2页

(54)发明名称

乙型肝炎病毒cccDNA转录的调控

(57)摘要

本公开涉及一类新型乙型肝炎病毒(HBV)共价闭环(ccc)DNA“首创”小分子抑制剂的发现，所述抑制剂可用作慢性HBV管理的治疗剂。提供了可提供cccDNA表观遗传修饰的药剂、组蛋白调节剂和继而调控HBV cccDNA的组蛋白去乙酰化活性抑制剂，所述调控HBVcccDNA从未用药理学药剂实现过。还提供了用于调控HBV cccDNA、治疗或预防受试者HBV以及调控受试者乙型肝炎的cccDNA转录的方法。

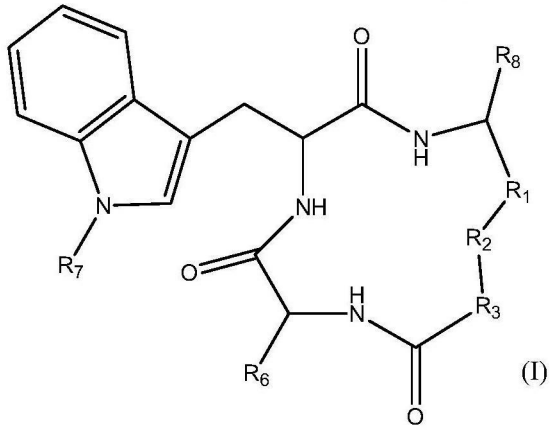


CN 104507488 A

1.一种调控受试者乙型肝炎的 cccDNA 转录的方法,其包括向所述受试者施用组蛋白去乙酰化活性抑制剂。

2.根据权利要求 1 所述的方法,其中所述组蛋白去乙酰化活性抑制剂是 I 类组蛋白去乙酰化活性抑制剂。

3.根据权利要求 1 所述的方法,其中所述组蛋白去乙酰化活性抑制剂是曲古抑菌素 A、辛二酰基双异羟肟酸、二甲氨基羟基 - 苯甲酰胺、阿匹西定或其类似物,或根据式 (I) 的化合物



(I)

其中

R_1是 $-(CH_2)_n-$ 或 $-C(=O)-$;

R_2是 $-C(=O)-$、3,5- 三唑基或 $-C(Z)N(R_4)-$;

R_4是氢、烷基、芳基、芳烷基、二烷基氨基烷基或羧基烷基;

R_3是 $-CH(R_5)-$,或 R_2是氮且 R_3是 -CH- 并且 R_2和 R_3一起形成哌啶基;

R_5是氢、$-CH_3$或 α 氨基酸 R 基团;

R_6是 $-(CH_2)_mC(X)Y$、$-(CH_2)_2CH_3$或 $-(CH_2)_q-$ 苯基 $-(CH_2)_mC(=O)NHOH$;

X 是 =O、H_2、$=N-NH_2$或 $=N-NH-C(=O)NH_2$;

Y 是 NHOH 或 $-CH_2CH_3$;

Z 是 H_2或 O;

R_7是氢或烷氧基;

R_8是烷基或羧基烷基;

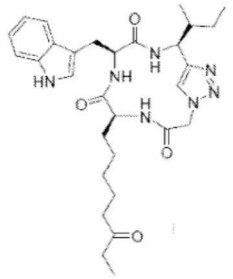
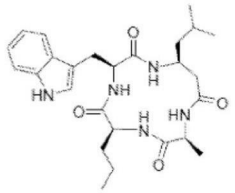
n 是 0-2;

m 是 0-6;并且,

q 是 0-3;

或其立体异构体或药学上可接受的盐。

4.根据权利要求 1 所述的方法,其中所述组蛋白去乙酰化活性抑制剂是阿匹西定,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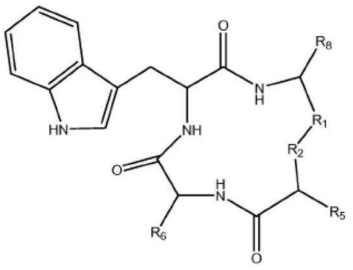

，

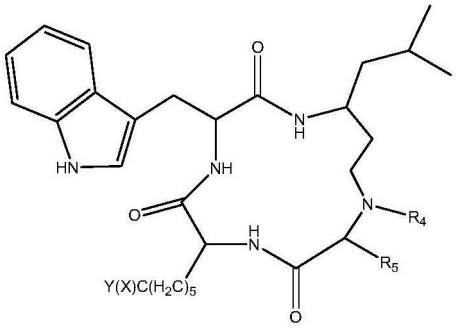
其中

R_1是 -(CH_2)-，

并且，

R_2是 -C(Z)N(R_4)-。

5. 根据权利要求 3 所述的方法，其中所述组蛋白去乙酰化活性抑制剂是



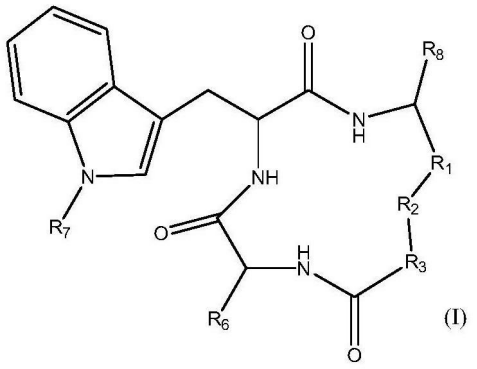
。

6. 根据权利要求 1 所述的方法，其进一步包括向所述受试者施用治疗有效量的调控乙型肝炎病毒的另一种药剂。

7. 一种治疗受试者乙型肝炎的方法，其包括向所述受试者施用组蛋白去乙酰化活性抑制剂。

8. 根据权利要求 7 所述的方法，其中所述组蛋白去乙酰化活性抑制剂是 I 类组蛋白去乙酰化活性抑制剂。

9. 根据权利要求 7 所述的方法，其中所述组蛋白去乙酰化活性抑制剂是曲古抑菌素 A、辛二酰基双异羟肟酸、二甲氨基羟基 - 苯甲酰胺、阿匹西定或其类似物，或根据式 (I) 的化合物



(I)

其中

R_1是 -$(CH_2)_n$- 或 -C(= O)- ;

R_2是 -C(= O)-、3,5- 三唑基或 -C(Z)N(R_4)- ;

R_4是氢、烷基、芳基、芳烷基、二烷基氨基烷基或羧基烷基 ;

R_3是 -CH(R_5)-,或 R_2是氮且 R_3是 -CH- 并且 R_2和 R_3一起形成哌啶基 ;

R_5是氢、-CH_3或 α 氨基酸 R 基团 ;

R_6是 -$(CH_2)_m$C(X)Y、-$(CH_2)_2CH_3$或 -$(CH_2)_q$- 苯基 -$(CH_2)_m$C(= O)NHOH ;

X 是= O、H_2、= N-NH_2或= N-NH-C(= O)NH_2;

Y 是 NHOH 或 -CH_2CH_3;

Z 是 H_2或 O ;

R_7是氢或烷氧基 ;

R_8是烷基或羧基烷基 ;

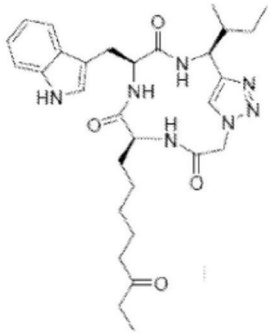
n 是 0-2 ;

m 是 0-6 ;并且,

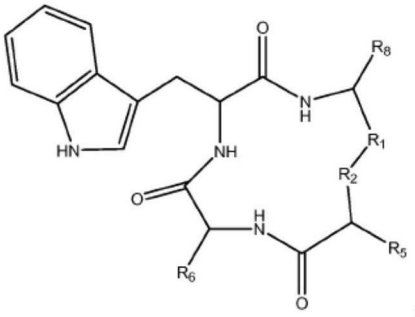
q 是 0-3 ;

或其立体异构体或药学上可接受的盐。

10. 根据权利要求 7 所述的方法,其中所述组蛋白去乙酰化活性抑制剂是阿匹西定,



，或



，

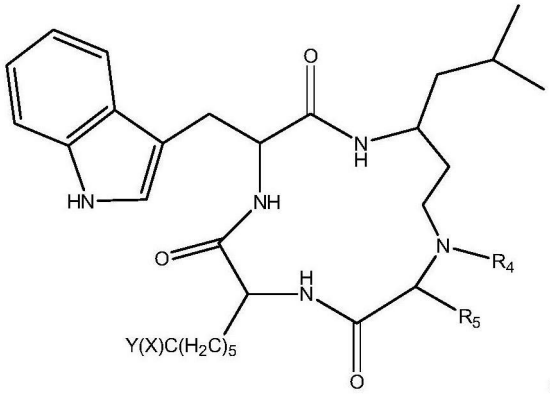
其中

R_1是 -(CH $_2$)-，

并且，

R_2是 -C(Z)N(R $_4$)-。

11. 根据权利要求 10 所述的方法，其中所述组蛋白去乙酰化活性抑制剂是



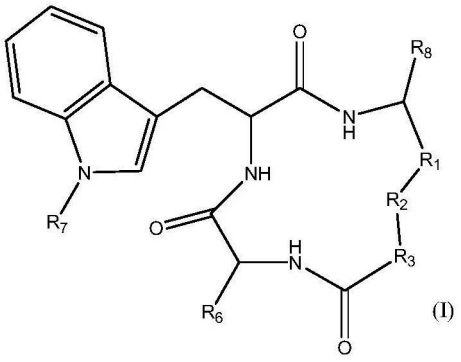
。

12. 根据权利要求 7 所述的方法，其进一步包括向所述受试者施用治疗有效量的调控乙型肝炎病毒的另一种药剂。

13. 一种调控乙型肝炎病毒共价闭环 DNA 的方法，其包括使乙型肝炎病毒与组蛋白去乙酰化活性抑制剂接触。

14. 根据权利要求 13 所述的方法，其中所述组蛋白去乙酰化活性抑制剂是 I 类组蛋白去乙酰化活性抑制剂。

15. 根据权利要求 13 所述的方法，其中所述组蛋白去乙酰化活性抑制剂是曲古抑菌素 A、辛二酰基双异羟肟酸、二甲氨基羟基 - 苯甲酰胺、阿匹西定或其类似物，或根据式 (I) 的化合物



(I)

其中

R_1是 -$(CH_2)_n$- 或 -C(= O)- ;

R_2是 -C(= O)-、3,5- 三唑基或 -C(Z)N(R_4)- ;

R_4是氢、烷基、芳基、芳烷基、二烷基氨基烷基或羧基烷基 ;

R_3是 -CH(R_5)-,或 R_2是氮且 R_3是 -CH- 并且 R_2和 R_3一起形成哌啶基 ;

R_5是氢、-CH_3或 α 氨基酸 R 基团 ;

R_6是 -$(CH_2)_m$C(X)Y、-$(CH_2)_2CH_3$或 -$(CH_2)_q$- 苯基 -$(CH_2)_m$C(= O)NHOH ;

X 是= O、H_2、= N-NH_2或= N-NH-C(= O)NH_2;

Y 是 NHOH 或 -CH_2CH_3;

Z 是 H_2或 O ;

R_7是氢或烷氧基 ;

R_8是烷基或羧基烷基 ;

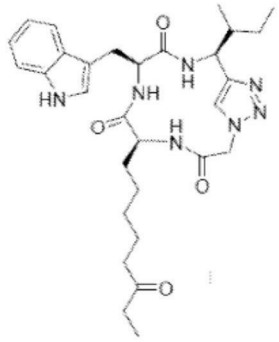
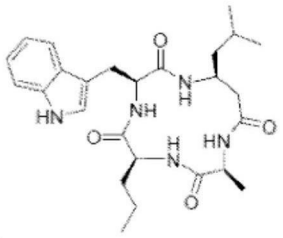
n 是 0-2 ;

m 是 0-6 ;并且,

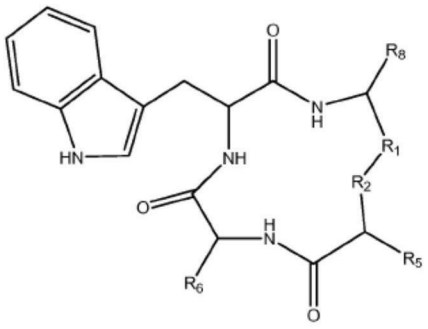
q 是 0-3 ;

或其立体异构体或药学上可接受的盐。

16. 根据权利要求 13 所述的方法,其中所述组蛋白去乙酰化活性抑制剂是阿匹西定,

或



，

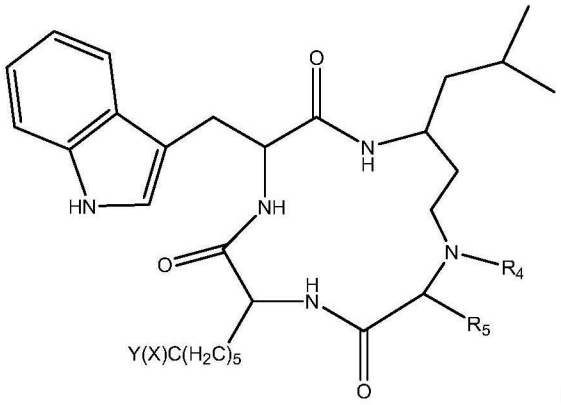
其中

R_1是 -(CH_2)-，

并且，

R_2是 -C(Z)N(R_4)-。

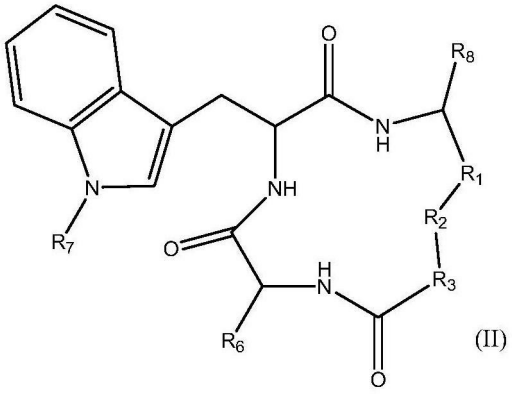
17. 根据权利要求 13 所述的方法，其中所述组蛋白去乙酰化活性抑制剂是



。

18. 根据权利要求 13 所述的方法，其进一步包括使所述乙型肝炎病毒与调控乙型肝炎病毒的另一种药剂接触。

19. 一种根据式 II 所述的化合物：



(II)

其中

R_1是 -$(CH_2)_n$- 或 -C(= O)- ;

R_2是 -C(= O)- 或 -C(Z)N(R_4)- ;

R_4是氢、烷基、芳基、芳烷基、二烷基氨基烷基或羧基烷基 ;

R_3是 -CH(R_5)- ;

R_5是氢、$-CH_3$或 α 氨基酸 R 基团 ;

R_6是 -$(CH_2)_m$C(X)Y、-$(CH_2)_2CH_3$或 -$(CH_2)_q$- 苯基 -$(CH_2)_m$C(= O)NHOH ;

X 是= O、H_2、= N-NH_2或= N-NH-C(= O)NH_2;

Y 是 NHOH 或 $-CH_2CH_3$;

Z 是 H_2或 O ;

R_7是氢或烷氧基 ;

R_8是烷基或羧基烷基 ;

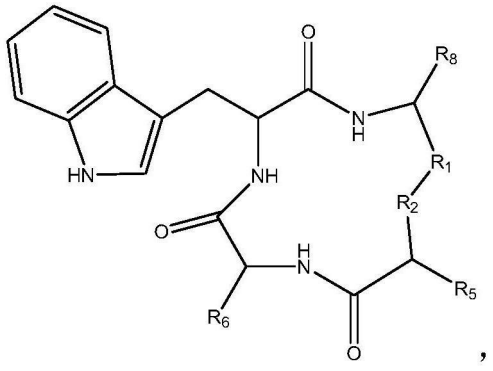
n 是 0-2 ;

m 是 0-6 ;并且,

q 是 0-3 ;

或其立体异构体或药学上可接受的盐。

20. 根据权利要求 19 所述的化合物,其中所述化合物是



,

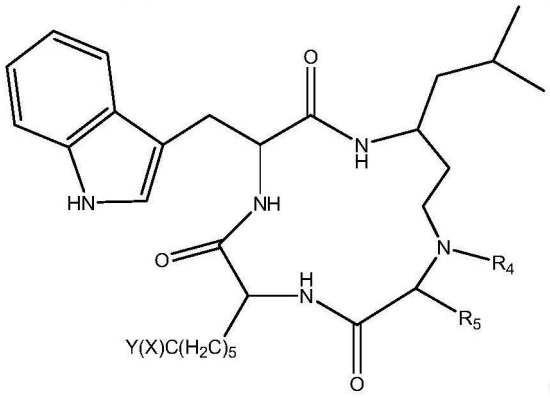
其中

R_1是 -(CH_2)-,

并且,

R_2是 -C(Z)N(R_4)-。

21. 根据权利要求 19 所述的化合物,其中所述化合物是


O
N
H
HN
NH
O
N
R4
H
N
R5
Y(X)C(H2C)5
O

。

乙型肝炎病毒 cccDNA 转录的调控

[0001] 相关申请的交叉引用

[0002] 本申请要求 2012 年 6 月 1 日提交的美国临时申请号 61/654,374 的优先权，所述申请的全部内容以引用方式并入本文。

技术领域

[0003] 本公开涉及优选具有组蛋白去乙酰化活性的药理学药剂调控乙型肝炎病毒的共价闭环 DNA 以及预防或治疗乙型肝炎的用途。

背景技术

[0004] 美国食品和药物管理局 (FDA) 现在批准七种药物用于慢性乙型肝炎的管理，这些药物属于两种类型中的一种：干扰素 (IFN) 和聚合酶抑制剂 (Lok，A.S. 和 B.J.McMahon.2007.Chronic Hepatitis B.Hepatology45:507-539)。这些药物被推荐在 3.5 亿以上感染人群中的大约 50％或更少人群中使用。虽然这是最高的风险人群，但是那些不属于治疗指南范围的人群可能也从干预中受益，因为他们也处于显著升高的肝病风险中。IFN 受限于显著的副作用。pol 抑制剂靶向相同的病毒生命周期阶段，并且因此组合治疗 (HIV 和治愈性 HCV 疗法的壁垒）的价值有限。他们需要终身使用，并且遭受最终的使用限制性毒性（正如 HIV 长期药物使用所见的），并且出现抗药性突变体。因此，需要对当前的药物组合 (portfolio) 做替换和补充。

[0005] 人们越来越相信“治愈”或至少维持停药 HBV 控制将需要或至少受益于控制病毒核基因组（共价闭环 DNA(cccDNA)）的药物。2006NIDDK 肝脏行动计划（由 2010 医学研究所报告加强）全体呼吁将 cccDNA 抑制作为 HBV 药物开发的重点。

[0006] 然而，HBV cccDNA 抑制剂的筛选是困难的，这是由于以下技术原因：HBV cccDNA 产生的量太低以致无法方便地检测，以及培养的常规转染的细胞中的大部分病毒基因产物来源于病毒基因组的转基因，而不是 cccDNA。本发明人建立了其中诸如 HbeAg 的 HBV 基因产物只从 cccDNA 产生而不从整合的病毒转基因产生并且产生的量可较好检测的细胞系，从而使筛选变得实际 (Cai，D. 等，2012.Identification of the Disubstituted Sulfonamide Compounds as Specific Inhibitors of Hepatitis B Virus Covalently Closed Circular DNA Formation.Antimicrobial Agents and Chemotherapy:In Press；Zhou，T. 等，2006.Hepatitis B virus e antigen production is dependent upon covalently closed circular(ccc)DNA in HepAD38cell cultures and may serve as a cccDNA surrogate in antiviral screening assays.Antiviral Research 72:116-124)。

[0007] 考虑到这些挑战，没有可使用的靶向 HBV cccDNA 的 HBV 治疗剂及有很少（如果有）程序以筛选和开发 cccDNA 抑制剂就不会让人吃惊了。这主要是由于技术困难（参见 Block，T.M. 等，2003.Molecular viral oncology of hepatocellular carcinoma. Oncogene 22:5093-5107；Locarnini，S.2005.Therapies for hepatitis B:where to from here？ Gastroenterology 128:789-792；Lok，A.S.2011.Does antiviral therapy for

hepatitis B and C prevent hepatocellular carcinoma？ J Gastroenterol Hepatol 26:221-227)。此外，宿主功能在调控 HBV cccDNA 转录和稳定性中的作用很少被人们所认识，这进一步阻碍了治疗剂的开发。因此，该领域中的任何工作都是创新的，并且会满足对控制乙型肝炎病毒核基因组的药物的突出和长期的需求而且还为 HBV 感染提供治疗。

[0008] 发明概述

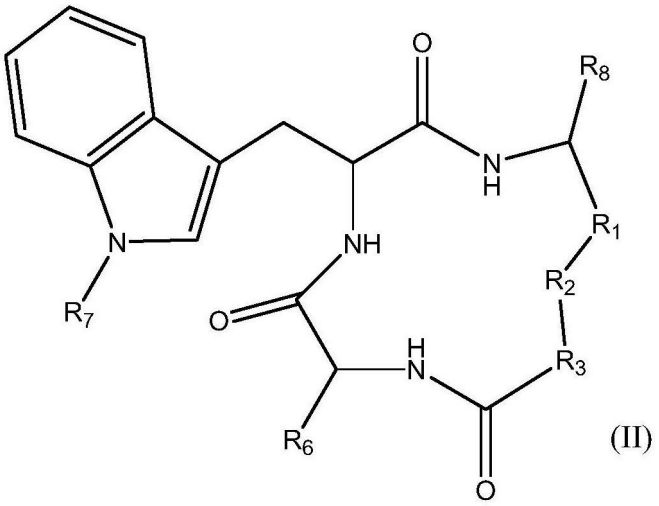
[0009] 提供调控受试者中乙型肝炎的 cccDNA 转录的方法，其包括向所述受试者施用提供 cccDNA 的表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化 (deacetylace) 活性抑制剂。例如，所述表观遗传调节剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂可以是药理学的诸如小分子。

[0010] 还提供治疗受试者乙型肝炎的方法，其包括向所述受试者施用组蛋白去乙酰化活性抑制剂。

[0011] 本公开还涉及调控乙型肝炎病毒共价闭环 DNA 的方法，其包括使乙型肝炎病毒与组蛋白去乙酰化活性抑制剂接触。

[0012] 还公开了根据式 II 的化合物：

[0013]



(II)

[0014] 其中 R_1-R_8如本文中提供的那样定义。

附图说明

[0015] 图 1 提供说明 HBV cccDNA 在 dstet5 细胞中有效形成并且有转录活性的数据。

[0016] 图 2 涉及说明 cccDNA 可以被 IFN-a 抑制的实验。

[0017] 图 3 涉及阿匹西定和 TSA 抑制 cccDNA 转录的本发现。

[0018] 图 4 涉及 HDAC 抑制剂剂量依赖地刺激从整合到宿主细胞染色体中的转基因合成 DHBV pgRNA 的发现。

[0019] 说明性实施方案详述

[0020] 通过参考与附图和实施例结合来进行的以下详细描述，可以更容易地理解本发明，所述附图和实施例构成本公开的一部分。应理解，本发明不限于本文中描述和 / 或展示的特定产物、方法、条件或参数，并且本文中使用的术语是为了仅通过举例的方式描述具体的实施方案并且不希望限制所要求保护的发明。

[0021] 在本文件中引用或描述的每件专利、专利申请和公布的公开内容在此以引用方式全部并入本文。

[0022] 如同以上和贯穿本公开所使用的那样，除非另外指出，否则以下术语和缩写应该被理解为具有以下含义。

[0023] 除非上下文另外明确指出，否则在本公开中单数形式“一个”、“一种”和“所述”包括复数引用，并且对特定数值的引用包括至少那个特定值。因此，例如，对“一种化合物”的引用是对一种或多种此类化合物以及本领域技术人员已知的其等价物等的引用。此外，在表明某个化学部分“可为”X、Y 或 Z 时，在一切情况下都不希望通过这种用法排除针对所述部分的其它选择；例如，有关 R_1“可为烷基、芳基或氨基”的叙述不一定排除 R_1的其它选择，诸如卤代、芳烷基等。

[0024] 当通过使用先行词“约”将值表达为近似值时，应理解所述特定值形成了另一个实施方案。如本文所使用，“约 X”（其中 X 是数值）优选是指引述值的 ±10%（包括端值）。例如，短语“约 8”是指 7.2 至 8.8 的值（包括端值）；再例如，短语“约 8%”是指 7.2%至 8.8%的值（包括端值）。存在时，所有范围都包括在内并且可以合并。例如，在引述“1 至 5”的范围时，引述的范围应该理解为包括“1 至 4”、“1 至 3”、“1-2”、“1-2 和 4-5”、“1-3 和 5”等的范围。此外，在积极地提供替换物的列举时，这种列举可以被解释为表示可以例如通过权利要求书中否定的限制排除任何替换物。例如，在引述“1 至 5”的范围时，引述的范围可以被理解为包括由此否定地排除的 1、2、3、4 或 5 的任一个的情况；因此，“1 至 5”的引述可以被理解为“1 和 3-5，但不是 2”，或简单的“其中不包括 2”。在另一个实例中，在提供包括“氢、烷基和芳基”或“氢、烷基或芳基”的可能的取代基的列举时，引述的列表可以被理解为包括由此否定地排除“氢、烷基和芳基”或“氢、烷基或芳基”的任一个的情况；因此，“氢、烷基和芳基”或“氢、烷基和芳基”的引述可以被理解为“氢和 / 或芳基，但不是烷基”或简单的“其中所述取代基不是烷基”。

[0025] 如本文所使用，术语“组分”、“化合物的组成”、“化合物”、“药物”、“药理学活性剂”、“活性剂”、“治疗剂”、“疗法”、“治疗”或“药剂”在本文中可互换使用，用于指向受试者（人或动物）施用时通过局部和 / 或全身作用引起期望的药理学和 / 或生理学作用的一种或多种化合物或物质组合物。

[0026] 本说明书中的缩写对应于如下计量单位、技术、性质或化合物：“min”表示分钟、“g”表示克、“mg”表示毫克、“μg”表示微克、“eq”表示当量、“h”表示小时、“μL”表示微升、“mL”表示毫升、“mM”表示毫摩尔、“M”表示摩尔、“mmol”或“mmole”表示毫摩尔、“cm”表示厘米、“SEM”表示平均标准误差及“IU”表示国际单位。“IC_{50}值”或“IC_{50}”表示导致观察的状态或作用减轻或抑制 50%的化合物的剂量。

[0027] “阿匹西定 (Apicidin)”是一种来源于镰刀菌属种真菌代谢物的化合物。其结构为环 (N-O- 甲基 -L- 色氨酰基 -L- 异亮氨酰基 -D- 哌可啉基 -L-2- 氨基 -8- 氧代癸酰基）。

[0028] “阿匹西定的天然类似物”是指粉白镰刀霉 (Fusarium pallidoroseum) 种 ATCC74322 和 ATCC47289 发酵产生的阿匹西定类似物（阿匹西定 A、B、C、D1、D2、D3，它们描述于 JOC 67，815(2002) 和 Tet Lett，37，8077(1996)，以及 WO 1996/9603428 中。

[0029] 如本文所使用，“烷基”是指具有约 1 至约 20 个碳原子的任选取代的、饱和直链或支链烃基（以及其中的碳原子的范围和具体数目的所有组合和子组合）。在适当的情况下，

“烷基”可以表示“亚烷基”;例如,如果 X 是 $-R_1R_2$,并且说 R_1是“烷基”,那么“烷基”可以被正确地解释为表示“亚烷基”。

[0030] “氨基”是指 $-NH_2$并且可以包括替代氢的一个或多个取代基。“氨基”可与胺互换使用并且还意在包括任何药学上可接受的胺盐。例如,氨基可以指 $-NH^+(X)(Y)Cl^-$,其中 X 和 Y 优选并且独立地是氢或烷基,其中烷基可以包括一种或多种卤代取代。

[0031] 如本文所使用,“芳基”“芳烃”和“芳香族”各自指具有约 3 至约 50 个环成员(以及其中的碳原子的范围和具体数目的所有组合和子组合)的任选取代的、饱和或不饱和、单环、多环或其它同素环、碳环或杂环芳香族环系统,优选约 5 至约 10 个环原子成员。此类部分涵盖(包括)如下文定义的“杂芳基”和“杂芳烃”。在适当的情况下,“芳基”可以表示“芳烃”;例如,如果 X 是 $-R_1R_2$,并且说 R_1是“芳基”,那么“芳基”可以被正确地解释为表示“芳烃”。

[0032] 如本文所使用,“烯基”是指具有约 2 至约 20 个碳原子和一个或多个双键(以及其中的碳原子的范围和具体数目的所有组合和子组合)的烷基,其中烷基如先前定义。在一些实施方案中,所述烯基具有约 2 至约 6 个碳原子是优选的。烯基可以被任选地取代。

[0033] 如本文所使用,“芳烷基”是指带有一个或多个芳基取代基并且具有约 4 至约 50 个碳原子(以及其中的碳原子的范围和具体数目的所有组合和亚组合)的烷基,其中芳基和烷基如先前定义。在一些优选实施方案中,所述芳烷基的烷基部分具有约 1 至约 4 个碳原子。在其它优选实施方案中,所述烷基部分具有约 1 至约 3 个碳原子。芳烷基可以被任选地取代。

[0034] “烷氨基”表示烷基 -(NH)-,其中烷基如先前描述并且 NH 是根据对氨基提供的定义进行定义的。“芳氨基”表示芳基 -(NH)-,其中芳基如本文定义并且 NH 是根据对氨基提供的定义进行定义的。同样地,“芳烷基氨基”是用来表示芳烷基 -(NH)-,其中芳烷基如先前定义并且 NH 是根据对氨基提供的定义进行定义的。“烷基酰胺基”是指烷基 -CH(=O)NH-,其中烷基如先前描述。本文中使用的“烷氧基”是指基团 R-O-,其中 R 是烷基,并且烷基如先前描述。“芳烷氧基”代表 R-O-,其中 R 是如先前定义的芳烷基。“烷基磺酰基”表示烷基 $-SO_2-$,其中烷基如先前定义。如本文中使用的“氨基氧基”是指基团氨基 -(O)-,其中氨基如上定义。如本文中使用的“芳烷基氨基氧基”用来表示芳基 - 烷基 - 氨基氧基 -,其中芳基、烷基和氨基氧基分别如先前提供的那样所定义。

[0035] 如本文所使用,“亚烷基”是指具有通式 $-(CH_2)_n-$ 的任选支链或取代的二价烷基,其中 n 是 1 至 10。非限制性实例包括亚甲基、三亚甲基、五亚甲基和六亚甲基。

[0036] “亚烷基氨基”是指 $-(CH_2)_n-NH-$,其中 n 是 1 至 10,并且其中所述二价烷基可以是任选支链或取代的,并且所述氨基可以包括替代氢的一个或多个取代基。

[0037] 如本文所使用,“杂芳基”或“杂芳烃”是指其中在至少一个环中,一个或多个碳原子环成员独立地被选自 S、O、N 和 NH 的杂原子基团替代的芳基,其中芳基如先前定义。具有总共约 3 至约 14 个碳原子环成员和杂原子环成员的杂芳基 / 杂芳烃基是优选的。同样地,“杂环”是其中一个或多个碳原子环成员可以(但不必然)独立地被选自 S、O、N 和 NH 的杂原子基团替代的芳基。具有总共约 3 至 14 个环成员和杂原子环成员的杂环是优选的,但是不一定存在;例如,“杂环己基”可以是有或没有杂原子基团的六元芳基。

[0038] “卤代”和“卤素”各自是指氟、氯、溴或碘部分,优选氟、氯或溴。

[0039] “卤代烷基”表示其中烷基和卤代分别如先前描述的卤代-烷基-。

[0040] 短语“[部分]不存在”可以表示所述部分连接的取代基彼此直接相连。

[0041] 通常，经取代的化学部分包括替代氢的一个或多个取代基。示例性取代基包括，例如，卤代（如，F、Cl、Br、I）、烷基、环烷基、烷基环烷基、环烷基烷基、烯基、炔基、芳烷基、芳基、杂芳基、杂芳烷基、螺烷基、杂环烷基、羟基（-OH）、硝基（$-NO_2$）、氰基（-CN）、氨基（$-NH_2$）、-N-取代的氨基（-NHR”）、-N,N-二取代的氨基（-N(R”)R”）、氧代（=O）、羧基（-COOH）、-O-C(=O)R”、-C(=O)R”、-OR”、-C(=O)OR”、-(亚烷基)-C(=O)-OR”、-NHC(=O)R”、氨基羰基（-C(=O)NH_2）、-N-取代的氨基羰基（-C(=O)NHR”）、-N,N-二取代的氨基羰基（-C(=O)N(R”)R”）、硫醇、硫醇根（thiolato）(-SR”)、磺酸（$-SO_3H$）、膦酸（$-PO_3H$）、-P(=O)(OR”)OR”、-S(=O)R”、$-S(=O)_2R$”、$-S(=O)_2NH_2$、$-S(=O)_2$NHR”、$-S(=O)_2$NR”R”、-NHS(=O)$_2$R”、-NR”S(=O)$_2$R”、$-CF_3$、$-CF_2CF_3$、-NHC(=O)NHR”、-NHC(=O)NR”R”、-NR”C(=O)NHR”、-NR”C(=O)NR”R”、-NR”C(=O)R”等。相对于上述取代基，例如，每个部分R”都可以独立地是H、烷基、环烷基、烯基、芳基、芳烷基、杂芳基或杂环烷基中的任何一种。

[0042] 如本文所使用，术语“治疗”或“疗法”（以及其不同词语形式）包括预防性（例如，防治性）、治愈性或缓和性治疗。

[0043] 如上以及本公开全文所使用，术语“有效量”是指就相关病症、疾患或副作用而论可以一定剂量并维持必要的一段时间实现期望效果的有效量。应理解本发明组分的有效量将会因不同患者而异，不仅与选择的特定化合物、组分或组合物、施用途径及组分在个体中引起期望的响应的能力有关，而且与以下因素有关：诸如待减轻的疾患的疾病状态或严重程度、个体的激素水平、年龄、性别、体重、患者的状态和所治疗的病理学病状的严重程度、特定患者遵循的并行药物或特殊饮食以及本领域技术人员了解的其它因素，其中合适的剂量最终由主治医师决定。剂量方案可进行调整以便提供改善的治疗响应。有效量还是其中治疗有益作用超过组分的任何毒性或有害作用的量。举例来说，将用于本发明方法的化合物以一定剂量施用一定时间以便与治疗开始之前的活性水平相比血小板的活性水平和粘附活性降低。

[0044] “药学上可接受的”是指在合理医学判断范围内，适于与人类和动物组织接触而没有过量毒性、刺激、过敏反应或其它问题并发症、与合理的利益/风险比相称的那些化合物、材料、组合物和/或剂型。

[0045] 在本发明内，公开的化合物可以药学上可接受的盐的形式制备。“药学上可接受的盐”是指所公开化合物的衍生物，其中所述母化合物通过制成其酸盐或碱盐进行修饰。药学上可接受的盐的实例包括但不限于诸如胺的碱性残基的无机或有机酸盐；诸如羧酸的酸性残基的碱盐或有机盐等。药学上可接受的盐包括例如由无毒无机酸或有机酸形成的母化合物的常规无毒盐或季铵盐。例如，此类常规无毒盐包括来源于诸如盐酸、氢溴酸、硫酸、氨基磺酸、磷酸、硝酸等的无机酸的那些盐；以及从有机酸制备的盐，所述有机酸诸如醋酸、丙酸、琥珀酸、乙醇酸、硬脂酸、乳酸、苹果酸、酒石酸、柠檬酸、抗坏血酸、双羟萘酸、马来酸、羟基马来酸、苯基乙酸、谷氨酸、苯甲酸、水杨酸、对氨基苯磺酸、2-乙酰氧基苯甲酸、富马酸、甲苯磺酸、甲烷磺酸、乙烷二磺酸、草酸、羟乙磺酸等。这些生理学上可接受的盐是通过本领域已知的方法制备的，例如，通过用在含水酒精中的过量的酸溶解游离胺碱，或用诸如氢氧

化物的碱金属碱或用胺中和游离羧酸。

[0046] 本文通篇描述的化合物都可以以其它形式使用或制备。例如，许多含氨基的化合物都可以作为酸加成盐酸使用或制备。此类盐往往改善所述化合物的分离和处理性质。例如，取决于试剂、反应条件等，如本文所描述的化合物可以例如作为其盐酸盐或甲苯磺酸盐使用或制备。同型结晶形式、所有手性和外消旋形式、N- 氧化物、水合物、溶剂化物和酸性盐水合物也涵盖在本发明的范围之内。

[0047] 本发明的某些酸性或碱性化合物可以作为两性离子存在。所述化合物的所有形式，包括游离酸、游离碱和两性离子，都涵盖在本发明的范围之内。本领域众所周知的是，同时含有氨基和羧基的化合物往往以与其两性离子形式平衡的形式存在。因此，本文通篇描述的含有例如氨基和羧基两者的任何化合物也包括对其相应的两性离子的引用。

[0048] “水合物”是指与分子形式的水缔合（即其中 H-OH 键不断裂）并且可以通过例如化学式 $R \cdot H_2O$ 表示的本发明化合物，其中 R 是本发明化合物。给定化合物可以形成一种以上水合物，包括例如一水合物 ($R \cdot H_2O$) 或多水合物 ($R \cdot nH_2O$，其中 n 是 >1 的整数）（包括，例如，二水合物 ($R \cdot 2H_2O$)、三水合物 ($R \cdot 3H_2O$) 等），或半水化合物（诸如，例如，$R \cdot n_{/2}H_2O$、$R \cdot n_{/3}H_2O$、$R \cdot n_{/4}H_2O$ 等，其中 n 是整数）。

[0049] “溶剂化物”是指与分子形式的溶剂缔合（即其中所述溶剂是配位结合的）并且可以通过例如化学式 R •（溶剂）表示的本发明化合物，其中 R 是本发明化合物。给定化合物可以形成一种以上溶剂化物，包括例如单溶剂化物 (R •（溶剂）) 或多溶剂化物 (R • n（溶剂）)，其中 n 是 >1 的整数）（包括，例如，二溶剂化物 (R •2（溶剂）)、三溶剂化物 (R •3（溶剂）) 等）或半溶剂化物（例如，$R \cdot n_{/2}$（溶剂）、$R \cdot n_{/3}$（溶剂）、$R \cdot n_{/4}$（溶剂）等，其中 n 是整数）。本文中的溶剂包括混合溶剂，例如，甲醇 / 水，并且因此，所述溶剂化物可以在溶剂化物内并入一种或多种溶剂。

[0050] “酸性水合物”是指复合物，它可以通过具有一个或多个碱部分的化合物与具有一个或多个酸部分的至少一种化合物缔合形成或通过具有一个或多个酸部分的化合物与具有一个或多个碱部分的至少一种化合物缔合形成，所述复合物进一步与水分子缔合以便形成水合物，其中所述水合物如先前定义并且 R 表示本文中的上述复合物。

[0051] 术语“立体异构体”是指具有相同化学组成但是原子或基团在空间中的排列不同的化合物。

[0052] “外消旋”表示具有拆分成相反光学活性形式的能力。

[0053] 如本文所使用，术语“部分立体异构体”是指具有两个或两个以上手性中心的立体异构体，其中至少一个手性中心具有确定的立体化学（即，R 或 S）并且至少一个具有不确定的立体化学（即，R 或 S）。当本文中使用术语“其部分立体异构体”时，其是指在描述的种类中的任何化合物，这类化合物在具有确定立体化学中心的手性中心处的构型被保留并且每个不确定的手性中心的构型独立地选自 R 或 S。例如，如果立体异构体具有三个手性中心并且第一个中心的立体化学构型确定具有“S”立体化学，那么术语“或其部分立体异构体”是指在所述三个手性中心处具有 SRR、SRS、SSR 或 SSS 构型的立体异构体和其混合物。

[0054] “同位素取代的类似物”是其中一个或多个原子已经被那个原子的同位素替代的本公开化合物。例如，氢（氕）可以被氘或氚取代。可以被其同位素替代以便形成其同位素取代的类似物的其它原子包括，例如，碳（被 C^{13} 替代）、氮（被 N^{15} 替代）、碘（被 I^{131} 替

代）、氟（被 F^{18} 替代）或硫（被 S^{31} 替代）。任何可用的同位素都可以用来形成其同位素取代的类似物，并且本领域的一般技术人员将会认识到由给定化合物形成此类类似物的可用技术。

[0055] “前药”是指对于期望的活性来说本身无活性或活性微乎其微，但是通过生物转化可以转化成生物学上活性的代谢物的化合物。例如，本发明的前药将尤其包括在体内通过代谢方式可转化成本公开中要求保护或描述的化合物的任何化合物。

[0056] “N- 氧化物”是指其中杂芳族环或叔胺的碱性氮原子被氧化从而得到带有正形式电荷的季氮和带有负形式电荷的连接的氧原子的化合物。

[0057] 当任何成分或任何式中的任何变量出现一次以上时，其每次出现时的定义与在每次另外出现的定义无关。只有当此类组合产生稳定化合物时取代基和 / 或变量的组合才是允许的。

[0058] 术语“施用”表示直接施用本发明的化合物或组合物，或者施用将在体内形成等量所述活性化合物或物质的前药、衍生物或类似物。

[0059] “剂量单位”是指适合作为待治疗的特定个体的单一剂量的物理上离散的单位。每个单位都可以含有与需要的药物载体结合的经计算产生期望的治疗效果的预定量的活性化合物。本发明的剂量单位形式的规格可以通过 (a) 所述活性化合物的独特特性和要获得的特定治疗效果，和 (b) 在合成这些活性化合物的领域中固有的限制来规定。

[0060] “受试者”或“患者”是指可用本发明的组合物和 / 或方法治疗的胚胎、不成熟或成年动物，包括人类。

[0061] 目前已经发现乙型肝炎病毒共价闭环 DNA(cccDNA)(在感染细胞的细胞核中作为“游离体”存在和表达）的调控不同于整合到宿主染色体中的 HBV DNA，并且与其它基因相比从 HBV cccDNA 表达 RNA 可以在药理学上被选择性地抑制 (如本文中更充分地描述那样)。实际上，本发明人已经鉴定出了众多以可重现和强力的方式抑制 DHBV cccDNA 转录的化合物，并且所述抑制在低浓度和没有明显毒性的条件下发生。这些结果第一次显示已经用小分子通过设计实现了选择性的药理学抑制。从 HBV cccDNA 表达基因的调控不同于整合到宿主染色体中的相同或相似 DNA 的这一结果是出人意料的并且是非常有用的观察结果，这在于它实现选择性地抑制 cccDNA DNA(例如，与整合的 HBV DNA 相比) 而不抑制或此外影响宿主染色体 DNA 的治疗。HBV cccDNA 可以在药理学上被抑制的本发现迄今为止是未知的，并且提供了先前叙述的有用概念的证据，并且证明此类药理学抑制是可能的。

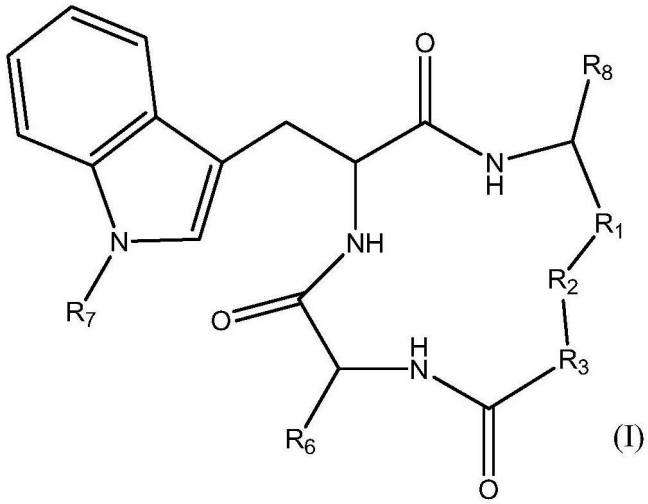
[0062] 因此，本公开尤其提供了调控受试者乙型肝炎的 cccDNA 转录的方法，其包括向所述受试者施用提供 cccDNA 的表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂。例如，所述表观遗传调节剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂可以是药理学的诸如小分子。表观遗传调节剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂对于 cccDNA 的抑制可能是选择性的，如与整合的 HBV DNA 相比，即不抑制整合的 HBV DNA，和 / 或如与细胞宿主 DNA 相比，即不抑制细胞宿主 DNA。组蛋白去乙酰化活性抑制剂可以是多类组蛋白去乙酰化酶的抑制剂，或者对于特定种类的组蛋白去乙酰化酶可以有选择性。例如，所述抑制剂可以是 I 类组蛋白去乙酰化活性、II 类组蛋白去乙酰化活性或两者的抑制剂。优选地，所述组蛋白去乙酰化活性抑制剂是 I 类组蛋白去乙酰化活性抑制剂。有大量的组蛋白去乙酰化活性抑制剂是已知的，并且可以按照本方法使用任何此类 HDAC 抑制剂。

[0063] 调控乙型肝炎的 cccDNA 转录的本方法还可以包括 - 除了向受试者施用提供 cccDNA 的表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂之外 - 向受试者施用治疗有效量的调控乙型肝炎病毒的其它药剂。所述其它药剂可以与提供 cccDNA 的表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂同时施用，或者仅仅作为与提供 cccDNA 的表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂相同的一般治疗方案的一部分施用。所述其它药剂可以是目前用于调控 HBV 的任何物质，其中大量类型对于本领域技术人员来说是已知的。例如，用于调控 HBV 的现有药物包括干扰素（例如，干扰素 α、聚乙二醇化干扰素）、核苷类似物（如，拉米夫定 (lamivudine)、阿德福韦酯 (adefovir dipivoxil)、恩替卡韦 (entecavir)、替比夫定 (telbivudine)、替诺福韦 (tenofovir)、克来夫定 (clevudine)、氨多索韦 (amdoxovir))、非核苷抗病毒剂（如，BAM 205、ANA380、myrcludex B、HAP 化合物 Bay 41-4109、REP 9AC、硝唑尼特 (nitazoxanide)、dd-RNAi 化合物、ARC-520、NVR-1221)、非干扰素免疫增强剂（如，胸腺素 α-1、白细胞介素 -7、DV-601、HBV 核抗原疫苗、GS-9620、GI13000) 以及暴露后和 / 或肝脏移植后治疗药物（如，hyperHEP S/D、Nabi-GB、Hepa Gam B)。

[0064] 具体来讲，所述其它药剂可以是任何其它直接作用抗病毒抗乙型肝炎剂（诸如聚合酶抑制剂 Barraclude、替诺福韦、拉米夫定、替比夫定和阿德福韦）和 / 或除了抑制 cccDNA 转录之外在病毒生命周期中的一个阶段起作用的任何其它直接作用抗病毒剂，诸如衣壳抑制剂、分泌抑制剂或进入抑制剂。所述其它药剂还可以是任何其它非直接作用抗病毒剂，诸如干扰素或其它免疫调节剂。

[0065] 根据调控乙型肝炎的 cccDNA 转录的本方法，所述组蛋白去乙酰化活性抑制剂可以是例如曲古抑菌素 A、辛二酰基双异羟肟酸、二甲氨基羟基 - 苯甲酰胺、阿匹西定、阿匹西定类似物（例如，阿匹西定的天然类似物或从头合成的类似物）或根据式 (I) 的化合物

[0066]



(I)

[0067] 其中

[0068] R_1是 $-(CH_2)_n-$ 或 -C(= O)- ；

[0069] R_2是 -C(= O)-、3,5- 三唑基或 $-C(Z)N(R_4)-$ ；

[0070] R_4是氢、烷基、芳基、芳烷基、二烷基氨基烷基或羧基烷基 ；

[0071] R_3是 $-CH(R_5)-$，或 R_2是氮并且 R_3是 -CH-，并且 R_2和 R_3一起形成哌啶基 ；

[0072] R_5是氢、-CH_3或 α 氨基酸 R 基团；

[0073] R_6是 -$(CH_2)_m$C(X)Y、-$(CH_2)_2CH_3$或 -$(CH_2)_q$- 苯基 -$(CH_2)_m$C(= O)NHOH；

[0074] X 是= O、H_2、= N-NH_2或= N-NH-C(= O)NH_2；

[0075] Y 是 NHOH 或 -CH_2CH_3；

[0076] Z 是 H_2或 O；

[0077] R_7是氢或烷氧基；

[0078] R_8是烷基或羧基烷基；

[0079] n 是 0-2；

[0080] m 是 0-6；并且，

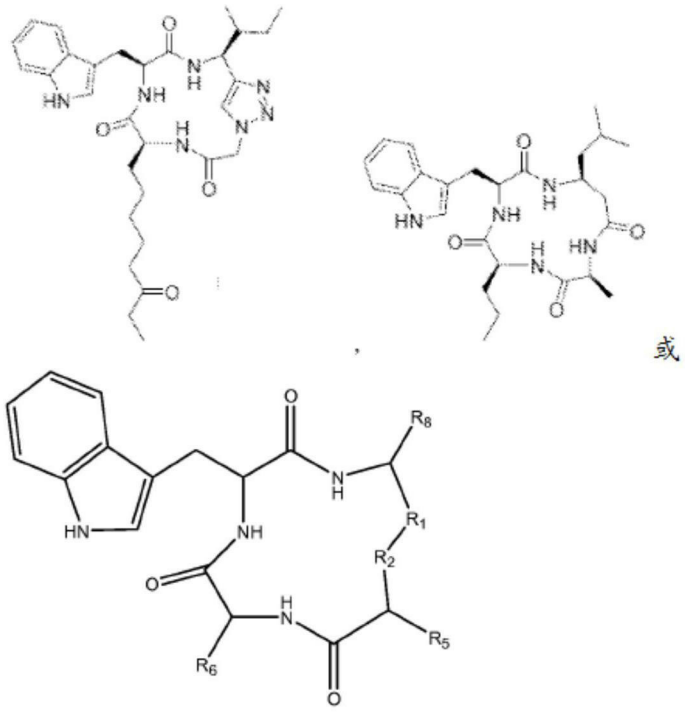
[0081] q 是 0-3；

[0082] 或其立体异构体或药学上可接受的盐。

[0083] 如本文所使用，短语“α 氨基酸 R 基团”是指来自天然或非天然氨基酸的侧链基团。

[0084] 在某些实施方案中，所述组蛋白去乙酰化活性抑制剂是阿匹西定，

[0085]



[0086] 其中

[0087] R_1是 -(CH_2)-，

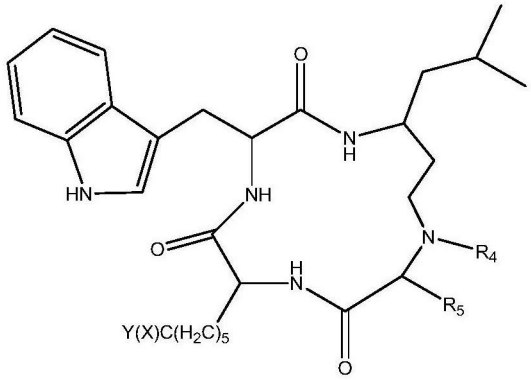
[0088] 并且，

[0089] R_2是 -C(Z)N(R_4)-

[0090] 或其立体异构体或药学上可接受的盐。

[0091] 在其它实施方案中，所述组蛋白去乙酰化活性抑制剂是

[0092]

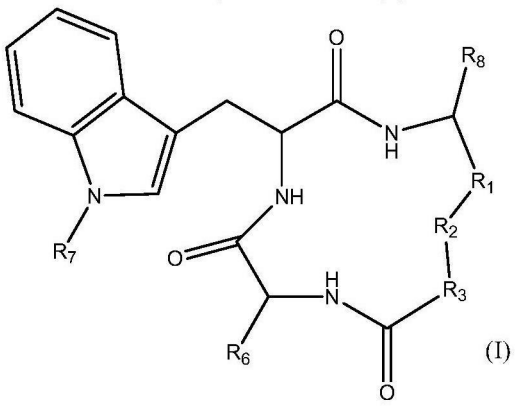


[0093] 或其立体异构体或药学上可接受的盐。

[0094] 本公开还涉及治疗受试者乙型肝炎的方法，它包括向所述受试者施用提供 cccDNA 的表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂。例如，所述表观遗传调节剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂可以是药理学的诸如小分子。表观遗传调节剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂对于 cccDNA 的抑制可能是选择性的，如与整合的 HBV DNA 相比，即，不抑制整合的 HBV DNA，和 / 或如与细胞宿主 DNA 相比，即，不抑制细胞宿主DNA。组蛋白去乙酰化活性抑制剂可以是多类组蛋白去乙酰化酶的抑制剂，或者可以对于特定种类的组蛋白去乙酰化酶有选择性。例如，所述抑制剂可以是 I 类组蛋白去乙酰化活性、II 类组蛋白去乙酰化活性或两者的抑制剂。优选地，所述组蛋白去乙酰化活性抑制剂是 I 类组蛋白去乙酰化活性抑制剂。大量的组蛋白去乙酰化活性抑制剂是已知的，并且可以按照本方法使用任何此类 HDAC 抑制剂。

[0095] 根据治疗受试者乙型肝炎的本方法，所述组蛋白去乙酰化活性抑制剂可以是例如曲古抑菌素 A、辛二酰基双异羟肟酸、二甲氨基羟基 - 苯甲酰胺、阿匹西定、阿匹西定类似物（例如，阿匹西定的天然类似物或从头合成类似物）或根据式 (I) 所述的化合物

[0096]



(I)

[0097] 其中

[0098] R_1是 $-(CH_2)_n-$ 或 -C(= O)- ；

[0099] R_2是 -C(= O)-、3,5- 三唑基或 $-C(Z)N(R_4)-$ ；

[0100] R_4是氢、烷基、芳基、芳烷基、二烷基氨基烷基或羧基烷基 ；

[0101] R_3是 $-CH(R_5)-$，或 R_2是氮并且 R_3是 -CH-，并且 R_2和 R_3一起形成哌啶基 ；

[0102] R_5是氢、$-CH_3$或 α 氨基酸 R 基团 ；

[0103] R_6是 $-(CH_2)_mC(X)Y$、$-(CH_2)_2CH_3$或 $-(CH_2)_q-$ 苯基 $-(CH_2)_mC(= O)NHOH$ ；

[0104] X 是＝ O、H_2、＝ N-NH_2或＝ N-NH-C(＝ O)NH_2；

[0105] Y 是 NHOH 或 -CH_2CH_3；

[0106] Z 是 H_2或 O ；

[0107] R_7是氢或烷氧基 ；

[0108] R_8是烷基或羧基烷基 ；

[0109] n 是 0-2 ；

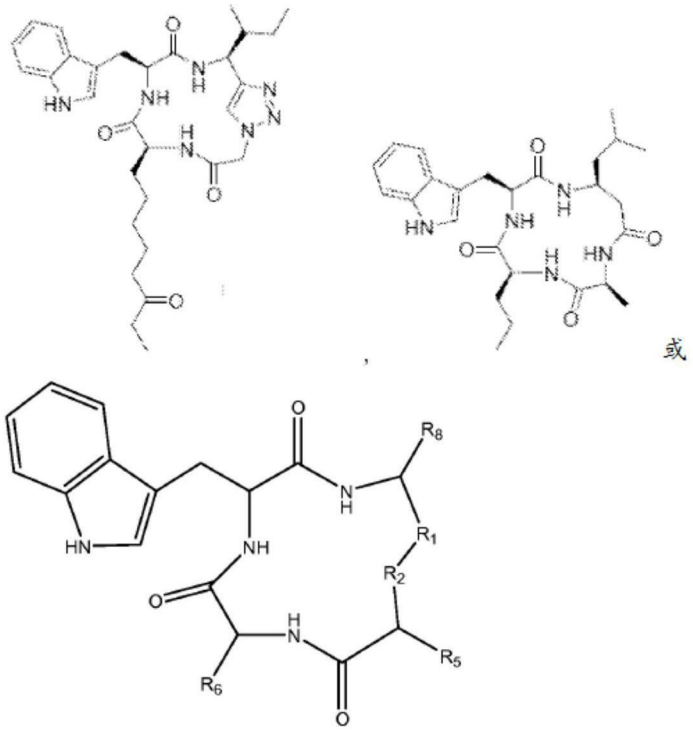
[0110] m 是 0-6 ；并且，

[0111] q 是 0-3 ；

[0112] 或其立体异构体或药学上可接受的盐。

[0113] 在某些实施方案中，所述组蛋白去乙酰化活性抑制剂是阿匹西定，

[0114]



[0115] 其中

[0116] R_1是 -(CH_2)-，

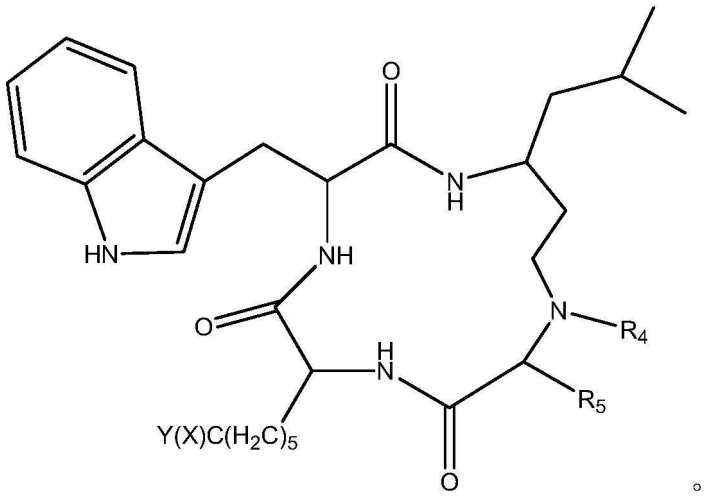
[0117] 并且，

[0118] R_2是 -C(Z)N(R_4)-

[0119] 或其立体异构体或药学上可接受的盐。

[0120] 在其它实施方案中，所述组蛋白去乙酰化活性抑制剂是

[0121]



。

[0122] 或其立体异构体或药学上可接受的盐。

[0123] 治疗受试者乙型肝炎的本方法还可以包括－除了向所述受试者施用提供 cccDNA 的表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂之外－向所述受试者施用治疗有效量的调控乙型肝炎病毒的其它药剂。所述其它药剂可以与提供 cccDNA 的表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂同时施用，或者仅仅作为与提供 cccDNA 的表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂相同的一般治疗方案的一部分施用。所述其它药剂可以是目前用于调控 HBV 的任何物质，其中大量的类型对于本领域技术人员来说是已知的。例如，用于调控 HBV 的现有药物包括干扰素（如，干扰素 α、聚乙二醇化干扰素）、核苷类似物（如，拉米夫定、阿德福韦酯、恩替卡韦、替比夫定、替诺福韦、克来夫定、氨多索韦）、非核苷抗病毒剂（如，BAM 205、ANA380、myrcludex B、HAP 化合物 Bay 41-4109、REP 9AC、硝唑尼特、dd-RNAi 化合物、ARC-520、NVR-1221）、非干扰素免疫增强剂（如，胸腺素 α-1、白细胞介素-7、DV-601、HBV 核抗原疫苗、GS-9620、GI13000）和暴露后和/或肝脏移植后治疗药物（如，hyperHEP S/D、Nabi-GB、Hepa Gam B）。

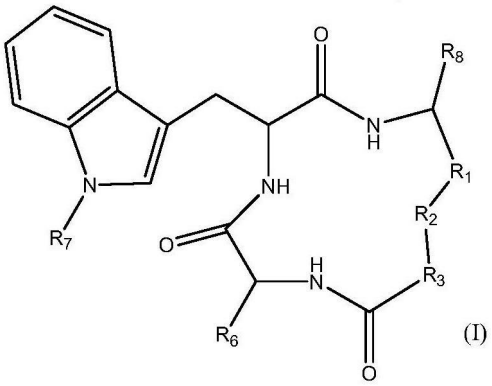
[0124] 具体来讲，所述其它药剂可以是任何其它直接作用抗病毒抗乙型肝炎剂（诸如聚合酶抑制剂 Barraclude、替诺福韦、拉米夫定、替比夫定和阿德福韦）和/或除了抑制 cccDNA 转录之外在病毒生命周期中的一个阶段起作用的任何其它直接作用抗病毒剂，如衣壳抑制剂、分泌抑制剂或进入抑制剂。所述其它药剂还可以是任何其它非直接作用抗病毒剂，如干扰素或其它免疫调节剂。

[0125] 还公开了调控乙型肝炎病毒共价闭环 DNA 的方法，它包括使乙型肝炎病毒与提供 cccDNA 的表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂接触。例如，所述表观遗传调节剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂可以是药理学的诸如小分子。表观遗传调节剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂对于 cccDNA 的抑制可能是选择性的，如与整合的 HBV DNA 相比，即，不抑制整合的 HBV DNA，和/或如与细胞宿主 DNA 相比，即，不抑制细胞宿主 DNA。组蛋白去乙酰化活性抑制剂可以是多类组蛋白去乙酰化酶的抑制剂，或者可以对于特定种类的组蛋白去乙酰化酶有选择性。例如，所述抑制剂可以是 I 类组蛋白去乙酰化活性、II 类组蛋白去乙酰化活性或两者的抑制剂。优选地，所述组蛋白去乙酰化活性抑制剂是 I 类组蛋白去乙酰化活性抑制剂。大量的组蛋白去乙酰化活

性抑制剂是已知的，并且可以按照本方法使用任何此类 HDAC 抑制剂。

[0126] 根据调控乙型肝炎病毒共价闭环 DNA 的本方法，所述组蛋白去乙酰化活性抑制剂可以是例如曲古抑菌素 A、辛二酰基双异羟肟酸、二甲氨基羟基 - 苯甲酰胺、阿匹西定、阿匹西定类似物（例如，阿匹西定的天然类似物或从头合成类似物）或根据式（I）的化合物

[0127]



(I)

[0128] 其中

[0129] R_1是 $-(CH_2)_n-$ 或 $-C(=O)-$；

[0130] R_2是 $-C(=O)-$、3,5- 三唑基或 $-C(Z)N(R_4)-$；

[0131] R_4是氢、烷基、芳基、芳烷基、二烷基氨基烷基或羧基烷基；

[0132] R_3是 $-CH(R_5)-$，或 R_2是氮并且 R_3是 $-CH-$，并且 R_2和 R_3一起形成哌啶基；

[0133] R_5是氢、$-CH_3$或 α 氨基酸 R 基团；

[0134] R_6是 $-(CH_2)_mC(X)Y$、$-(CH_2)_2CH_3$或 $-(CH_2)_q-$ 苯基 $-(CH_2)_mC(=O)NHOH$；

[0135] X 是 =O、H_2、$=N-NH_2$或 $=N-NH-C(=O)NH_2$；

[0136] Y 是 NHOH 或 $-CH_2CH_3$；

[0137] Z 是 H_2或 O；

[0138] R_7是氢或烷氧基；

[0139] R_8是烷基或羧基烷基；

[0140] n 是 0-2；

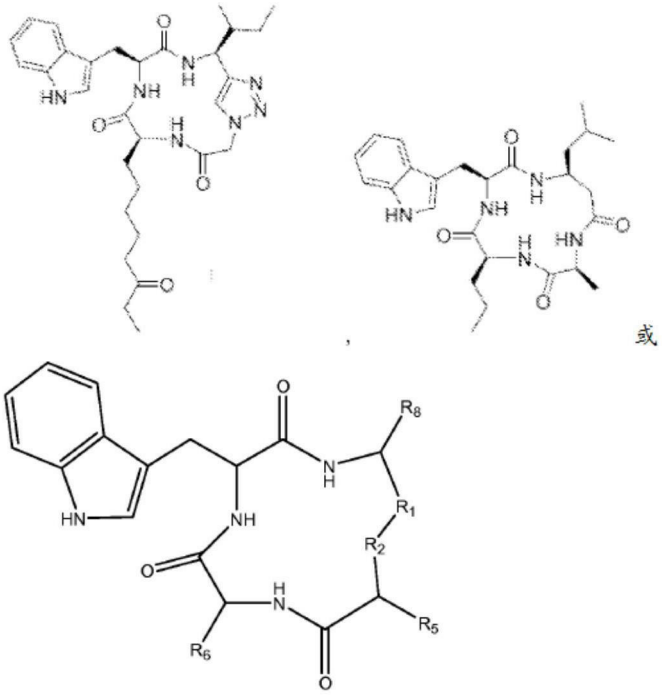
[0141] m 是 0-6；并且，

[0142] q 是 0-3；

[0143] 或其立体异构体或药学上可接受的盐。

[0144] 在某些实施方案中，所述组蛋白去乙酰化活性抑制剂是阿匹西定，

[0145]



[0146] 其中

[0147] R_1是 $-(CH_2)-$，

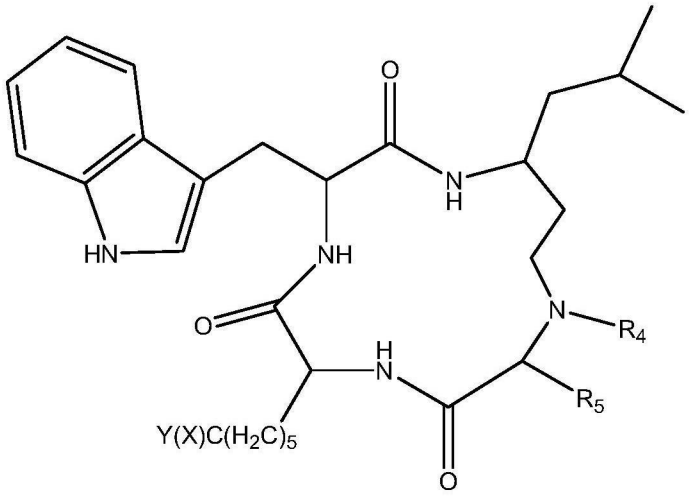
[0148] 并且，

[0149] R_2是 $-C(Z)N(R_4)-$

[0150] 或其立体异构体或药学上可接受的盐。

[0151] 在其它实施方案中，所述组蛋白去乙酰化活性抑制剂是

[0152]



[0153] 或其立体异构体或药学上可接受的盐。

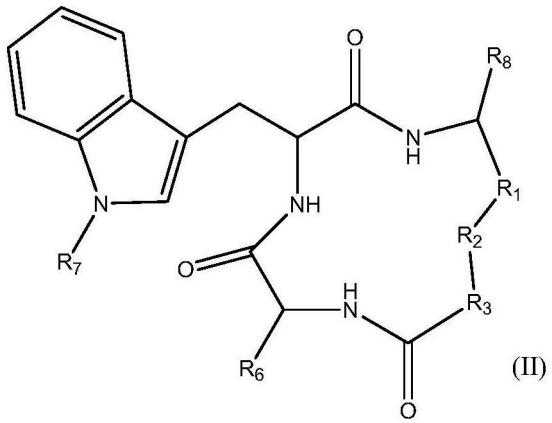
[0154] 调控乙型肝炎病毒共价闭环 DNA 的本方法还可以包括－除了使乙型肝炎病毒与提供 cccDNA 的表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂接触之外－使所述乙型肝炎病毒与治疗有效量的调控乙型肝炎病毒的其它药剂接触。所述其它药剂与所述 HBV 的接触可以与涉及使 HBV 与提供 cccDNA 的表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂接触同时发生，或者仅仅作为涉及使 HBV 与提供 cccDNA 的

表观遗传修饰的药剂、组蛋白调节剂或组蛋白去乙酰化活性抑制剂接触相同的操作的一部分。所述其它药剂可以是目前用于调控 HBV 的任何物质，其中大量类型对于本领域技术人员来说是已知的。例如，用于调控HBV的现有药物包括干扰素（如，干扰素 α 、聚乙二醇化干扰素）、核苷类似物（如，拉米夫定、阿德福韦酯、恩替卡韦、替比夫定、替诺福韦、克来夫定、氨多索韦）、非核苷抗病毒剂（如，BAM 205、ANA380、myrcludex B、HAP 化合物 Bay41-4109、REP 9AC、硝唑尼特、dd-RNAi 化合物、ARC-520、NVR-1221)、非干扰素免疫增强剂（如，胸腺素 α -1、白细胞介素 -7、DV-601、HBV 核抗原疫苗、GS-9620、GI13000) 和暴露后和 / 或肝脏移植后治疗药物（如，hyperHEP S/D、Nabi-GB、Hepa Gam B)。

[0155] 具体来讲，所述其它药剂可以是任何其它直接作用抗病毒抗乙型肝炎剂（诸如聚合酶抑制剂 Barraclude、替诺福韦、拉米夫定、替比夫定和阿德福韦）和 / 或除了抑制 cccDNA 转录之外在病毒生命周期中的一个阶段起作用的任何其它直接作用抗病毒剂，诸如衣壳抑制剂、分泌抑制剂或进入抑制剂。所述其它药剂还可以是任何其它非直接作用抗病毒剂，诸如干扰素或其它免疫调节剂。

[0156] 本公开还涉及根据式 II 的化合物：

[0157]



(II)

[0158] 其中

[0159] R_1是 $-(CH_2)_n-$ 或 -C(= O)- ；

[0160] R_2是 -C(= O)- 或 -C(Z)N(R_4)- ；

[0161] R_4是氢、烷基、芳基、芳烷基、二烷基氨基烷基或羧基烷基 ；

[0162] R_3是 -CH(R_5)- ；

[0163] R_5是氢、$-CH_3$或 α 氨基酸 R 基团 ；

[0164] R_6是 $-(CH_2)_mC(X)Y$、$-(CH_2)_2CH_3$或 $-(CH_2)_q-$ 苯基 $-(CH_2)_mC(= O)NHOH$ ；

[0165] X 是 = O、H_2、= N-NH_2或 = N-NH-C(= O)NH_2；

[0166] Y 是 NHOH 或 $-CH_2CH_3$；

[0167] Z 是 H_2或 O ；

[0168] R_7是氢或烷氧基 ；

[0169] R_8是烷基或羧基烷基 ；

[0170] n 是 0-2 ；

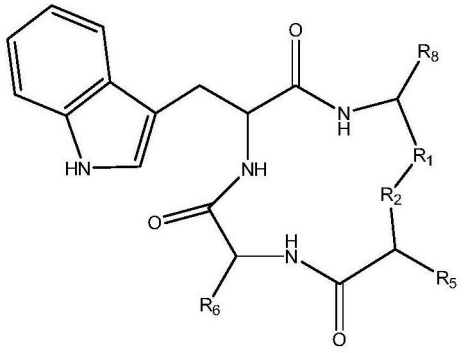
[0171] m 是 0-6 ；并且，

[0172] q 是 0-3 ；

[0173] 或其立体异构体或药学上可接受的盐。

[0174] 例如，所述化合物可以是

[0175]



[0176] 其中

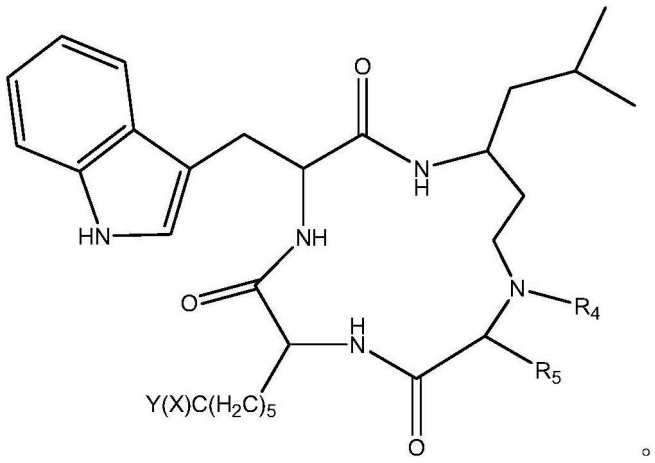
[0177] R_1是 $-(CH_2)-$，

[0178] 并且，

[0179] R_2是 $-C(Z)N(R_4)-$。

[0180] 在其它实施方案中，所述化合物可以是

[0181]



。

[0182] 如将易于理解，在合成的过程中存在的官能团可以含有保护基。保护基已知是本身作为可以是选择性地附加到诸如羟基和羧基的官能团以及可以从这些官能团移除的化学官能团。化合物中存在这些基团是为了在所述化合物暴露的室温化学反应条件下提供这些官能团。各种保护基中的任何一种都可以与本发明一起使用。根据本发明可以使用的保护基可以在 Greene，T.W. 和 Wuts，P.G.M.，Protective Groups in Organic Synthesis，第 2 版，Wiley&Sons，1991 中描述。

[0183] 在另一方面中，本公开涉及包含根据式 (I) 或 (II) 的化合物或其药学上可接受的盐、同位素取代的类似物或立体异构体与药学上可接受的载体、稀释剂或赋形剂的药物组合物。所述适用的载体、稀释剂或赋形剂可以根据选择的施用途径和例如在 Remington's Pharmaceutical Sciences(Mack Pub.Co.，Easton，PA，1985) 中描述的标准药学实践进行选择，所述文献的公开内容在此以引用方式全部并入。所述药物组合物还可以包含治疗有效量的调控乙型肝炎病毒的其它药剂。例如，所述调控病毒的其它药剂可以是已知的抗病毒剂。在某些实施方案中，本组合物包含治疗有效量的根据式 (I) 或 (II) 的化合物，其是

与有效预防或减轻HBV症状的免疫或疫苗组合施用的。实例包括抗体、免疫抑制剂、抗炎剂等。

[0184] 如本文所使用，术语“接触”是指在体外系统或体内系统中将指定部分一起形成物理或化学联系。例如，使HBV病毒与本发明中的化合物“接触”可以包括向感染HBV的个体或患者（诸如人）施用本发明中的化合物，以及，例如向包含含有cccDNA的细胞或纯化制剂的样品中引入本发明化合物。

[0185] 如本文所使用，可互换使用的术语“个体”或“患者”是指包括哺乳动物的任何动物，诸如小鼠、大鼠、其它啮齿类动物、兔、犬、猫、猪、牛、绵羊、马，或诸如人的灵长类动物。

[0186] 如本文所使用，短语“治疗有效量”是指研究者、兽医、医学博士或其它临床医师在组织、系统、动物、个体或人中寻求的引起生物或医药响应的活性化合物或药剂的量，所述生物或医药响应包括以下一种或多种：

[0187] (1)预防所述疾病；例如，预防个体中的疾病、疾患或病症，所述个体可能易感染所述疾病、疾患或病症但是还没有遭受或显示所述疾病的病变或症状；

[0188] (2)抑制所述疾病；例如，抑制个体中的疾病、疾患或病症，所述个体正遭受或显示所述疾病、疾患或病症的病变或症状（即，包括中止所述病变和/或症状的进一步发展）；和

[0189] (3)改善所述疾病；例如，改善个体中的疾病、疾患或病症，所述个体正遭受或显示所述疾病、疾患或病症的病变或症状（即，包括使所述病变和/或症状逆转）。

[0190] 对于疾病或病症施用所述治疗化合物是有效的治疗方案的受试者或患者优选是人，但是可以是任何动物，包括在临床试验或筛选或活性实验情况中的实验室动物。因此，如本领域一般技术人员可以容易理解的那样，本发明的方法、化合物和组合物特别适于向任何动物施用，特别是哺乳动物，并且包括但决不限于人、家畜（诸如猫科动物或犬科动物受试者）、农场动物（诸如但不限于牛科动物、马科动物、山羊、绵羊和猪科动物受试者）、野生动物（无论是野生或在动物园中）、研究动物（诸如小鼠、大鼠、兔、山羊、绵羊、猪、犬、猫等）、禽类物种（诸如鸡、火鸡、鸣禽等，即用于兽医学用途）。

[0191] 本发明的化合物可以单独或与常规药物载体、稀释剂或赋形剂（可以是液体或固体）组合口服或肠胃外施用。其中，适用的固体载体、稀释剂或赋形剂可以起粘合剂、崩解剂、填充剂、润滑剂、助流剂、压缩助剂、加工助剂、着色剂、甜味剂、防腐剂、悬浮/分散剂、片剂崩解剂、包封材料、成膜剂或涂层、调味剂或印刷油墨的作用。当然，用于制备任何单位剂型的任何材料都优选是药用纯的并且在使用的量下基本上无毒。此外，活性化合物可并入持续释放制剂和配方中。在这方面的肠胃外施用包括尤其通过以下途径施用：静脉内、肌内、皮下、眼内、滑膜内、经上皮（包括透皮）、经眼部、舌下和经颊；局部，包括经眼部、经真皮、经眼、经直肠和经由吹入剂、气溶胶的经鼻吸入以及经直肠全身。

[0192] 在粉末剂中，载体、稀释剂或赋形剂可以是与细碎活性成分混合的微细固体。在片剂中，活性成分与具有必要压缩性质的载体、稀释剂或赋形剂以合适的比例混合并且压制成所需的形状和尺寸。对于经口治疗施用，活性化合物可掺入载体、稀释剂或赋形剂中并且以可摄取片剂、口含片剂、锭剂、胶囊、酏剂、混悬剂、糖浆剂、干胶片等形式使用。此类治疗有用组合物中的活性化合物的量是优选的使得将获得适合的剂量。所述治疗组合物优选含有多达约99％的活性成分。

[0193] 液体载体、稀释剂或赋形剂可以用于制备溶液、混悬液、乳液、糖浆、酏剂等。本发明的活性成分可以溶解或悬浮在药学上可接受的液体（诸如水、有机溶剂、两者的混合物或者药学上可接受的油或脂肪）中。液体载体、赋形剂或稀释剂可以含有其它合适的药物添加剂，诸如增溶剂、乳化剂、缓冲剂、防腐剂、甜味剂、调味剂、助悬剂、增稠剂、着色剂、粘度调节剂、稳定剂或渗透调节剂。

[0194] 合适的固体载体、稀释剂和赋形剂可以包括，例如，磷酸钙、二氧化硅、硬脂酸镁、滑石粉、糖、乳糖、糊精、淀粉、明胶、纤维素、甲基纤维素、乙基纤维素、羧甲基纤维素钠、微晶纤维素、聚乙烯基吡咯烷、低熔点石蜡、离子交换树脂、交联羧甲纤维素碳(croscarmellose carbon)、阿拉伯胶、预胶化淀粉、交聚维酮、HPMC、聚维酮、二氧化钛、多晶纤维素、甲基氢化铝(aluminum methahydroxide)、琼脂、黄芪胶或其混合物。

[0195] 用于经口和胃肠外施用的液体载体、稀释剂和赋形剂的合适实例包括水（特别是含有如上添加剂，如纤维素衍生物，优选羧甲基纤维素钠溶液）、醇（包括一元醇和多元醇，如二醇）及其衍生物，以及油（如，分馏的椰子油和花生油），或其混合物。

[0196] 对于胃肠外施用，载体、稀释剂或赋形剂还可以是油性酯，诸如油酸乙酯和肉豆蔻酸异丙酯。还涵盖了无菌液体载体、稀释剂或赋形剂，它们在用于肠胃外施用的无菌液体形式组合物中使用。作为游离碱的活性化合物或药理学上可接受的盐的溶液可以在适当地与表面活性剂（诸如羟丙基纤维素）混合的水中制备。分散液也可在甘油、液体聚乙二醇和其混合物中以及油中制备。在正常贮藏和使用条件下，这些制剂可以含有防腐剂以防止微生物生长。

[0197] 适于注射用途的药用形式包括例如无菌水溶液或分散液和用于临时制备无菌可注射溶液或分散液的无菌粉末。在所有情况下，形式优选是无菌和液体的，以便能够容易地注射。它在制造和贮藏条件下优选是稳定的并且优选被保存以防止诸如细菌和真菌的微生物的污染作用。载体、稀释剂或赋形剂可以是含有例如水、乙醇、多元醇（例如，甘油、丙二醇、液体聚乙二醇等）、其合适的混合物和植物油的溶剂或分散介质。可以例如通过使用包覆剂（诸如卵磷脂）、在分散液的情况下通过维持所需的粒径以及通过使用表面活性剂来维持适当的流动性。可通过各种抗细菌剂和抗真菌剂，例如，对羟基苯甲酸酯、氯丁醇、苯酚、山梨酸、硫柳汞等获得预防微生物的作用。在许多情况下，优选包括等渗剂，例如糖或氯化钠。可注射的组合物的延长吸收可以通过使用延缓吸收的药剂（例如单硬脂酸铝和明胶）来实现。

[0198] 无菌可注射溶液可以通过将将所需量的活性化合物视需要与以上列举的各种其它成分掺入至适当溶剂中，随后通过过滤灭菌来制备。一般来讲，可以通过将灭菌活性成分掺入到含有基本分散介质和来自以上列举的那些的所需其它成分的无菌媒介物中来制备分散液。在用于制备无菌可注射溶液的无菌粉末的情况下，优选制备方法可以包括真空干燥和冻干技术，其产生一种或多种活性成分外加来自其先前无菌过滤溶液的任何其它所需成分的粉末。

[0199] 本发明化合物可以通过医药领域中任何成熟的常规技术以有效量施用。可以通过使活性剂与患者体内的所述药剂的一个或多个作用位点接触的任何方式施用包括式(I)或(II)化合物的本发明的方法中使用的化合物。所述化合物可以通过任何可用的常规方式施用。

[0200] 所述药物组合物优选是单位剂型，如片剂、口含片剂、锭剂、胶囊、酏剂、粉末剂、溶液、混悬剂、乳剂、糖剂、干胶片、颗粒剂、栓剂等。在此类形式中，所述组合物被再次分成含由适当量的活性成分的单位剂量；所述单位剂型可以是包装的组合物，例如包装的粉末剂、小瓶、安瓿、预装注射器或含液体的囊剂。所述单位剂型可以是例如胶囊或片剂本身，或者它可以是适当数量的包装形式的任何此类组合物。此外，本发明的剂型可以是其中一种活性成分被压制成片剂的胶囊的形式，或者是多个微片剂、颗粒剂、粒剂或非危险(non-peril)的形式。这些微片剂、颗粒剂、粒剂或非危险形式随后被放入胶囊中或被压制成胶囊，可能伴随着另一种活性成分的颗粒化。

[0201] 最适合于预防或治疗的本发明化合物的剂量将随施用形式、选择的特定化合物和治疗中的特定患者的生理特征而变化。一般来讲，开始可以使用小剂量，并且必要时以小增量增加，直到达到在这些情况下的期望效果。一般来讲，经口施用可能需要更高的剂量。

[0202] 所需剂量可方便地以单个剂量提供或以适当时间间隔施用的分次剂量提供，例如以每天两次、三次、四次或更多次亚剂量提供。亚剂量本身可以如进一步分成多次分开不精确间隔的施用。所述剂量还可以通过本领域熟知的技术对所述化合物的控制释放来提供。

[0203] 关于用于施用的本发明化合物的制备以及根据本发明所述的组合物的配方的附加信息在下文中提供。

[0204] 用于本发明的方法中的化合物可以本领域技术人员熟知的若干方式制备。所述化合物例如可以如由技术人员所理解的那样通过如下所述的方法或其变型合成。制备本发明化合物中使用的试剂可以是商购获得的或者可以通过文献中描述的标准程序制备。期待联合本发明的公开的所有方法以任何规模实践，包括毫克、克、克级、千克、千克级或商业同业规模。

[0205] 对于变量不止一次出现的化合物而言，每个变量都可以是选自限定所述变量的马库什(Markush)组的不同部分。例如，在结构被描述为具有同时存在于同一化合物上的两个R基团的情况下，所述两个R基团可以表示选自为限定R的马库什组的不同部分。

[0206] 应进一步理解，为清晰起见，在独立实施方案的情况下描述的本发明的某些特征也可以在单个实施方案中以组合形式提供。相反地，为简洁起见，在单个实施方案的情况下描述的本发明的各种特征也可以单独提供或以任何合适的亚组合形式提供。

[0207] 在以下实施例中进一步描述本发明。应理解，这些实施例虽然表明本发明的优选实施方案，但是只通过说明方式给出，并且不应该理解为限制附加的权利要求书。从上文的讨论和这些实施例中，本领域技术人员可以确定本发明的必要特性，并且在不背离其精神和范围的情况下，可以对本发明做出各种变化和修改以便使其适应于各种用法和条件。

实施例

[0208] HBV cccDNA的调控

[0209] LMH源dstet5细胞中的DHBV cccDNA有效地产生并且有转录活性。大部分产生HBV的细胞系由整合到宿主染色体中的HBV转基因产生HBV基因产物，并且因此cccDNA不是病毒产物的主要来源。这使得难以筛选靶向cccDNA的药物。产生其中病毒基因产物依赖于cccDNA的细胞系。为了这个目的，用四环素(tet)调控的HBV/DHBV建立了人Hep G2和鸡肝细胞瘤(LMH)-稳定细胞系。如图1所示，在不存在tet和存在2mM膦甲酸(PFA)条件

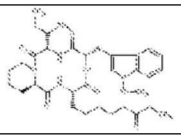
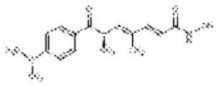
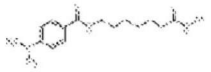
下培养以阻断病毒逆转录之后，DHBV RNA 积聚，但是 DHBV 复制在含 pgRNA 的核衣壳的阶段中止（泳道 0）。重新向培养基中添加 tet 以阻断转基因转录并且移除 PFA 以允许含 pgRNA 的衣壳中的病毒 DNA 合成进行之后，病毒 RNA 有快速的下降（第 1 天和第 2 天），在第 3 天之后产生 cccDNA 时最终升至更高的水平。

[0210] 这些结果意味着 cccDNA 在 dstet5 细胞中有效地形成并且有转录功能。图 2 中更加彻底地说明了这些结果，其中，在其中规定的用 tet 阻断转基因转录的条件下，新 HBV RNA 的出现与 cccDNA 的出现密切相关（图 2 组 II，展示核 DNA），然而病毒转录物在 cccDNA 合成和新的转基因转录都被阻断（图 2，组 I）的细胞中迅速降解（1/2 寿命约 3 小时）

[0211] 有效抑制 DHBV cccDNA 转录的化合物的鉴定。使用系统以及在以依赖 cccDNA 的方式产生病毒转录物的条件下（图 1，2B），筛选了大约 100 种化合物，包括来自本发明人自有的小化合物库的那些、在本发明人天然产物收集品中存在的那些，及选择的化合物包括细胞表观遗传修饰酶抑制剂（包括 HDAC、HAT、长寿蛋白 (Sirtuins)、组蛋白甲基转移酶、组蛋白去甲基酶和 DNA 甲基转移酶）。许多化合物（包括表 1 中展示的四种化合物）显著地降低了 cccDNA 源 DHBV pgRNA 的量。全部都具有 HDAC I 类抑制活性。

[0212]

表1：抑制HBV cccDNA功能的化合物及其对HDAC[1]的活性

命中物	阿匹西定	曲古抑菌素A (TSA)	辛二酰基双异羟肟酸 (SBHA)	二甲氨基羟基苯甲酰胺(M344)[5]
结构				
HBVcccDNA (EC50, uM)	0.183	0.480	2.50	6.25
毒性(CC50,um)[2]	>20.00	>20.00	>40.00	>100.00
选择性指数 (SI)[3]	>100	>40	>16	>16
HDAC-I 抑制剂?	是[4,5]	是[5,6,9]	是[4,6,7,10]	是[4,7]
HDAC- II 抑制剂?	否[3,7]	是[4,5,6,9]	是 & HDAC III[11]	是 & HDAC III[7,11]

[1] 发现在 dstet5 系统中抑制 HBV cccDNA 功能的化合物，在 Prelim Evid 中描述，如图 2 中说明。

[2] 来自我们对dstet5细胞进行的试验的毒性，如正文；选择性指数(SI)是毒性CC50除以有效性EC50，参见正文。

[3] 选择性指数(SI)是降低50%细胞生存力的浓度(CC_{50})除以降低50% HBV特定信号(RNA和/或HBeAg)的浓度(EC_{50})。

[4] (7); [5](15)(26); [6](45); [7]反应生物学专著：[8] (33);[9](13); [10](14); [11](38)

[0213] 如图 3 所示，因为阿匹西定有效地抑制 cccDNA(EC50 约 180nM) 且在多达 20uM 的情况下持续五天没有毒性，并且对 I 类而非 II 类 HDAC 有纳摩尔活性，所以似乎 HDAC II 抑制对于抑制这个系统中的 HBV 不是必要的。

[0214] 阿匹西定和 TSA 抑制 HBV cccDNA 转录。图 3 示出了阿匹西定和 TSA 抑制 Dstet5 细胞中的 cccDNA 转录。此外得到了证明这些化合物还抑制 HepG2 细胞中的 HBV cccDNA 转

录的证据。在鲜明的对比之下，观察到阿匹西定和 TSA 剂量依赖性刺激 DHBV pgRNA 从整合到宿主细胞染色体中的转基因的转录（图 4）。这对于 HDAC 抑制来说是更加典型的细胞基因响应并且表明不像染色体 DNA，从 cccDNA“微型染色体”的转录调控也不同。此外，甚至还有 cccDNA 水平降低的证据，这表明了如在鸭系统中看到的那样转录抑制伴随着不稳定。

[0215] 化合物对人肝细胞瘤细胞中 HBV cccDNA 转录的作用。

[0216] 将 HepDE19 细胞接种到 6 孔板中，在四环素存在下培养直到融合。从培养基中移除 tet 从而允许发生 pgRNA 转录、DNA 合成和 cccDNA 形成。将 tet 添加回培养基中以停止转基因转录。第 3 天之后，不处理或用不同浓度（即，0.1 至 10.0uM）的各种“测试”化合物（来自表 1 的四种“命中物”和约 20 种类似物）处理不同孔中的细胞 2 天。通过如上所述的 DNA/RNA 印迹杂交测定 (Southern/Northern blot hybridization assay) 并且用已知的操作定量胞内 HBV cccDNA、病毒 RNA 和核 DNA。分别用蛋白印迹 (Western blot) 和 ELISA 测定定量胞内全长 HBeAg 前体和分泌的 HBeAg。使用 HepG2.2.15 细胞作为对照，因为所有 HBV 表达主要是来自这些细胞中的 HBV 转基因。将纳入干扰素 α（已经证明抑制 cccDNA 转录）和抑制 cccDNA 形成的二取代磺胺 (DSS)CCC-0975（来自我们的筛选，Guo 2012) 作为阳性药物对照。在一些实验中，在从所述培养基移除“测试”药物之后将培养物维持不同时间（天），以确定 HBV cccDNA 的任何药物诱发的抑制的持久性。为了确定测试化合物对 cccDNA 转录的选择性，还通过定量 RT-PCR 或 RNA 印迹杂交测量了测试化合物对一组细胞基因（包括但不限于 α 1 抗胰蛋白酶、白蛋白）表达的作用。化合物的细胞毒性是通过在平行培养物中的 MTT 试验确定的。

[0217] 用 HBeAg 和 HBV 转录物降低的量 (0-100％) 作为 HBV cccDNA 转录抑制的衡量尺度。用 HBV cccDNA 降低的量 (0-100％) 作为 HBV cccDNA 不稳定和降解的衡量尺度。用 A1AT 和 / 或白蛋白 mRNA 降低的抑制的量 (0-100％) 作为特异性测定中的细胞功能抑制的衡量尺度。用 MTT(0-100％) 活性的量作为细胞生存力和细胞细胞毒性 (cell cytoxicity) (CC) 基准的衡量尺度。选择性指数 (SI) 如表 1 图例所示。

[0218] 化合物对原代旱獭肝细胞中 WHV cccDNA 转录的作用。旱獭肝炎病毒 (WHV) 是评价 HBV 治疗剂的有用模型。因此，为了计划了解所述先导化合物是否对 WHV 有活性是有用的。通过在 50-90％的肝细胞含有 WHV 的条件下接种胶原酶处理的组织（来源于从慢性感染的旱獭获得的小切片活检组织）制备原代旱獭肝细胞培养物 (PWHC)，并且所述培养物 (90％或更高）是肝细胞并如先前完成的那样可在培养基中维持至少两个月（参见 Fletcher SP 等，2012.Transcriptomic analysis of the woodchuck model of chronic hepatitis B.Hepatology:In press)。在接种 7 天内，在测试化合物不存在或存在下孵育培养物，并且使用与先前使用和公开类似的方法测定培养基中 (WHV 病毒粒子相关 DNA ;WH) 和细胞内 (WHV DNA,WHV RNA 转录物)WHV 基因产物的量 (Guo,H. 等，2010.Production and function of the cytoplasmic deproteinized relaxed circular DNA of hepadnaviruses.J Virol 84:387-396 ;Guo, 等，2011.Alkylated porphyrins have broad antiviral activity against hepadnaviruses, flaviviruses, filoviruses, and arenaviruses.Antimicrob Agents Chemother 55:478-486)。

[0219] 如果在与禽和人系统中的 SI 类似的 SI 下 WHV 对阿匹西定和其它候选 cccDNA 抑制剂敏感（正如怀疑的那样），那么可使用慢性感染的旱獭作为功效研究的体内证据。

[0220] 所述抑制剂是通过以下方面分级的(i)其选择性指数(SI),其中最大的选择性在于抑制HBV cccDNA转录相对细胞生存力,随后是细胞功能,是最有吸引力的;(ii)其抑制cccDNA转录的效力(最低EC50),和最后是(iii)“关键化学”(可缩放性类型/配方)问题。具有最低EC_{50}值(抑制50%cccDNA转录的RNA的浓度)和最大SI的化合物是最有吸引力的。

[0221] HDAC同工型的鉴定

[0222] 在初次筛选中鉴定的各种化合物都有HDAC抑制活性的性质(参见,如,表1)。HDAC抑制很可能是这些化合物的HBV抗病毒作用机制的一部分或核心。虽然没有必要精确地了解所述化合物的机制,但是这种信息对于选择或设计修饰的化合物以及预测和降低可能的体内毒性和设计临床研究是有益的。此外,因为对于许多HDAC来说晶体结构是可得到的,所以可以帮助将来的药物设计。总地来说,HDAC抑制剂在研究中和在人体中不断增加的经验能够为目前的临床设计和将来的计划提供指导。

[0223] 根据功能和DNA序列同源性,HDAC去乙酰化多肽(即,组蛋白)可分为四种类型。I类和II类HDAC可被曲古抑菌素A(TSA)抑制。阿匹西定有效地抑制I类HDAC,但是不抑制II类HDAC。III类HDAC(称为长寿蛋白)是NAD+依赖性蛋白的家族,不受TSA影响。根据DNA序列,IV类被认为是非典型的类型。因为阿匹西定和TSA有效地抑制cccDNA转录,I类和II类中的HDAC同工酶是最相关的。然而,因为阿匹西定只抑制I类,所以最初只关注这一类。

[0224] 实验细节:用shRNA使HDAC同工酶转录物沉默以及对HBV cccDNA功能的影响。从慢病毒转导载体表达的短发夹RNA(shRNA)现在是抑制与shRNA同源的转录物的翻译的标准工具。焦点放在I类HDAC同工酶上。因此,在转导混合物中用100ul慢病毒(约$5x10^7$/ml)转导以cccDNA依赖性方式(如上文所述,tet抑制之后)表达HBV基因产物的HepDE19细胞的融合单层,从而在至少95%的所述细胞接受并且表达shRNA的条件下表达对于I类-1、2、3或8同工酶有选择性的shRNA。这是通过从逆转录病毒转基因表达报告基因确定的。shRNA慢病毒表达载体是由供应商作为准备好转导提供的,并且每个载体靶向不同的亚I类的HDAC。它们购自供应商(如,Santa Cruz Bio,OpenBio),并且含有并且表达具有与要靶向的每个HDAC同工酶转录物同源的19-25nt的短发夹。例如,一种HDAC 1特异性shRNA含有5′ -GAT CCC CGC AGA…ATC TGC TTT TTG GAA A-3′,并且其它的是类似设计的但是对于其它shRNA有特异性,如由供应商以及先前的研究提供的那样。对于每种HDAC同工酶有4和5倍的覆盖。对照载体含有扰码序列(scrambled sequence),并且用作阴性对照。shRNA慢病毒转导5天之后,通过RNA分析和蛋白印迹定量特异性HDAC的抑制(用由供应商提供的HDAC特异性探针和单克隆抗体),并且如同前述描述中进行的那样测量HBV cccDNA和cccDNA依赖性转录物以及HBeAg的量。在瞬时转导手段不能令人满意时,虽然涉及得多一点,但使用稳定转导,因为使用具有可选择标志物的shRNA构建体。如上所述,还作为特异性对照定量了细胞基因表达(A1AT和白蛋白mRNA)的量。阳性对照包括将HepDE19细胞于1000nM的阿匹西定孵育,这个浓度抑制HBV cccDNA(并且将已在HepDE19上验证)。不同HDAC的每个都与特定细胞功能(即1,p53的上调,2&3p21、8,H4的去乙酰化)有关,所述细胞功能是作为成功抑制HDAC亚类的证据进行定量的,那应该是期望的。

[0225] 鉴于阿匹西定的效力,期望使至少一种I类HDAC沉默将导致HBV cccDNA功能的

显著抑制。据了解，作用于多种 HDAC 的 HDAC 抑制剂可能比通过单一 HDAC 转录物敲低可实现的更佳的作用。然而，我们还使用覆盖所有 I 类酶的慢病毒组合进行敲低实验，因为如果涉及 I 类酶（正如阿匹西定显示的）这应该抑制 HBV cccDNA，但是为了检测 HBV cccDNA 抑制必须抑制多种酶。

[0226] 在使特定的或一组 HDAC 转录物沉默导致 HBV cccDNA 功能抑制时，这作为 HBV 抗病毒作用的靶标被验证，并且证实所鉴定的化合物的抗 HBV cccDNA 作用的机制涉及 HDAC 抑制这一发现。所述化合物当然可以使用 HBV cccDNA 抑制的其它机制，但是至少要知道 HDAC 抑制确实抑制 HBV cccDNA，这类新的 HBV 治疗策略的大门现在打开了。

[0227] 确定哪种经鉴定的化合物对负责 HBV cccDNA 抑制的 HDAC 同工酶具有最大的抑制作用。已经鉴定了负责调控 HBV cccDNA 的特异性 HDAC 同工酶后，这有助于鉴定对于抑制 HBV cccDNA 调控同工酶具有最大选择性的化合物。这允许推进具有最大选择性的化合物并且帮助避免由不必要抑制调控 HBV cccDNA 中没有涉及的 HDAC 同工酶产生的脱靶作用。我们注意到在表 1 中指出的四种化合物中，阿匹西定具有最大的选择性指数，并且还具有最窄的 HDAC 抑制曲线（对 I 类 HDAC 有选择性）。因此，通过避免宽的 HDAC 抑制剂并且接近足以抑制 HBV cccDNA 的特异性 HDAC 亚同工酶可能达到甚至更佳的选择性。

[0228] 各种 I 类 HDAC(1、2、3、8) 同工酶的酶测定都可作为商业化试剂盒得到，并带有阳性和阴性竞争性抑制剂对照。试剂盒是对应于如以上确定的相关同工酶从 BioTeK，BPS Bioscience 或其它可利用的来源购买的。简单地说，使用 BioTek 系统，提供了亚类特异性纯化的 HDAC 酶（重组，以约 10-50ng/ 容器），带有用预混合的反应中的显色剂在去乙酰化之后检测的荧光底物。购买的是通过沉默证明在 HBV cccDNA 抑制中涉及的酶。将不同量的对照或各种实验化合物与酶反应混合物一起孵育。

[0229] 针对同时作为时间和酶浓度的函数的线性对测定读数进行优化。来自抑制 HDAC 同工型的 50％去乙酰化酶活性需要的测试化合物的浓度（即 IC50）的试剂盒是通过使用 SigmaPlot 软件 (Systat Software，Inc.，San Jose，CA) 进行回归分析计算的。

[0230] 理论上，以及最逻辑地讲，根据上述程序发现有活性的化合物对发现的大多数涉及 HBV cccDNA 调控的 HDAC 有活性，并且这些代表偏好的化合物。有活性但是广泛地抑制 HDAC 的化合物（发现其中一些与 HBV cccDNA 调控无关）是有些不太受偏好的，因为它们可能产生不必要的副作用。其中，另一方面，有一个脱节，并且在前述试验中有活性的化合物不抑制发现的对 HBV cccDNA 调控最重要的 HDAC，根据 HBV cccDNA 抑制活性而非 HDAC 抑制分级，所述化合物是高级 (advanced) 的。

[0231] 先导化合物体外吸收、分布、代谢和毒性 (ADMET) 性质的评价产生 HBV 的细胞和不产生 HBV 的细胞

[0232] 介绍和原理体内实验是昂贵的并且受伦理学限制。因此，在动物中进行测试之前，需谨慎开始（尽可能）表征预示体内表现的化合物潜在毒性和其它细胞－血清－相互作用的性质。这些研究已经成为本领域中的标准。在复制细胞中的毒性也已经发现是就毒性对化合物分级的一个好方法。最后，在推进到体内研究之前，通常还需要不同的配方，因为在组织培养环境中使用的溶剂并不总是与体内施用相容。如下使用它们。除了常规的 ADMET 之外，目前提出了体外“ADMET”中的一个革新，其中所述表征是在目前批准的抗病毒疗法存在下用产生 HBV 的细胞进行的。

[0233] 可能的是，新的抗 HBV 药物将与目前正在使用的其它 HBV 抗病毒药物组合使用。组合治疗对于 HIV 和 HCV 以及其它感染性疾病是标准的。了解治疗 HBV 的新药在当前的护理标准存在下是否具有毒性或其它改变的性质是重要的，因为有证据说明许多另外良好耐受的药物在慢性感染个体中有选择性毒性。HBV 产生细胞可能比非产生性细胞对一些药物 (emdications) 更敏感（阻断，进行中）。因此，以下毒性实验是在不存在和存在 HBV 聚合酶抑制剂以及（在一些情况下）干扰素 α (IFNa) 下进行的。

[0234] 一些本发明先导化合物可能已经在动物中使用（通过其它人），可能有相当多的可用信息。另一方面，一些先导化合物可能是没有动物数据的新型化合物。由于上述原因，还在 HBV 感染的情况下研究了化合物的性质。

[0235] 最后，测试了抑制野生型HBV cccDNA功能并且在体外良好耐受的化合物抑制来自对 HBV 聚合酶抑制剂耐受的 HBV 的 cccDNA 的能力。根据前述研究的结果，使用人和 / 或鸭 HBV 转染（并且对于鸭来说是感染）系统。

[0236] 对于以下描述的每个实验，包括了具有已知毒性、代谢、蛋白质渗透性、膜转运和确定的配方性质的对照。例如，包括了 Barraclude 和 FIAU 分别作为在产生 HBV 的细胞中没有可检测毒性的化合物以及具有可检测毒性化合物的对照，并且具有可以产生比较的报道的 PK 和 TK 性质。

[0237] 实验细节 :体外“施用、分布、代谢、“消除”和毒性”(ADMET) 研究。这些实验中的一些是由供应商（即 Absorption Systems) 根据合同进行的，而其它的实验（特别是使用产生 HBV 的细胞和材料时）是由以下指出的本发明人进行的。

[0238] 标准细胞毒性测定 :以每孔 2x 10^4个细胞的密度将人肝细胞瘤 (HepG2、Huh7、HepRG) 和 HepG2 来源的细胞系支撑组成型 (HepG2.2.15) 和四环素诱导型 HBV 复制 (HepDE19 和 HepDES19) 接种到 96 孔板中。用测试化合物的连续稀释物处理细胞。每隔一天换一次培养基。处理开始后在第 2、4、6、8 和 10 天进行 MTT 测定。

[0239] 对增殖细胞的毒性 :将不同浓度的先导化合物与以低密度 (32mm 培养皿，每孔 100 个细胞）接种的 HepRG 细胞在产生 HBV 和不产生 HBV 的条件下一起孵育并且培养 10 天，每 3 天换一次培养基。

[0240] 在人和小鼠肝微粒体中的代谢稳定性 :在 NADPH 的存在下将所述化合物与来自产生 HBV 和不产生 HBV 的细胞（如上所述的组织培养来源）的人和小鼠肝微粒体一起孵育。此外，在人模拟胃液和模拟肠液的存在下评价化合物的稳定性。这套实验的目的也是确定所述化合物是否被消化酶代谢。因为寻求口服可利用的化合物，找出在 GI 道中可能产生什么代谢物（如果有）是重要的。

[0241] 毒性和代谢稳定性研究是在不存在和存在浓度等于和几倍于通常在人体中达到的血清水平（约 0.1ug/ml，对于 barraclude，-10ug/ml，对于拉米夫定）的拉米夫定、barraclude、替比夫定、替诺福韦和 / 或阿德福韦或干扰素 α (IFNa) 下进行的。如在上述测定中确定的，cccDNA 抑制性测试化合物是以其 IC50 的 10 倍使用的。每个小组中也包括（具有确定毒性和确定代谢性质）的对照化合物（即 FIAU、他汀类等）。

[0242] 血浆蛋白结合 :在这个测定中使用平衡透析确定结合人血浆蛋白的化合物的百分比（通过供应商）。

[0243] 双向渗透性 :这个测定用来确定化合物以顶端到底外侧和底外侧到顶端方向通过

Caco-2 细胞单层的渗透性（承包商）。

[0244] 在干扰素 (IFN) 存在下先导化合物的抗病毒活性。以上实验研究先导化合物与聚合酶抑制剂或干扰素在未感染细胞中组合使用时的体外 ADMET。确定先导化合物是否对确定的抗病毒剂的抗病毒性质有影响是同样重要的。与 pol 抑制剂相比，IFNα (a) 较少用来处理 HBV。在使用时，不同于 pol 抑制剂可使用几年并且更可能与 cccDNA 抑制剂共同施用，它只持续几个月的时间。然而，鉴于抗病毒作用和毒性的 IFNa 机制可能涉及 HDAC 这一事实，就这可以体外评价而言，评价目前公开的 cccDNA 抑制剂的与 IFNa 相互作用的性质确实是有意义的。因此，在不存在和存在不同浓度候选药物 cccDNA 抑制剂以及不存在和存在一定量的已知体外抑制 HBV 的禽 IFN 或人 IFNa 的情况下，孵育在克隆密度（用于生长研究）和半融合（用于抗病毒 /cccDNA 转录研究）下接种的计划从 HBV cccDNA 产生转录物的 dSTET 细胞和 AD38 细胞（如在初步证据和 Cai 2012 中的）。细胞生存力和产生的 HBV cccDNA 来源基因产物（转录物）的量是按照先前描述的程序和文献中已知的那些确定的。

[0245] 还测试了所述化合物在目前使用的聚合酶抑制剂存在下的体外活性。在慢性感染的管理中，对 HBV 聚合酶的核苷 / 核苷酸抑制剂耐受的突变型病毒的出现确实是个问题，尽管所述问题随使用的聚合酶抑制剂而不同。因此，测试了抑制野生型 HBV cccDNA 功能的化合物抑制来自对 HBV 聚合酶抑制剂耐受的 HBV 的 cccDNA 的能力。需要的所有突变型病毒 (DHBV 和 WHV) 都是可得到的。使用人和 / 或鸭 HBV 转染（并且对于鸭来说，是感染）系统。鉴于不同的作用机制，本发明化合物保留抗病毒活性。

[0246] 配方优化 :对于选择的化合物来说，评价了适合于经口灌胃的给药媒介物的开发。测试媒介物包括 1)pH 处理，2) 共溶剂（诸如甘氨酸 (glycin)、聚乙二醇丙二醇、乙醇等），3) 表面活性剂（诸如聚山梨醇酯、泊洛沙姆、聚氧乙烯蓖麻油 (polyoxyl castor oil)、甘油酯和 PEG 酯），4) 非水性系统（诸如芝麻油、中链甘油三酯、大豆油、油酸），5) 复合剂（诸如环糊精）。

[0247] 从 ADMET 角度来说，优选的化合物是就耐受性而论具有类似于 Barraclude 的性质的那些化合物。还优选的化合物是不存在 HBV 聚合酶抑制剂（拉米夫定、barraclude、干扰素等）时的毒性和代谢稳定性性质与其存在时相同的那些化合物。对产生 HBV 的细胞具有选择性毒性的化合物是不受欢迎、不合格或特别谨慎推进的。已经增强或增强当前 HBV 抗病毒剂毒性或拮抗所述抗病毒剂、活化那些化合物的化合物仍然被推进，但是应谨慎并且在体内实验中测试以组合形式增强毒性的可能性。可能建议 cccDNA 活性化合物不组合使用（或只是谨慎使用）。

[0248] 将具有良好体外性质的先导化合物放大并且测试体内毒性、药代动力学 (PK) 和功效

[0249] 药代动力学、毒理动力学 (TK) 和剂量范围调查研究。在进行体内功效研究（其昂贵、受伦理学限制并且消耗大量化合物）之前，有必要确定候选药物在未感染动物中的体内最大耐受剂量 (MTD) 和药代动力学性质 (PK)。这允许鉴定出值得改进和建立适当的给药和施用途径的化合物。在慢性嗜肝 DNA 病毒感染的鸭和 / 或旱獭模型之一（或两者）中测试化合物的功效，因为它们是成熟和预测性的动物模型。鸭对比旱獭的原理如下所述。对于阿匹西定本身，关于其在动物中的 PK/TK，已经有很多是已知的，因为它已经用于小鼠中。然而，即使对于阿匹西定，并且当然对于本发明的任何其它化合物而言，仍需要鸭和旱獭的

新 PK、TK 研究。因此，如下进行了一系列鼠科动物和大鼠的 PK 和 TK 研究。

[0250] 实验细节—在小鼠、鸭和（如果指出）旱獭中的单剂量药代动力学研究。这个研究的目的是获得分布容积、全身清除率、半衰期 (T1/2)、最大血浆浓度 (Cmax) 和生物利用度。这些参数用来评价每种亚氨基糖的清除率和生物利用度以便可以通过其保持血浆浓度的能力分级。一般来讲，对于要推进的化合物来说大于 50％的生物利用度是优选的。

[0251] 如上所述，借助于静脉内注射 (5mg/kg) 或口服 (25mg/kg) 向小鼠 (6 周龄 Balb/c ；6 只小鼠 / 组）；北京鸭 (6 周龄）或旱獭（每组 3 只）施用候选药物。给药之后在几个时间间隔记录临床观察。在给药前和在给药后 5、15 和 30 分钟、1、2、4、6、8、16 和 24 小时采集用于药代动力学的血液和尿液样品。分析样品中施用的药物（药物或前药）的存在和量，并且在施用前药的情况下，也分析“药物代谢物”的存在和量。通过 Absorption Systems 分析样品，所述 Absorption Systems 具有为我们的其它化合物建立的小鼠血浆分析。

[0252] 组织分布（鼠科动物）。在施用之后的各种时间从接受单次口服或静脉内施用化合物的小鼠（每个剂量组 3 只）获取组织。对化合物组织分布的认识可以显著有助于评价作为成功药物候选物的潜力。虽然其它体外参数诸如血浆蛋白结合和分布容积对于向血管外组织的分布速率和程度有预测值，但是药物的肝组织浓度可能是与功效最有关的。与血清、肾脏和腹部脂肪组织 / 淋巴结相比，通过腹膜内和口服途径在小鼠中单次施用所述化合物之后，焦点保持在肝脏的候选物的组织浓度（使用终点样品）的上。一个感兴趣的地方是活性化合物是否在关键组织中积聚，这就其在组织中的有效半衰期提供洞察力。也就是说，虽然药物的血清半衰期可能是约 2 小时，但是它可以在肝脏中具有数倍时间的组织半衰期，这解释了大于预期功效（对于给定的给药方案）或大于预期毒性。

[0253] 剂量调查最大耐受剂量 (MTD) 研究。因为在鼠科动物模型中评价所述化合物的抗病毒活性，所以了解所述化合物在小鼠中的耐受性是重要的。通过经口灌胃（因为我们寻求口服可利用的化合物）单独的“媒介物”或化合物已经溶解其中的媒介物对 Balb/c 小鼠 (6 周龄，6 只每组）；鸭 (6 周龄，3 只每组）给药。根据先前的经验，施用化合物的范围可能在 100mg/kg 至 500mg/kg 之间，每个剂量组 5 只小鼠。对动物观察多达 14 天，每天读取重量和存活性终点。进行常规组织学和临床化学研究。不导致任何死亡 / 毒性的化合物的最高剂量被认为是 MTD。旱獭不能用于这个 MTD 研究；结合 PK 旱獭研究对鼠科动物研究外推将是必要的。

[0254] 根据化合物的口服生物利用度、耐受性和半衰期对化合物分级。理想的化合物用可溶、口服、单日给药能够达到并且维持至少 10 倍组织培养物 IC50 浓度的血清或肝脏组织水平，并且具有大于组织培养物 IC50100 倍的 MTD。根据这些品质将化合物分级，并且最好和次好的将被推进。

[0255] 先导化合物在慢性感染动物模型（体内）中有效吗？已经证明体外功效并且确定安全和体内研究的给药原理之后，了解所述先导化合物是否能够在验证的慢性 HBV 动物模型中控制病毒水平是重要的。这表示靶向 cccDNA 的小分子药物将第一次在动物中进行测试。与安全、选择性和 cccDNA 靶向药剂一致的结果是感兴趣的。功效终点包括：将提示 cccDNA 抑制的病毒血症、抗原血症以及肝脏内 cccDNA 和复制形式的量的快速和协同降低。这些目标决定了使用的动物模型和研究的治疗时长。

[0256] 现有慢性 HBV 感染的若干动物模型，并且每种都有其优点和缺点。鸭和旱獭分别

可在实验上慢性感染鸭和旱獭嗜肝 DNA 病毒。现在有若干鼠科动物模型，但是因为带有 HBV 转基因的转基因小鼠不从 cccDNA 模板产生 HBV 以测试 cccDNA 靶向化合物，所以具有人肝细胞的嵌合小鼠是必要的，诸如 uPA 小鼠。实际考虑需要做出选择。实验被设计成在慢性 HBV 鸭模型中评价，因为已经知道所述化合物对培养中的禽细胞中的鸭病毒是有活性的。还准备了慢性感染的旱獭中的研究，因为这是对于测试 HBV 治疗剂的成熟模型并且是自然感染。uPA 小鼠是非常昂贵的，但是如果旱獭对药物不敏感而人 HBV 敏感就要使用。

[0257] 因此，将优选的化合物放大到必需的量并且如以下定义在以下鸭和旱獭（如果适当）中测试功效。

[0258] 实验细节 - 优选化合物的放大制备。阿匹西定是在镰刀菌属（即种 ATCC 74322）的发酵中产生的。将菌株接种到被称为 MED5 的营养培养基中，在控制湿度的气氛中以 220rpm 振摇 12-16 天。收获时，用甲乙酮提取全肉汤并且在 Sephadex 上通过凝胶分级分离提取物，随后通过 RP-HPLC 最终纯化分级分离提取物。产率约为 250mg/L，因此放大到克量是常规的。

[0259] 鸭嗜肝 DNA 病毒功效研究。因为已知阿匹西定对 DHBV 具有高活性，所以，在培养中，在慢性感染的鸭中对其进行测试。这个研究的目标是确定优选化合物的抗病毒潜力。血清学和组织学是次要的。

[0260] 使用六周龄慢性感染 16 型 DHBV(Alberta 菌株）的北京鸭。在 6 周时，鸭中的病毒血症和肝脏肿块倾向于已经稳定。通过肌肉内或经口灌胃（取决于 Aim 4PK/TK 结果）给予鸭测试化合物 (3 个剂量组，给药量和频率取决于 PK 结果，但是目的在于达到至少 10 倍组织培养物 IC50 的稳定血清水平）。有三个剂量组，每个剂量组 5-6 只动物。对照剂量组 (6 只动物每组）包括安慰剂治疗动物和每天用 barraclude(1mg/kg) 或拉米夫定 (40mg/kg) 治疗的动物。所有剂量组中的至少三个动物参加了至少一个治疗前和一个治疗后肝脏活检。治疗持续 10 周，因为这超过了拉米夫定抑制病毒血症至可检测水平之下的时间以及 cccDNA 在鸭中报告的 1/2 寿命。撤回药物之后再每周收集鸭血清持续 4 周。血清将在每周采集。

[0261] 测试每周血清的标准“实验室值”（血液学、白蛋白、AST、ALT)。确定了循环中的 DHBV 病毒 DNA、sAg、sAb 的量。检查了来源于活检（来自相同动物的治疗前和治疗结束的一些活检）的肝脏组织的 DHBV DNA(cccDNA，复制形式）和 DHBV 核（免疫染色）。

[0262] WHV 感染的旱獭研究。所述研究使用 10 组，每组 5 只动物，药物治疗持续 10 周，随后 10 周停止药物（为了测试影响的持久性）。由于在病毒血症和抗原血症水平上的变异性，通过如研究开始之前七天所确定的 WHV 病毒血症和抗原血症水平将动物分层到组中，以便两种病毒标志物的平均水平均匀分布在所有组的动物中。在这个研究中不使用 WHVsAg 水平异常低的动物。通过在啮齿类动物中进行生物利用度研究之后最后确定的途径和频率每天施用化合物。研究中给药第一天是研究第 1 天。研究第 1 天剂量水平是根据预先测试的体重计算的，并且每周获取体重以用于剂量施用。给药范围按照四种剂量的小鼠研究，组 10 用作为参比化合物（坦能 (Tennant)）的 Barraclude 处理。

[0263] 主要终点是病毒血症和抗原血症给药和停药时获得持久的停药降低的剂量依赖性降低。

[0264] 生存力和动物健康。每天进行并记录一次发病率和死亡率的临床观察。此外经由

血液学、血清化学和组织学检查研究毒理学。为了确保病毒血症或抗原血症的降低或其它假定有益的结果不是次要的推论方案(secondary consequence protocol)(化合物)毒性,在一般动物健康的情况下考察所有生化和免疫终点是同样重要的。在每周的基础上确定总的身体特性(体重、屎尿量和特征)。此外,为了评价毒性和功效,还对肝功能测试(对按月采集的样品进行)、血液学和化学(对治疗前、中和结束的样品进行)(如表中所述)进行评价,以及对于选择的动物(在给药前、给药中和治疗结束时),还进行了源自穿孔活检的肝脏切片的组织学分析。

[0265] 肝功能测试作为肝脏生存力的标志在每月一次的样品中由商业服务确定

[0266] 体液响应性的证据。通过 ELISA 确定识别 WHsAg 的抗体的存在。即使 WHs Abs 与抗原复合这种分析法也能检测。

[0267] 毒理学。如对小鼠研究所述,经由血液学和血清化学仔细研究毒理学。此外,进行肝脏穿孔活检的组织学检查,包括炎症、胆管增生以及门脉肝炎和小叶肝炎。

[0268] 血清中的 WHV 病毒水平。每周进行评价(条形斑点杂交和 PCR)或每两月进行评价(DNA 印迹)。

[0269] 活检。在治疗开始前、中间、结束和研究结束时采集肝脏活检组织并且用于组织学和细胞内 WHV DNA 检查。基于 Hirt 提取定量地测定了复制形式和肝内共价闭环 WHV DNA(WHV cccDNA)的水平。对于免疫染色来说,使用分离的组织并且确定治疗对比未治疗动物中的核和 WHsAg 的积累。

[0270] 对于鸭和旱獭研究来说,预计没有技术难度,因为这些研究是相当常规的,评价需要的所有方法和试剂都是现成的。有关鸭的一个可能的问题是没有药物时出现的病毒血症/抗原血症的变化。这通过使用其中的病毒学通常稳定化了的 6 周龄之后的鸭来减轻。

[0271] 阳性活性的基准是 LFMAU 治疗的动物。相对于预治疗和未治疗的组,预期这些动物通过在鸭和旱獭中分别进行 3 周和 10 周的治疗一致地丧失 HBV 病毒血症并且甚至是抗原血症,其中感染 HBV 的肝细胞的数量大大降低。

[0272] cccDNA 转录(和稳定性)的抑制应该降低所有病毒基因产物(以受其血清半衰期影响的速率)的胞内和胞外的量,甚至在 HBV 感染的细胞数量降低之前(并且也许,和 HBV 感染的细胞损失的数量不成比例)。实际上,我们的新型化合物功效的最清晰的证据是 HBV DNA 病毒血症和 sAg 抗原血症中的时间和剂量依赖性静态显著的降低。鉴于本发明化合物的体外功效,预期在模型之一或两者中有至少十倍的血清表面抗原降低。

[0273] 还测量了 DHBsAgWHsAb 水平。对照(慢性感染的动物)预期没有可检测的(或极少可检测的)Ag。慢性感染的人(和旱獭)有能力并且确实产生 sAb 的证据在不断增加,但是它受循环的 sAg 抑制或与循环的 sAg 结合。因此如果 Ag 下降并且随着 Ag 下降,sAb 表露出自身是可能的。

[0274] 在治疗前后,使用固定的肝脏组织对免疫染色的 HBV 核(sAg)进行活检分析,并且用提取物检测 HBV 核酸的量。理论上,感染细胞的数量将随着药物治疗起作用而下降。有用信息包括这是否出现在肝炎(细胞浸润)增加的情形中。

[0275] 在药物治疗已经停止之后还评价了来自 10 和 4 周(分别是旱獭和鸭)的动物血清。为了达到所有主要目的,考虑了在 sAb 出现的情况下抗原血症、病毒血症的稳定停药抑制。在不存在任何有害反应或事件的情况下,用超过安慰剂的量的药物抑制病毒血症和抗

原血症被认为是药物特异性影响的证据。

[0276] 以上列出的动物研究允许就所述化合物是否在体内环境中有效降低抗原血症做出明确的结论。

[0277] 在确定 HDAC 的抑制是抑制 HBV cccDNA 转录时，结果出人意料的有用，因为 HDAC 抑制通常与基因活化有关，包括整合到宿主染色体中的 HBV DNA。所述结果可能提供来自大部分细胞基因的HBV cccDNA的调控有多么不同的一个实例，并且即使本文中鉴定的抑制剂最终没有在人类系统中使用，但证明了用药理学活性小化合物进行非催化性抑制 cccDNA 是可能的。

[0278] 总地来说，这个研究提供两个非常关键的答案。首先，它指出 HBV cccDNA 功能在人和旱獭培养物中的选择性抑制。其次，它确定哪种 HDAC（阿匹西定的靶标）调控 HBV cccDNA。我们理解在 HBV 感染的人中的 HDAC 抑制必须谨慎进行，而这个研究提供了关于如何用革命性的新治疗策略前进的方向。

[0279] 一般合成

[0280] 本发明的化合物可以使用以下一般方法和程序从易得的起始原料制备。应理解，除非另外说明，否则在给出典型的或合适的工艺条件（即，反应温度、时间、反应物的摩尔比、溶剂、压力等）的情况下，还可以使用其它工艺条件。最佳反应条件可根据所使用的特定反应物或溶剂变化，但这类条件可由本领域的技术人员通过常规优化程序来确定。

[0281] 本文中描述的工艺可以根据本领域已知的任何合适的方法监测。例如，可通过诸如核磁共振光谱法（如，^{1}H 或 ^{13}C NMR）、红外光谱法（IR）、分光光度法（如，UV- 可见）或质谱法的光谱手段，或通过诸如高效液相色谱法（HPLC）或薄层色谱法的色谱法来监测产物的形成。

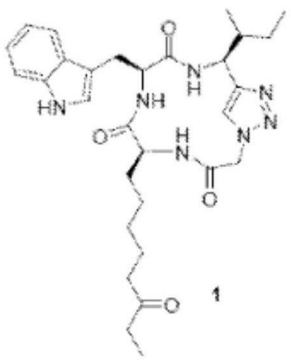
[0282] 化合物的制备可涉及各种化学基团的保护和脱保护。对保护和脱保护的需求和合适的保护基的选择可以由本领域技术人员容易地确定。保护基的化学性质例如可以在 P.G.M.Wuts 和 T.Greene，Greene's Protective Groups in Organic Synthesis，第 4 版，Wiley&Sons，2006 中找到，其内容以引用方式全部并入本文。

[0283] 本文中描述的方法的反应可在合适的溶剂中进行，所述溶剂可由有机合成领域中的技术人员容易地选择。合适的溶剂可以在反应所进行的温度（即，可在溶剂的冷冻温度至溶剂的沸腾温度范围内的温度）下与起始原料（反应物）、中间体或产物基本上不反应。给定反应可在一种溶剂或多于一种溶剂的混合物中进行。取决于具体反应步骤，可针对具体反应步骤选择合适的溶剂。本发明化合物例如可以使用如下所述的反应路径和技术制备。

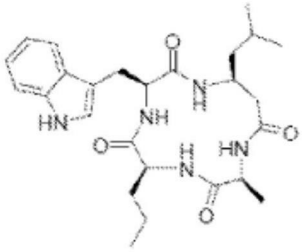
[0284] 化合物合成

[0285] 阿匹西定已经被衍生化并且最新的类似物 1 和 2

[0286]



1

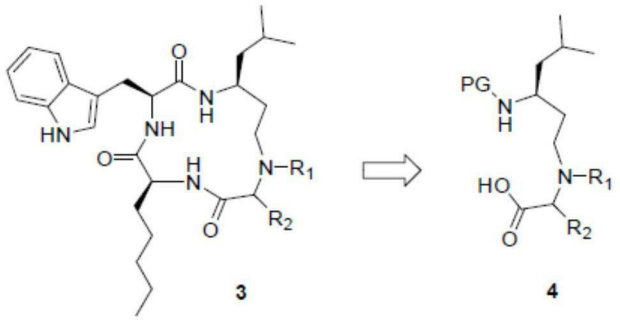


2

[0287] （参见 Horne，W.S.，C.A.Olsen，J.M.Beierle，A.Montero 和 M.R.Ghadiri.2009. Probing the bioactive conformation of an archetypal natural product HDAC inhibitor with conformationally homogeneous triazole-modified cyclic tetrapeptides.Angew Chem Int Ed Engl48:4718-4724；Vickers，C.J.，C.A.Olsen，L.J.Leman 和 M.R.Ghadiri.2012.Discovery of HDAC Inhibitors That Lack an Active Site Zn^{2+}-Binding Functional Group.ACS Medicinal Chemistry Letters）表明可以修饰阿匹西定结构而不丧失抗 HDAC 效力。

[0288] 制备了其它类似物，着重于相对于具有非常差的水溶性、口服生物利用度和体内半衰期的阿匹西定改善了药物性质。尤其通过标准固相和溶液相方法制备了阿匹西定衍生物。在某些实施方案中，源自还原的 β－异亮氨酸氨基酸的片段 4

[0289]



3 4

[0290] 是以适当保护的形式（PG ＝合适的保护基，诸如 Fmoc 或 Boc）通过溶液相方法制备的，并且通过溶液相或固相方式引入至所述氨基酸序列中，随后使用建立的方法环化制备的。

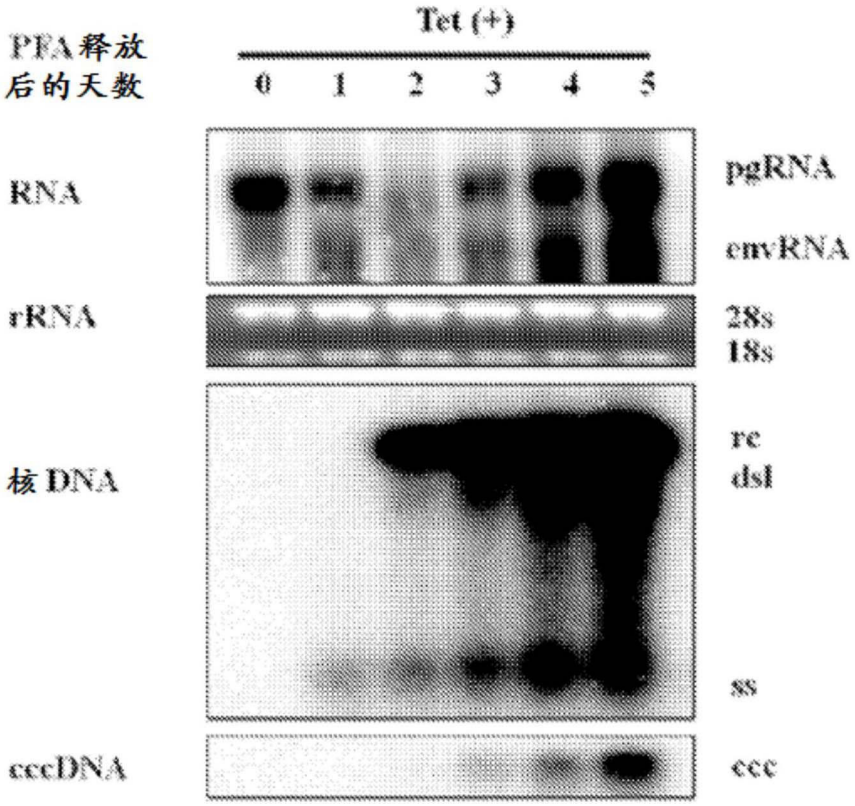

Tet (+)
PFA释放
后的天数
0 1 2 3 4 5
RNA
pgRNA
cmvRNA
rRNA
28s
18s
核 DNA
rc
dsl
ss
cccDNA
ccc

图 1

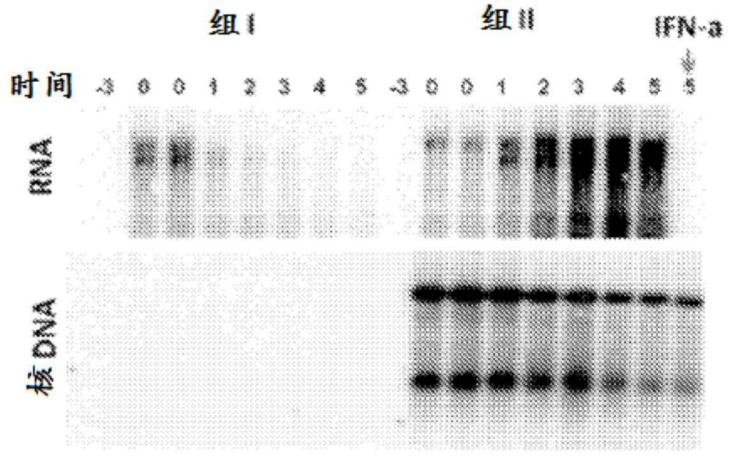

组 I
组 II
IFN-a
时间
-3 0 0 1 2 3 4 5 -3 0 0 1 2 3 4 5 5
RNA
核 DNA

图 2

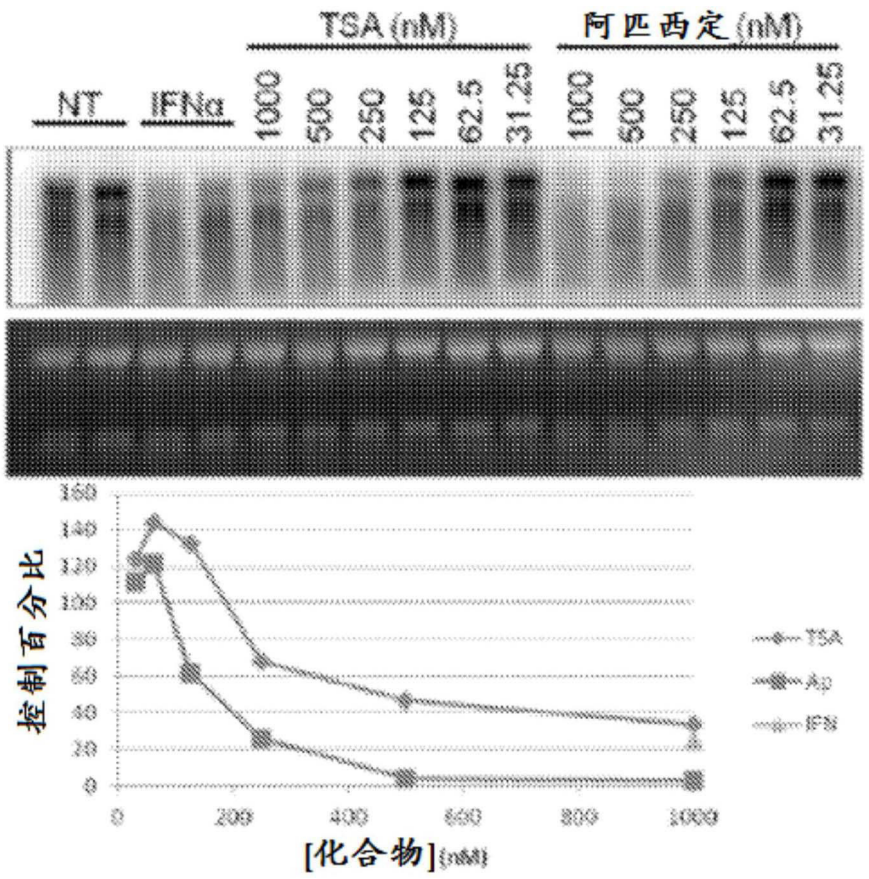

TSA (nM)
阿匹西定 (nM)
NT
IFNα
1000
500
250
125
62.5
31.25
控制百分比
[化合物] (nM)
TSA
Ap
IFN

图 3

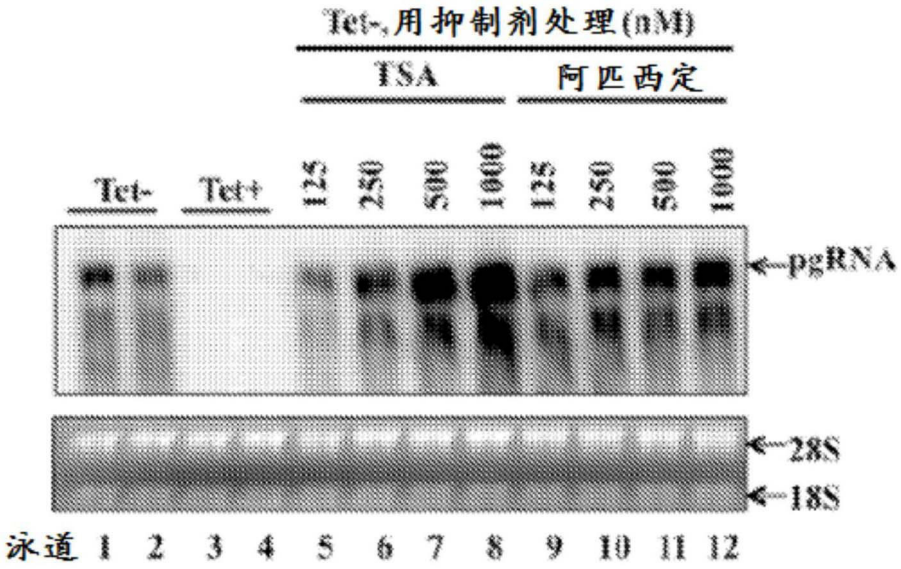

Tet-,用抑制剂处理 (nM)
TSA
阿匹西定
Tet-
Tet+
125
250
500
1000
pgRNA
28S
18S
泳道 1 2 3 4 5 6 7 8 9 10 11 12

图 4

Abstract

The present disclosure pertains to the discovery of a new family of "first in class" small molecular inhibitors of hepatitis B virus (HBV) covalently closed circular (ccc) DNA for use as therapeutics in the management of chronic HBV. Provided are agents that provide epigenetic modification of the cccDNA, histone modifying agent, and inhibitors of histone deacetylace activity that in turn modulate HBV cccDNA, which has never been achieved with a pharmacological agent. Also provided are methods for modulating HBV cccDNA, for treating or preventing HBV in a subject, and for modulating cccDNA transcription of hepatitis B in a subject.