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(54) Title: SMALL MOLECULE INHIBITION OF RNA/LIGAND BINDING

(57) Abstract

Disclosed is a method for the inhibition of binding of a ligand to an RNA, the inhibition being mediated by a small organic molecule which binds to the RNA, thereby inhibiting ligand binding. A preferred class of small organic molecules are the 2-deoxystreptamine (2-DOS) aminoglycosides. Disclosed herein are members of the 2-DOS class that are useful for the inhibition of binding of an RNA to a trans-activating protein.

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SMALL MOLECULE INHIBITION OF RNA/LIGAND BINDING

Background

Replication of human retroviruses, such as human immunodeficiency virus type 1 (HIV-1), entails an ordered pattern of viral gene expression. This regulated gene expression is dependent upon the sequence-specific interactions of two viral regulatory proteins, Tat and Rev, with their respective sites on viral RNA. Tat is a transcriptional activator, whereas Rev acts posttranscriptionally to increase the cytoplasmic accumulation of the viral *gag-pol* and *env* messenger RNAs (mRNAs).

Because Tat and Rev are critical for viral replication they are attractive targets for therapeutic intervention. To date, three strategies have been attempted to reduce the levels of these viral regulatory proteins or to block their action. First, antisense nucleic acids directed against Rev mRNA have been used to decrease the steady-state level of Rev protein. A second strategy is to sequester (titrate) the regulatory protein by the introduction of a large excess of small "decoy" RNA that contains a high affinity protein-binding site. For example, retroviral vectors expressing the Tat-binding site TAR can inhibit viral replication in cultured cells. A third approach is to express dominant negative mutants of the viral regulatory proteins. For example, Rev derivatives bearing mutations within a discrete C-terminal region abolish Rev activity, and these mutants can inhibit wild-type Rev in a cotransfection assay. A major difficulty inherent in these three strategies is the problem of delivering the therapeutic agent, which is either a nucleic acid or a protein.

Summary of the Invention

The present invention relates to the use of small organic molecules to inhibit the binding of a ligand to an RNA. In a preferred embodiment, the ligand is a trans-activating regulatory protein and the RNA is MRNA or pre-MRNA which contains a cis-acting sequence.

A preferred class of small organic molecules are the 2-deoxystreptamine (2-DOS) aminoglycosides. Disclosed herein are members of the 2-DOS class that are useful for the inhibition of binding of a trans-activating regulatory protein to a mRNA. The invention also relates to a variety of structural modifications which have been found to affect the ability of the 2-DOS aminoglycoside to inhibit binding of a ligand to an RNA.

Detailed Description of the Figures

Figure 1. Sisomycin Inhibits Binding of a Tat Peptide to the TAR RNA.

A) A 26-nucleotide ^{32}P-Labeled TAR RNA probe (+18 to +44) was incubated with a synthetic peptide containing amino acids 48-60 of the HIV-1 Tat protein (Calnan et al., *Genes Rev.*, 5:201-210 (1991)) in the absence (-) or presence of 250μM of the indicated antibiotic. The presence (+) of the Tat peptide is indicated above each lane. Control reactions are shown at the left. The Tat-TAR complex and unbound RNA probe are indicated by the arrows. Antibiotics which inhibit Tat binding are denoted by an asterisk (*).

B) The same TAR probe was incubated with decreasing concentrations (micromolar) of sisomycin in the presence (+) of the Tat peptide. The absence (-) of peptide or antibiotic is indicated above the lane. The Tat-TAR complex and unbound probe is indicated by the arrows.

Figure 2. Inhibition of the Tat-TAR Interaction by Sisomycin Is Specific

A) Sisomycin does not affect Rev-RRE Interactions. A ^{32}P-labeled RNA probe (Zapp et al., *Cell*, 74:969-978 (1993)) containing the high affinity Rev-binding site was incubated with purified *E. coli*-derived Rev protein in the presence of increasing concentrations of sisomycin. The concentration of antibiotic (micromolar) is indicated above each lane. Control reactions in the absence of antibiotic are shown at the left. The Rev-XRE complex and unbound probe are indicated by the arrows.

B) Inhibitory Aminoglycosides Do Not Affect PremRNA Splicing. A ^{32}P-labeled human β-globin pre-mRNA (Krainer et al., *Cell*, 36:993-1005 (1984) and Ruskin et al., *Cell*, 38:317-331 (1984)) was incubated in a HeLa nuclear extract in the absence (-) or presence (+) of several aminoglycosides. The concentration (micromolar) is shown at the left. The structure of the reaction products are indicated at the right. The control reaction is shown at the left.

Figure 3. Sisomycin Binds to a Discrete Region of the TAR RNA. Kethoxal modification/protection experiments. A 46-nucleotide RNA containing a high-affinity TAT binding site was incubated in the absence (-) or with increasing concentrations of sisomycin (10 μM, 100 μM, or 1 Mm) and treated with kethoxal as indicated. The reverse-transcribed RNAs were separated on a denaturing polyacrylamide gel. Nucleotide positions are as indicated to the left of the autoradiogram. Guanosines protected selectively by sisomycin are indicated by the asterisk (*).

Figure 4. Dihydrostreptomycin Inhibits Binding of the HTLV-1 Rex Protein to the XRE RNA. A 100-nucleotide ^{32}P-labeled RNA probe containing the high affinity Rex-binding site was incubated with purified *E. coli*-derived GST-Rex protein in the absence (-) or presence (+) of various aminoglycosides. The antibiotic tested is shown above each lane. The concentration (micromolar) or antibiotic is indicated at the left. The control reactions are shown at the left. The Rex-XRE complex and unbound probe are indicated by the arrows.

Figure 5. Inhibition of Rex-XRE Interaction is Specific. A 67-nucleotide ^{32}P-labeled RNA probe (Zapp et al., *Cell*, 74:969-978 (1989)) containing a high affinity Rev binding site was incubated with purified *E. coli*-derived Rev protein in the absence (-) or presence (+) of increasing concentrations of dihydrostreptomycin. The concentration of antibiotic is indicated above each lane. Control reactions are shown at the left. The Rex-XRE complex and unbound probe are indicated by the arrows.

Figure 6. 6DEE Inhibits Binding of a Tat Peptide to the TAR RNA.

A) A 26-nucleotide ^{32}P-labeled TAR RNA probe (+18 to +44) was incubated with a synthetic peptide containing amino acids 40-60 of the HIV-1 Tat protein in the absence (-) or presence (+) of 250 μM of the indicated compound. (Calnan et al., *Genes Rev.*, 5:201-210 (1991)). The presence (+) of the Tat peptide is indicated above each lane. Control reactions are shown at the left. The Tat-TAR complex and unbound probe are indicated by the arrows. The Tat binding reaction in the presence of 6DEE is indicated by the asterisk (*).

B) The same probe was incubated with decreasing concentrations (micromolar) of 6DEE in the presence (+) or absence (-) of the Tat peptide. The absence (-) of

peptide or 6DEE is indicated above the lane. That Tat-TAR complex and unbound probe is indicated by the arrows.

Figure 7. Inhibition of the Tat-TAR Interaction by 6DEE is Specific. A ^{32}P-labeled RNA probe (Zapp et al., *Cell*, 74:969-978 (1993)) containing the high affinity Rev-binding site was incubated with purified *E. coli*-derived Rev protein in the presence (+) of increasing concentrations of 6DEE. The concentration of 6DEE (micromolar) is shown in each lane. Control reactions in the absence of 6DEE are shown at the left. The Rev-RRE complex and unbound probe are indicated by the arrows.

Figure 8. The Inhibitory Compound 6DEE Does not Affect Pre-mRNA Splicing. A ^{32}P-labeled human β-globin pre-mRNA (Krainer et al., *Cell*, 36:993-1005 (1984) and Ruskin et al., *Cell*, 38:317-331 (1984)) was incubated in a HeLa nuclear extract in the absence (-) or presence (+) of 6DEE. The concentration (micromolar) is shown at the left. The structure of the reaction products are indicated at the right. The control reaction is shown at the left.

Detailed Description of the Invention

The present invention is based on the discovery that the specific binding of a ligand to an RNA can be inhibited by the presence of a small organic molecule. The small organic molecule binds to the RNA thereby inhibiting ligand binding to the RNA. The expression "small organic molecule", as used herein, refers to compounds other than peptides, oligonucleotides, or analogs thereof having a molecular weight of less than about 2,000 daltons. The RNA member of the RNA/ligand pair can be any ribonucleic acid polymer. The ligand member of the RNA/ligand pair is preferably a protein, nucleic acid, lipid or carbohydrate.

In one embodiment, the ligand member of the ligand/RNA pair is the HIV Rev protein. The Rev protein acts posttranscriptionally to facilitate the transport of *gag-pol* and *env* mRNAs from the nucleus to the cytoplasm of infected cells. Without Rev, these structural gene transcripts fail to accumulate in the cytoplasm, and the virus cannot replicate. A cis-acting element within Rev-responsive transcripts is required for activation by Rev. This element, termed the Rev-responsive element (RRE), has been mapped to a 234 nucleotide fragment (see Malim et al., *Nature* 338: 254 (1989)).

Rev binds specifically to the RRE in vitro. In chemical and RNAase protection experiments, binding of Rev primarily protects nucleotides within a 66 nucleotide fragment, referred to as domain II of the RRE. This fragment has been shown to be necessary and sufficient for high affinity Rev binding. Described in the Exemplification below are experiments in which small organic molecules were tested for their ability to block Rev binding to a labeled 67-nt high affinity Rev-binding site (see Bartel et al., *Cell* 67: 529 (1991)).

As discussed in detail below, successful inhibition of binding of Rev to the high affinity RRE was observed with a preferred class of small organic molecules, the 2-deoxystreptamine (2-DOS) aminoglycoside class. The 2-DOS class comprises a group of aminoglycosides that are characterized by the presence of a 2-deoxystreptamine nucleus joined by glycosidic linkages to one or more sugar moieties.

The experiments set forth in the Exemplification section below describe the testing of 13 members of the 2-DOS aminoglycoside class in a Rev/RRE binding inhibition assay. Specifically, the 2-DOS compounds which were tested were neamine, amikacin, sisomycin, ribostamycin, butirosin, kanamycin B, kanamycin A, tobramycin,

gentamycin, neomycin B, paromomycin 1, lividomycin A and hygromycin B. The chemical structure of each of these compounds, with the exception of lividomycin A, is provided in *The Merck Index* (Eleventh Edition, Budavari et al. eds., Rahway (1989)). The structure for lividomycin A is provided in the J. Berdi, CRC Handbook of "Antibiotic Compounds", Volume I (Carbohydrate Antibiotics), CRC Press Inc. Boca Raton, FL (1980).

The 2-DOS class of compounds can be divided into subclasses based on structural features. One method for subclassification is based upon the number of sugar moieties attached to the 2-deoxystreptamine nucleus. Another conventional method for subclassification is based upon the position of substituents on the 2-deoxystreptamine nucleus. The table below summarizes the nature of these structural features for each of the 2-DOS compounds tested.

Table 1

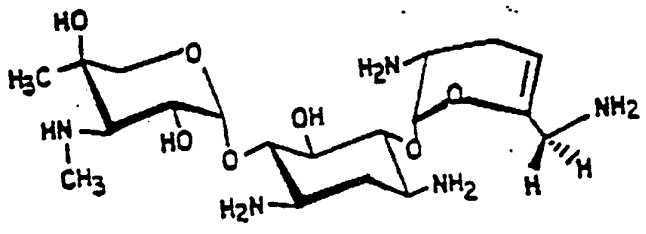
2-DOS Aminoglycoside	Position of 2-DOS Substituents	Number of Sugar Moieties
Neamine	4	1
Amikacin	4-6	2
Sisomycin	4-6	2
Ribostamycin	4-5	2
Butirosin	4-5	2
Kanamycin B	4-6	2
Kanamycin A	4-6	2
Tobramycin	4-6	2
Gentamycin	4-6	2
Hygromycin B	5	2
Neomycin B	4-5	3
Paromomycin 1	4-5	3
Lividomycin A	4-5	4

Surprisingly, of the 13 2-DOS aminoglycosides tested in the Rev/RRE binding inhibition assay, 8 were effective in inhibiting the binding of Rev to the RRE (neomycin B, neamine, ribostamycin, lividomycin A, kanamycin B, amikacin, gentamicin C, and tobramycin). Furthermore, three 2-DOS aminoglycosides (neomycin B, tobramycin, and lividomycin A) had exceptional inhibitory potential. These data clearly demonstrate that the 2-DOS aminoglycosides are characterized by a yet to be determined structural feature which results in this Rev/RRE binding inhibition. It appears as though the effective 2-DOS aminoglycosides contain the proper orientation of amino and hydroxyl groups to provide hydrogen bond donors and acceptors for the RNA bases and to provide positively charged amino groups to interact with the negatively charged phosphate groups of RNA.

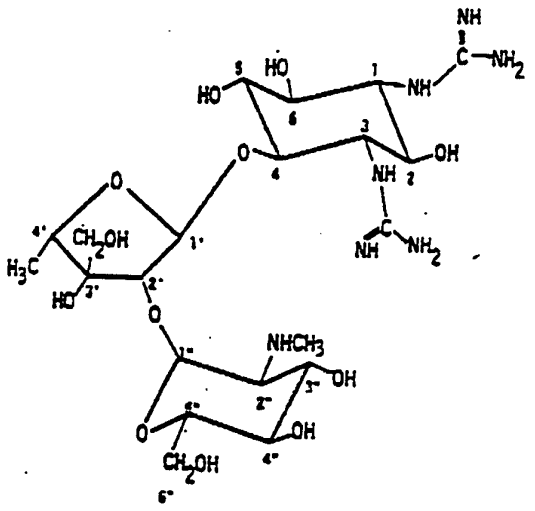
Therefore, this invention also relates to small organic molecules that are able to mimic this structural feature and thereby inhibit the Rev/RRE interaction.

In another embodiment, the ligand member of the ligand/RNA pair is the HIV Tat protein. The tat protein is known to enhance the expression of viral genes by acting at the level of transcription. It accomplishes this function through an arginine-rich RNA-binding motif which binds to the *trans*-acting responsive element (or TAR). The TAR is included in the mRNA transcript of every HIV gene.

Described in the Exemplification below are experiments in which 32 small organic molecules were tested for their ability to block Tat binding to a labeled 26 nucleotide TAT RNA probe (Calnan et al., *Genes Dev.* 5: 201-210 (1991)). As seen with Rev binding to RRE, the chemical properties of the 2-DOS compounds are essential for Tat/TAR binding. The aminoglycoside antibiotic sisomycin (Structure I) resulted in greater than 80% inhibition of Tat/TAR binding at concentrations of 50 μM. Ribostamycin, dihydrostreptomycin (Structure II), lincomycin and thiostrepton showed no inhibition. The chemical structure of each of these compounds is provided in The Merck Index (Eleventh Edition, Budavari et al., eds., Rahway (1989)).



Structure I



Structure II

In chemical and RNAase protection experiments described in the Exemplification, it was demonstrated that sisomycin inhibition of Tat/TAR interaction is highly specific. Also, sisomycin binding was located to a discrete region of TAR, wherein sisomycin distorts the TAR RNA structure.

In another embodiment, the ligand member is the Rex protein of the Human T-cell Leukemia Virus Type 1 (HTLV-1). Rex protein is a sequence-specific RNA binding protein that is essential for HTLV-1 replication. Rex function requires a cis-acting RNA element designated the Rex Responsive Element or "XRE", which is located in the 3′ portion of the U3 region. Analogous to the HIV-1 Rev protein, the HTLV-1 Rex protein acts post-transcriptionally to facilitate the nuclear-cytoplasmic redistribution of unspliced and singly spliced mRNAS that encode the viral structural proteins.

Described in the Exemplification below are experiments in which small organic molecules were tested for their ability to inhibit the binding of a glutathione-S-transferase (GST)-Rex fusion protein with a 100 nucleotide labeled RNA probe containing a high affinity

affinity Rex binding site (Ohta, et al., *J. Virol.*, 62: 4445-4451 (1988)). In particular, 32 aminoglycoside and nonaminoglycoside antibiotics were tested tested in this fashion. As with Rev and Tat protein, structural elements contained within the 2-DOS nucleus appear to be essential for ligand/RNA inhibition. Specifically, dihydrostreptomycin (Structure II) completely inhibited Rex binding at concentrations as low as 50 μM. Hygromycin B, lincomycin, kasugamycin, olivomycin and chromomycin A showed no inhibition up to 250 μM. The structure of each of these compounds with the exception of kasugamycin, is provided in The Merck Index (Eleventh Edition, Budavari et al., eds., Rahway (1989)). The structure of kasugamycin is provided in the J. Berdi, (RC Handbook of "Antibiotic Compounds", Volume I (Carbohydrate Antibiotics), CRC Press Inc. Boca Raton, FL (1980).

The binding of dihydrostreptomycin is specific for the Rex-XRE interaction. This was shown when concentration up to 100 μM were shown to be ineffective in inhibiting Rev/RRE binding. The specificity of dihydrostreptomycin for Rex-XRE was also demonstrated by RNAase protection experiments. Further details about these experiments are provided in the Exemplification below.

The method of inhibition described herein is useful in a variety of contexts. In an in vitro context the method of inhibition can be used, for example, as a positive control for Rev/RRE binding inhibition within an experiment designed to identify other compounds having this desirable property. As shown in the Exemplification section, the method of inhibition can be applied to treat HIV infected cells thereby resulting in the inhibition of replication of the HIV virus.

The 2-DOS aminoglycoside can be functionalized and further tested for enhancement of the desired properties

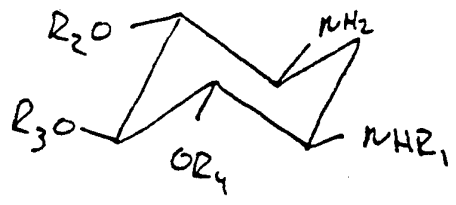
disclosed herein. The amine and hydroxy functionalities are preferred locations for derivatization. The functionalization of the 2-DOS aminoglycosides can serve to increase the affinity/specificity of the compounds for the RRE. The modifications can also serve to increase the membrane permeability and cellular uptake of the compounds. Derivitization with lipophilic moieties or groups that decrease basicity of the amines may have this desired effect. In a therapeutic context, the modified aminoglycosides may show decreased toxicity. It is also the case that the modified 2-DOS aminoglycosides may not themselves be inhibitors of the Rev RRE interaction and may be converted to the active inhibitors inside the cell.

In one embodiment, the amines of the 2-deoxystreptamine aminoglycoside with three attached sugars are functionalized with modifying groups that increase the molecular weight of the 2-DOS aminoglycoside by no more than 500 daltons. In another embodiment the amines of 4,5-disubstituted 2-deoxystreptamine aminoglycoside are also functionalized with modifying groups that increase the molecular weight of the 2-DOS aminoglycoside by no more than 500 daltons. In a further embodiment the amines of 2-deoxystreptamine aminoglycosides with one, two or four attached sugars and of 4-monosubstituted and 4,6-disubstituted 2-deoxystrepamine aminoglycoside are functionalized with modifying groups in which there is no molecular weight restriction. Common modifying groups which would satisfy this limitation include, for example, benzyl, substituted benzyl, N-*tert*-butoxycarbonyl, carbobenzyloxy, alkyl, substituted alkyl, acyl, benzoyl, substituted benzoyl alkanoyl or substituted alkanoyl. Preferably the modifying group is one which tends to increase the overall lipophilicity of the 2-DOS aminoglycoside.

In another embodiment, the amine modifying group is a methyl group provided that all amines in the 2-DOS aminoglycoside are either methyl substituted or primary amines. In this instance, the hydroxyl groups on the 2-DOS molecule may be non-substituted, partially substituted or completely substituted.

The hydroxy functionalities of the 2-DOS aminoglycosides can also be functionalized with modifying groups. Common modifying groups which are useful for hydroxyl derivation include, for example, benzyl, substituted benzyl, benzoyl, substituted benzoyl, alkyl, substituted alkyl, alkanoyl, substituted alkanoyl or silyl. As was discussed for amine derivitization, the choice of the modifying group for the hydroxyl modification is preferably one having a tendency to increase of the overall lipophilicity of the 2-DOS aminoglycoside.

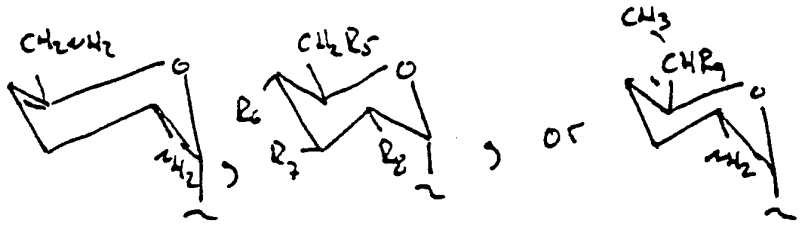
The following detailed structural information is presented in an effort to describe particularly useful 2-DOS aminoglycoside derivatives. These include 4-substituted, 4,5 disubstituted, or 4,6 disubstituted 2-deoxystreptamine aminoglycosides having the structural features set forth in Structures III-IV below.



Structure III

wherein R_1 is chosen from H or L-(-)-4-amino-2-hydroxybutyrl;

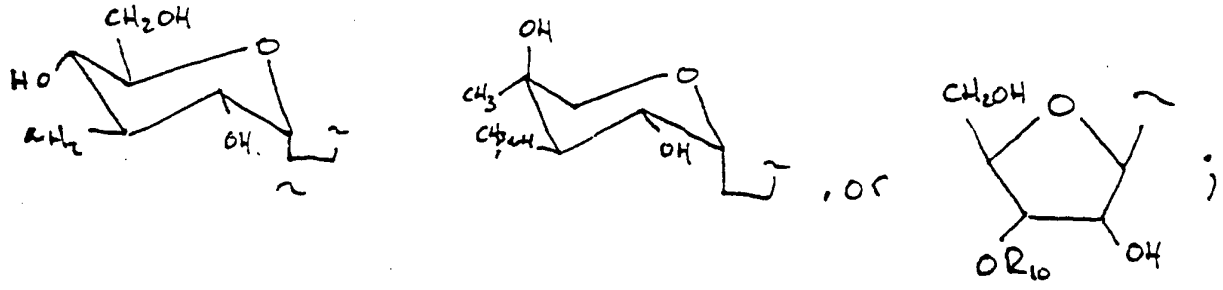
wherein R_2 is chosen from the group of hexoses having the structures according to Structure IV:



Structure IV

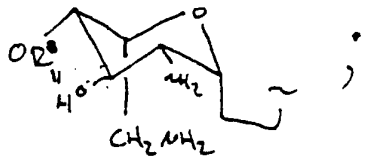
wherein R_5 can be either amino or hydroxyl;
wherein R_6 and R_7 can be either H or hydroxyl;
wherein R_8 can be either amino or hydroxyl;
wherein R_9 can be either amino or methylamino;

wherein R_3 and R_4 are independently selected from H, R_{10}, or moieties of the structures of Structure V:



Structure V

wherein R_{10}, as shown in Structure VI, is selected from the group consisting of H or



;

Structure VI

wherein R_{11} is selected from the group consisting of H or mannosyl;

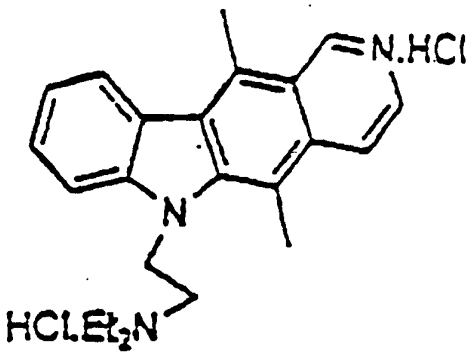
but wherein at least one of R_3 or R_4 must be H.

It should be recognized that the amine and hydroxyl functionalities of the structures shown as Structures I-IV above can be modified as described earlier in the Detailed Description of the Invention.

As shown in the Exemplification, inhibition of Rev/RRE binding <u>in vitro</u> by a 2-DOS aminoglycoside serves as a predictor of that aminoglycoside's ability to inhibit the replication of HIV in infected cells. Therefore, another aspect of the invention relates the inhibition of HIV replication in HIV infected cells. Inhibition of replication is affected by contacting infected cells with an amount of the aminoglycoside sufficient to inhibit replication. Conventional modes for the administration of small organic molecules can be used in a therapeutic context.

In addition to the preferred class of small organic molecules discussed above (the 2-DOS aminoglycoside class) the Exemplification section which follows describes experimental work which demonstrates the effective use of a small organic molecule which is not an non-aminoglycoside, or even an antibiotic. More specifically, the experimental work described below shows that the ellipticine derivative, 6-2[2-(diethylamino)ethyl]

ellipticine dihydrochloride (6DEE) (Structure VII), inhibits the binding of the Tat peptide to the TAR RNA.



Structure VII

EXAMPLES

Example 1: Aminoglycoside Antibiotics that Inhibit Rev Binding to the RRE of HIV RNA

To test whether antibiotics can block binding of Rev to the RRE, a 67 nt ^{32}P-labeled RNA probe containing a high affinity Rev-binding site was incubated with purified Escherichia coli-derived Rev protein in the presence of various antibiotics. Binding of Rev to the RNA probe was quantitated by an RNA gel mobility shift assay.

A total of 32 aminoglycoside and nonaminoglycoside antibiotics were initially surveyed for their ability to inhibit Rev binding. Several conclusions follow from this data. First no nonaminoglycoside antibiotic (erythromycin, tetracycline, thiostrepton, chloramphenicol, chromomycin A, olivomycin, lincomycin, clindamycin A, vineomycin, capreomycin, viomycin, distamycin A, ampicillin, carbenicillin and tunicamycin) was inhibitory at any concentration tested. Second, three of the aminoglycoside antibiotics (neomycin B, tobramycin, and lividomycin A) had significant inhibitory potential. In particular, neomycin B completely inhibited Rev binding at concentrations as low as 1 μM. This same concentration of drug also inhibited binding of Rev to a 33 nt RNA probe

that contained a high affinity binding site and to a 234 nt RRE that supports a Rev response in vivo. Tobramycin and lividomycin A both required a slightly higher concentration (10 μM) to achieve inhibition of Rev binding. The third group of aminoglycosides were inhibitory at 100 μm (gentamicin, kanamycin B, ribostamycin, neamine, amikacin and sisomycin). The remaining aminoglycoside antibiotics tested had no inhibitory activity at any concentration tested (butirosin, kanamycin A, paromomycin, spectinomycin, and streptomycin, kasugamycin, dihydrostreptomycin and hygromycin B).

Association of aminoglycosides with RNA may involve ionic interactions between the positively charged amino groups of the drugs and negatively charged phosphate groups of RNA. The importance of the amino groups for RNA binding is revealed by several important structure-activity relationships: in the cases of both kanamycin A versus kanamycin B and neomycin B versus paromomycin, changing an amino to a hydroxyl group eliminated inhibitory activity.

The Rev-RRE inhibition data suggests that a 2-deoxystreptamine (2-DOS) core structure is necessary for inhibition. For example, drugs lacking 2-DOS, such as streptomycin and spectinomycin, were inactive, as were nonaminoglycoside antibiotics. The most active aminoglycosides contain a 4,5-disubstituted 2-DOS (neomycin B and lividomycin A) and 4,6-disubstituted 2-DOS (tobramycin) moiety.

The most striking structure activity relationship involves neomycin B and paromycin. These two molecules are almost identical, differing only in the nature of the C6 substituent of the aminoglucose at the 4 position of 2-DOS. Neomycin B, the most potent inhibitor has an amino group whereas paromomycin, which is completely inactive,

has a hydroxyl group at this position.

Example 2: Neomycin B Specifically Inhibits the Rev-RRE Interaction

To determine whether inhibition was specific for Rev-RRE interaction, the effect of the inhibitory antibiotics on several unrelated sequence-specific RNA and DNA-binding proteins was examined. Initially, two well characterized proteins involved in pre-mRNA splicing were examined: the essential splicing factor U2AF^{65} and Drosophila splicing regulator Sex lethal.

In these binding experiments, Sex lethal protein or the 65 kD subunit of U2AF^{65}, each purified as fusion protein with glutathione-S-transferase (GST) were incubated with a ^{32}P-labelled RNA which contains the well-characterized polypyrimidine tract-3′ splice site found in the Drosophila tra gene in the presence of 100 μM neomycin B or 100 μM tobramycin.

The binding of GST-Sex lethal to the tra RNA is unaffected after incubation with 100 μM neomycin B. Similarly, in vitro binding of GST-Sex U2AF^{65} is not inhibited by the presence of 100 μM neomycin B. Thus, these experiments demonstrate that neomycin B has no effect on the in vitro binding properties of these two RNA binding proteins.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme that also binds to a subset of transfer RNAs (tRNAs) in a sequence-specific manner, was next analyzed. The binding of GAPDH to tRNA^{Tyr} was unaffected by 100 μM neomycin B or 100 μM tobramycin.

Rev is a member of a class of RNA-binding proteins containing an arginine-rich RNA-binding motif. Whether aminoglycoside antibiotics could inhibit binding of

another member of this class, the HIV-1 Tat protein, was examined next. A 12 amino acid peptide containing the Tat arginine-rich region possesses an RNA binding activity comparable to that of the full-length protein. Binding of the Tat peptide was not affected by any of the aminoglycoside antibiotics. Thus, of all the RNA-protein interactions tested, only the Rev-RRE interaction was inhibited.

Example 3: Inhibition by Neomycin B on DNA Binding Proteins

To provide additional evidence that the inhibition by neomycin B is specific for the Rev:RRE interaction, the affect of neomycin B on the in vitro binding properties of DNA binding proteins was examined.

In these studies, the DNA binding activity of two eukaryotic transcription factors, Oct-1 and ATF-2, was analyzed after incubation in 100 μM neomycin B using a gel mobility shift assay. Purified Oct-1 was incubated in the absence or presence of 100 μM neomycin B with a ^{32}P-labelled 70 nucleotide DNA probe which contained an HSV ICPO octamer binding site (Stern and Herr, *Genes and Development*, 5:2555-2566 (1991)). Additionally, the affect of 100 μM neomycin B on the binding of ATF-2 to a 35 nucleotide DNA probe homologous to nucleotides -251 to -232 of the HTLV-1 LTR was analyzed. The binding of Oct-1 to the HSV ICPO octamer binding site is unaffected by 100 μM neomycin B. The level of DNA binding activity for ATF-2 remains unchanged after incubation with 100 μM neomycin B. Thus, neomycin B has no affect on the in vitro DNA binding properties of Oct-1 or ATF-2.

Example 4: Inhibition of Precursor mRNA Processing

Previous studies have reported that aminoglycoside antibiotics such as neomycin B and tobramycin effectively inhibit self-splicing of Group I introns in vitro (von Ahsen et al., *J. Mol. Biol.*, 226:935-941 (1992). However, whether these same antibiotics have an inhibitory effect on precursor mRNA splicing, a process which involves many RNA:protein interactions, has not previously been determined. To determine whether these aminoglycoside antibiotics inhibit precursor mRNA splicing, as well as further demonstrate that the inhibition by neomycin B is specific for the Rev:RRE interaction, the ability of kanamycin A, neomycin B or tobramycin to inhibit precursor mRNA processing in vitro was tested. In these studies, a 220 nucleotide adenovirus major late precursor mRNA (MXSVL) was incubated in HeLa cell nuclear extracts which contained kanamycin A, neomycin B, or tobramycin. The addition of these aminoglycoside antibiotics at concentrations up to 100 μM to in vitro mammalian splicing extracts had no effect on either step of precursor mRNA splicing. Taken together with the results described in the previous section, these data demonstrate that the inhibition of Rev binding to the RRE by neomycin is highly specific.

Example 5: Neomycin B Binds to the Core Binding Site of RRE

Experiments were next performed to determine the basis for inhibition. Given the background information on aminoglycoside antibiotics, one possibility was that neomycin B could complex with the RRE and block Rev binding. To test this hypothesis chemical footprinting experiments were performed. The 67nt RRE RNA was incubated alone, with Rev, or with increasing concentrations of neomycin B, tobramycin, or kanamycin A,

(0.1, 1.0 and 10 μM) and treated with either dimethyl sulfate (DMS), which methylates adenosine at N1 and cytosine at N3, or kethoxal, which modifies N1 and N2 of guanosine. Modification of specific bases was detected by primer extension. Under these conditions DMS and kethoxal are single strand-specific, and thus the modification pattern of RNA alone provides an indication of secondary structure.

Results from the chemical modification/protection experiments performed in the presence of neomycin B have demonstrated that neomycin B binds to nucleotides within the RRE which are contained within the high affinity recognition/binding site for the Rev protein. Tobramycin produced similar protection patterns, although protection by tobramycin was weaker, consistent with its weaker inhibiting activity. For example, Rev, neomycin B and tobramycin all strongly protected nucleotides 46-48 of the 67 nt RNA. Similar concentrations of the non-inhibiting aminoglycoside kanamycin A did not protect the RRE from chemical modification.

Example 6: Rev Competes with Neomycin B for Binding to the RRE

The chemical modification/protection experiments of Example 5 revealed that the inhibitory antibiotics protected bases within the core of the high affinity Rev-binding site. This suggested that the drugs inhibited Rev binding by a competitive mechanism. A prediction of this hypothesis is that inhibition could be overcome by high concentrations of Rev. To test this possibility, increasing amounts of Rev were added to a reaction mixture containing 100 μM neomycin B. At the lowest protein amounts (1.3-2 ng), Rev binding was completely inhibited by the drug. However, as the amount of Rev was increased, RNA binding was restored (10-20 ng). The combined results

of Examples 5 and 6 indicate that inhibition of Rev binding by neomycin B occurs by a competitive mechanism.

Example 7: Neomycin B Antagonizes Rev Function In Vitro

The experiments described above measured the ability of the antibiotics to interfere with binding of purified Rev to RNA. This suggested that inhibitory antibiotics could also interfere with Rev function. This hypothesis was tested by analyzing Rev function in vitro. In this assay, inhibition of splicing requires both a Rev derivative that can bind RNA and a pre-mRNA containing the RRE. Thus, splicing repression is dependent upon the Rev-RRE interaction. Because neomycin B inhibited Rev binding it would be expected that the drug would reverse splicing repression.

The results of this experiment showed that the Rev derivative inhibited splicing of the RRE-containing pre-mRNA. Significantly, neomycin B counteracted the Rev-mediated repression of splicing at concentrations similar to those that inhibited Rev binding (1.0-2.5 μM). In contrast, a 40-fold higher concentration of the noninhibitory antibiotic kanamycin A failed to reverse splicing repression. This demonstrates that neomycin B inhibits Rev function in vitro. Because a 400-fold higher concentration of neomycin B did not inhibit the RNA-based pre-mRNA splicing reaction (See Example 4) it is evident that inhibition is highly specific.

Example 8: Neomycin B Selectively Antagonizes Rev Function In Vivo

Experiments were next performed to determine whether the inhibitory antibiotics could also antagonize Rev function in vivo. Rev function was measured in a cotransfection assay using the chloramphenicol

acetytransferase (CAT) reporter plasmid pCM128 which has a single intron containing both the RRE and the bacterial CAT coding sequence (Hope et al., *Proc. Natl. Acad. Sci. USA*, 87:7787-7791 (1990)). The intron is normally efficiently excised by splicing and therefore cells transfected with pCM128 express only trace amounts of CAT enzyme activity. Cotransfection with a Rev expression plasmid permits unspliced transcripts to enter the cytoplasm, and thus CAT expression is increased. There resulted dose-dependent inhibition of Rev function by neomycin B and tobramycin, whereas kanamycin A had no inhibitory effect. These findings parallel the results obtained in the in vitro Rev binding and splicing inhibition assays described above.

Three considerations indicate that the inhibition of Rev function was specific and not due to a more general inhibitory effect on gene expression. First, at all concentrations of antibiotics tested, cellular viability and growth was normal, and total cellular translation (incorporation of [^{35}S]methionine) was not inhibited. Second, concentrations of neomycin B and tobramycin that inhibited Rev function did not inhibit expression of the cotransfected β-galactosidase reporter gene. Third, comparable concentrations of neomycin B and tobramycin did not inhibit expression of a cytomegalovirus (CMV)-CAT reporter, or the HIV Tat-mediated activation of an HIV long terminal repeat-CAT reporter. Thus, inhibition of Rev function is not due to interference with CAT gene expression at the transcriptional or posttranscriptional level. The failure to inhibit Tat is particularly relevant because transcription activation involves an RNA-protein interaction between Tat and trans-acting

responsive sequence. From this work it can be concluded that neomycin B and tobramycin selectively inhibit Rev function in vivo.

Example 9: Neomycin B Inhibits Production of HIV

Previous studies have shown that Rev is essential for replication of HIV. Thus, a prediction for the results presented above is that neomycin B should inhibit viral production. To test this prediction, the effect of neomycin B on viral production in a chronically infected cell line, U1 was analyzed. This cell line has been previously used as a model to study Rev-dependent activation of viral production. (Pomerantz, et al., *Cell*, 61:1271-1276 (1990); Michael, et al., *J. Virol.*, 55:1291-1303, (1991)).

Viral production was assessed at increasing concentrations of neomycin B using a standard assay that quantitates the amount of a viral structure protein, p24, in the culture medium. The results of this experiment clearly show that neomycin B inhibited viral production in a dose-dependent fashion (0-2.5 mM); at the highest concentration tested, inhibition was approximately 85%. Significantly, at all of these drug concentrations, cellular growth was 94%-108% of the untreated control. Neomycin B therefore clearly inhibits production of HIV in chronically infected cells.

Example 10: Sisomycin Inhibits Bindings of a TAT Peptide to the TAR RNA

A total of 32 aminoglycoside and non-aminoglycoside antibiotics were tested for their ability to inhibit Tat binding. Some of these data are shown in Figure 1A. A 26-nucleotide ^{32}P-Labeled TAR RNA probe (+18 to +44) was incubated with a synthetic peptide containing amino acids

48-60 of the HIV-1 Tat protein (Calnan et al., *Genes Rev.*, 5:201-210 (1991)) in the absence or presence of 250 μM of the indicated antibiotic. The results indicate that only the aminoglycoside antibiotic sisomycin inhibited binding of the Tat peptide to TAR RNA. Ribostamycin, an aminoglycoside that has been shown to inhibit Rev binding at concentrations of 100 μM or greater has no effect on Tat binding at 250 μM. The same TAR probe was incubated with decreasing concentrations (micromolar) of sisomycin in the presence of the TAT peptide. The data are presented in Figure 1B. The data indicate that at a concentration as low as 50 μM, binding of the TAT peptide is inhibited >80%.

Example 11: Sisomycin Specifically Inhibits the Tat-TAR Interaction

Tat is a member of a class of RNA-binding proteins containing an arginine-rich RNA-binding motif. To determine whether sisomycin inhibition is specific for Tat/TAR a ^{32}P-labeled RNA probe (Zapp et al., *Cell*, 74 969-978 (1993)) containing the high affinity Rev-binding site was incubated with purified *E. coli*-derived Rev protein in the presence of increasing concentrations of sisomycin. As shown in Figure 2A, binding of Rev was not affected by any concentration of sisomycin tested.

Example 12: Sisomycin Does Not Inhibit Mammalian Pre-mRNA Splicing

Splicing of pre-mRNAs involves numerous RNA-protein and RNA-RNA interactions and, therefore, provides a stringent test for inhibitors of RNA-binding proteins. A ^{32}P-labeled human β-globin pre-mRNA (Krainer, et al., *Cell*, 36:993-1005 (1984) and Ruskin et al. *Cell*, 38:317-331 (1984)) was incubated in a HeLa nuclear extract in the

absence or presence of several aminoglycosides. The splicing was unaffected by 100 μM sisomycin (Figure 2B). In conjunction with the results described above, these data indicate that inhibition of the Tat-TAR interaction by sisomycin is highly specific.

Example 13: Sisomycin Binds to a Discrete Region of TAR

To test whether sisomycin binds to a discrete region of TAR, chemical footprinting experiments were performed with kethoxal, which modifies the N1 and N2 positions of guanosine. A 46-nucleotide RNA containing a high-affinity TAT binding site was incubated in the absence or with increasing concentrations of sisomycin (10 μM, 100 μM or 1 Mm) and treated with kethoxal as indicated. The reverse-transcribed RNAs, along with dideoxy-sequencing reactions (C, U, G, and A) and a control reaction with untreated RNA (K), were separated on a denaturing poly-acrylamide gel. The results (Figure 3) show that sisomycin protects two guanosines (G33 and G43), suggesting that sisomycin induces a conformational change in TAR RNA. Taken together these data indicate that sisomycin binds directly to TAR RNA and distorts its structure. This direct binding in induced conformational change explain the inhibition of Tat binding described above.

Example 14: A Small Molecule that Inhibits Binding of the HTLV-1 Rex Protein

To test whether antibiotics block binding of the HTLV-1 Rex protein to its cognate RNA binding site, the XRE, a 100 nucleotide ^{32}P-labeled RNA probe containing a high affinity Rex binding site (Ohta et al., *J. Virol.* 62:4445-4451 (1988)) was incubated with purified Escherichia coli-derived glutathione-S-transferase (GST)-Rex fusion protein in the presence of various antibiotics.

Binding of GST-Rex to the RNA probe was quantitated by an RNA mobility shift assay.

A total of 32 aminoglycoside and nonaminoglycoside antibiotics were tested for their ability to inhibit Rex binding. Some of these data are shown in Figure 4. In particular, dihydrostreptomycin completely inhibited Rex binding at a concentration of 50 μM. The remaining antibiotics shown in Figure 4 had no inhibitory activity (hygromycin B, lincomycin, kasugamycin, olivomycin, chromomycin A) at the highest concentration tested (250 μM).

Example 15: Dihydrostreptomycin Specifically Inhibits the Rex-XRE Interaction

To determine whether inhibition was specific for the Rex-XRE interaction, the effect of dihydrostreptomycin on an unrelated sequence-specific RNA binding protein, the HIV-1 Rev protein was examined. Rex is a member of a class of RNA-binding proteins containing an arginine-rich RNA binding motif. Whether dihydrostreptomycin could inhibit binding of another member of this class, the HIV-1 Rev protein was therefore tested. A 67-nucleotide ^{32}P-labeled RNA probe (Zapp et al., *Cell*, 74:969-978 (1989)) containing a high affinity Rev binding site was incubated with purified *E. coli*-derived Rev protein in the absence or presence of increasing concentrations of dihydrostreptomycin. As shown in Figure 5, binding of Rev to the XRE was not affected by the highest concentration (100 mM) of dihydrostreptomycin.

Example 16: Dihydrostreptomycin Does Not Inhibit Mammalian Pre-mRNA Splicing

As described above, pre-mRNA splicing provides a stringent specificity test for inhibitors of RNA-protein

and RNA-RNA interactions. As shown in Figure 2B and described in Example 12, 100 μM dihydrostreptomycin did not affect splicing of a human β-globin pre-mRNA substrate in a HeLa cell nuclear extract. In conjunction with the results described above, these data indicate that inhibition of the Rex-XRE interaction by dihydrostreptomycin is highly specific.

Example 17: A Small Molecule, Which is Not an Aminoglycoside Antibiotic, Selectively Inhibits Binding of the HIV TAT Protein

The ellipticines are a class of compounds that are known to have antitumor activity. They are believed to exert their antitumor effect by interaction with DNA, presumably through an intercalative mechanism. Whether any member of this class of molecules could inhibit the interaction of an RNA binding protein with its cognate RNA binding site, TAR, was therefore tested.

A 36-nucleotide ^{32}P-labeled RNA probe (+18 to +44) containing the high affinity Tat binding site was incubated with a synthetic peptide containing the Tat arginine-rich RNA binding region (Calnan et al., *Genes Rev.*, 5:201-210 (1991)). This particular Tat peptide (amino acids 48-60) has been shown previously to possess an RNA binding activity comparable to that of the full-length Tat protein. Binding of the Tat peptide to the TAR RNA probe was quantitated by an RNA gel mobility shift assay.

Figure 6A show that an ellipticine derivative, 6-[2-(diethylamino)ethyl]ellipticine dihydrochloride (6DEE), at 250 μM inhibited binding of the Tat peptide to the TAR RNA. Figure 6B presents a titration experiment wherein the same probe was incubated with decreasing concentrations of 6DEE in the presence or absence of the

TAT peptide. The data indicate that at a concentration of 50 μM, 6DEE inhibited binding of the Tat peptide >85%.

Whether 6DEE could inhibit binding of another member of this class, the HIV-1 Rev protein was tested next. A ^{32}P-labeled RNA probe (94.7 Zapp et al. *Cell* 74:969-978 (1993)) containing the high affinity Rev-binding site was incubated with purified *E. coli*-derived Rev protein in the presence of increasing concentration of 6DEE.

Example 18: 6DEE Does not Inhibit Mammalian Pre-mRNA Splicing

A ^{32}P-labeled human β-globin pre-mRNA (Krainer et al.19:4, Ruskin et al. 19:4 was incubated in a HeLa nuclear extract in the presence or absence of 6DEE. Figure 8 shows that 100 μM 6DEE did not affect splicing of a human ^{32}P-labelled β-globin pre-mRNA substrate in HeLa cell nuclear extract. In conjunction with the results described above, these data indicate that inhibition of the Tat-TAR interaction by 6DEE is highly specific.

CLAIMS

The invention claimed is:

1. A method for inhibiting an RNA-ligand interaction, comprising contacting the RNA with a small organic compound other than a peptide, oligonucleotide or analog thereof, the small organic compound having a molecular weight of less than about 2,000 daltons that binds directly with the RNA, thereby inhibiting the ligand binding to the RNA.

2. A method of Claim 1 wherein the ligand is selected from the group consisting of a protein, nucleic acid, lipid or carbohydrate.

3. A method of Claim 2 wherein the small organic compound is an aminoglycoside.

4. A method of Claim 3, wherein the aminoglycoside is a 2-deoxystreptamine aminoglycoside.

5. A method for inhibiting the binding of a trans-activating regulatory protein to a cis-acting RNA sequence, comprising contacting the RNA with a small organic compound other than a peptide, oligonucleotide or analog thereof, the small organic compound having a molecular weight of less than about 2,000 daltons that binds directly with the RNA, thereby inhibiting the protein binding to the RNA.

6. A method of Claim 5 wherein the RNA is a mRNA or a precursor mRNA.

7. A method of Claim 6 wherein the mRNA is encoded by a virus.

8. A method of Claim 7 wherein the virus is an RNA virus.

9. A method of Claim 8 wherein the RNA virus is a retrovirus.

10. A method of Claim 9 wherein the virus is HIV-1.

11. A method of Claim 10 wherein the trans-activating regulatory protein is Rev.

12. A method of Claim 10 wherein the trans-activating regulatory protein is Tat.

13. A method of Claim 9 wherein the virus is HTLV-1.

14. A method of Claim 13 wherein the trans-activating regulatory protein is Rex.

15. A method of Claim 10 wherein the small organic compound is an aminoglycoside.

16. A method of Claim 13 wherein the small organic compound is an aminoglycoside.

17. A method of Claim 15 wherein the aminoglycoside is a 2-deoxystreptamine aminoglycoside.

18. A method of Claim 16 wherein the aminoglycoside is a 2-deoxystreptamine aminoglycoside.

19. A method for inhibiting HIV Rev protein binding to RNA containing a Rev-responsive element, comprising contacting the RNA with a 2-deoxystreptamine aminoglycoside that binds directly to the RNA, thereby inhibiting HIV Rev protein binding to the Rev-responsive element.

20. A method of Claim 19, wherein the 2-deoxystreptamine aminoglycoside contains three sugar moieties.

21. A method of Claim 20, wherein one or more amino groups of the 2-deoxystreptamine aminoglycoside are functionalized with modifying groups whose combined molecular weight is less than 500 daltons.

22. A method of Claim 21, wherein the modifying groups increase the lipophilicity of the 2-deoxystreptamine aminoglycoside.

23. A method of Claim 20, wherein one or more of the hydroxyl groups of the 2-deoxystreptamine aminoglycoside are functionalized with modifying groups.

24. A method of Claim 23, wherein the modifying groups increase the lipophilicity of the 2-deoxystreptamine aminoglycoside.

25. A method of Claim 20, wherein the amino functionalities of the 2-deoxystreptamine aminoglycoside are independently methyl substituted or are primary amines.

26. A method of Claim 25, wherein no hydroxyl moieties of the 2-deoxystreptamine aminoglycoside are functionalized.

27. A method of Claim 19, wherein the 2-deoxystreptamine aminoglycoside contains one, two or four sugar moieties.

28. A method of Claim 27, wherein one or more amino groups of the 2-deoxystreptamine aminoglycoside are functionalized with modifying groups.

29. A method of Claim 28, wherein the modifying groups increase the lipophilicity of 2-deoxystreptamine aminoglycoside.

30. A method of Claim 27, wherein one or more hydroxyl groups of the 2-deoxystreptamine aminoglycoside are functionalized with modifying groups.

31. A method of Claim 30, wherein the modifying groups increase the lipophilicity of the 2-deoxystreptamine aminoglycoside.

32. A method of Claim 27, wherein the amino functionalities of the 2-deoxystreptamine aminoglycoside are independently methyl substituted or are primary amines.

33. A method of Claim 32, wherein no hydroxyl moieties of the 2-deoxystreptamine aminoglycoside are functionalized.

34. A method of Claim 19, wherein the 2-deoxystreptamine aminoglycoside is tobramycin.

35. A method of Claim 19, wherein the 2-deoxystreptamine aminoglycoside is neomycin B.

36. A method of Claim 19, wherein the 2-deoxystreptamine aminoglycoside is neamine.

37. A method of Claim 19, wherein the 2-deoxystreptamine aminoglycoside is ribostamycin.

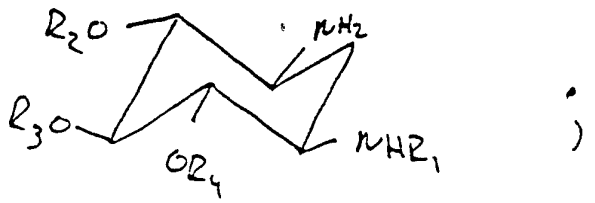
38. A method of Claim 19, wherein the 2-deoxystreptamine aminoglycoside is lividomycin A.

39. A method of Claim 19, wherein the 2-deoxystreptamine aminoglycoside is kanamycin B.

40. A method of Claim 19, wherein the 2-deoxystreptamine aminoglycoside is amikacin.

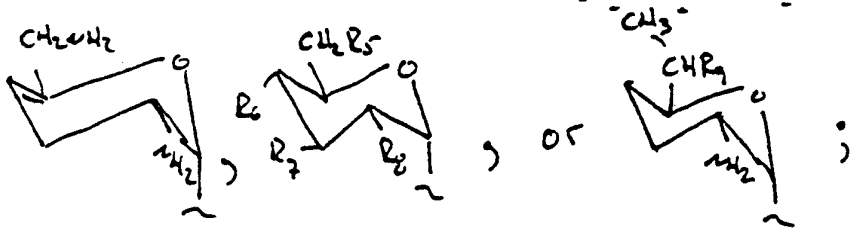
41. A method of Claim 19, wherein the 2-deoxystreptamine aminoglycoside is gentamicin C.

42. A method for inhibiting HIV Rev protein binding to a RNA containing a Rev-responsive element, comprising contacting the RNA with a 4-monosubstituted, 4,5-disubstituted or 4,6-disubstituted-2 deoxystreptamine aminoglycoside that binds directly to the RNA, thereby inhibiting HIV Rev protein binding to the Rev-responsive element, wherein the 2-deoxystreptamine aminoglycoside has the following structure:



wherein R_1 is selected from the group consisting of H or $COCHOHCH_2CH_2NH_2$;

wherein R_2 is selected from the group consisting of



wherein R_5 is selected from the group consisting of NH_2 or OH;

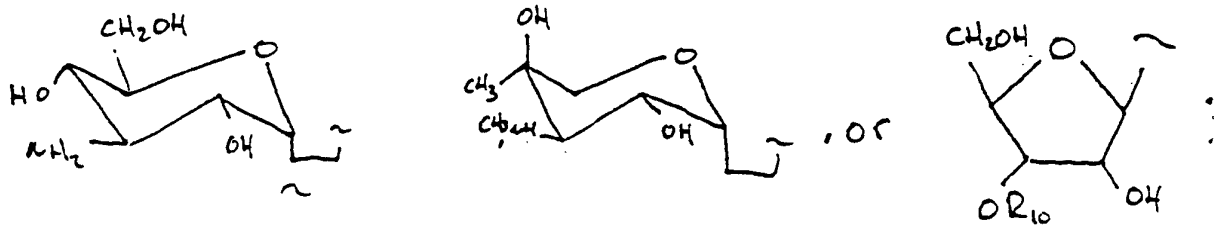
wherein R_6 is selected from the group consisting of H or OH;

wherein R_7 is selected from the group consisting of H or OH;

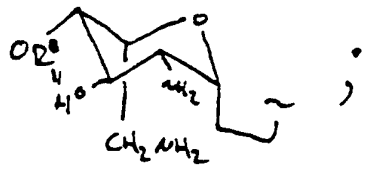
wherein R_8 is selected from the group consisting of NH_2 or OH;

wherein R_9 is selected from the group consisting of NH_2 or $NHCH_3$;

wherein R_3 and R_4 are independently selected from H, R_{10},



wherein R_{10} is selected from the group consisting of H or



wherein R_{11} is selected from the group consisting of H or mannosyl, but wherein at least one of R_3 or R_4 must be H.

43. A method of Claim 42, wherein one or more of the hydroxyl groups of the 2-deoxystreptamine aminoglycoside are functionalized with modifying groups.

44. A method of Claim 43, wherein the modifying groups increase the lipophilicity of the 2-deoxystreptamine aminoglycoside.

45. A method of Claim 42, wherein the amino functionalities of the 2-deoxystreptamine aminoglycoside are independently methyl substituted or are primary amines.

46. A method of Claim 45, wherein no hydroxyl moieties of the 2-deoxystreptamine aminoglycoside are functionalized.

47. A method of Claim 42, wherein the 2-deoxystreptamine aminoglycoside is a 4-monosubstituted 2-deoxystreptamine wherein R_3 and R_4 are H or 4,6-disubstituted 2-deoxystreptamine whereby R_3 is H.

48. A method of Claim 47, wherein one or more amino groups of the 2-deoxystreptamine aminoglycoside are functionalized with modifying groups.

49. A method of Claim 48, wherein the modifying groups increase the lipophilicity of the 2-deoxystreptamine aminoglycoside.

50. A method of Claim 42, wherein the 2-deoxystreptamine aminoglycoside is a 4,5-disubstituted 2-deoxystreptamine whereby R_4 is H.

51. A method of Claim 50, wherein one or more amino groups of the 2-deoxystreptamine aminoglycoside are functionalized with modifying groups whose combined molecular weight is less than 500 daltons.

52. A method of Claim 51, wherein the modifying groups increase the lipophilicity of the 2-deoxystreptamine aminoglycoside.

53. A method for inhibiting retroviral replication, comprising contacting retroviral infected cells with a 2-deoxystreptamine aminoglycoside which binds to a retroviral encoded RNA and inhibits the binding of a protein to the RNA, thereby inhibiting retroviral replication.

54. A method of Claim 53 wherein the retrovirus is HIV; wherein the HIV encoded RNA contains a Rev-responsive element; and
wherein the protein whose binding to HIV RNA is being inhibited is Rev.

55. A method of Claim 54, wherein the 2-deoxystreptamine aminoglycoside contains three sugar moieties.

56. A method of Claim 55, wherein one or more amino groups of the 2-deoxystreptamine aminoglycoside are functionalized with modifying groups whose combined molecular weight is less than 500 daltons.

57. A method of Claim 56, wherein the modifying groups increase the lipophilicity of the 2-deoxystreptamine aminoglycoside.

58. A method of Claim 55, wherein one or more of the hydroxyl groups of the 2-deoxystreptamine aminoglycoside are functionalized with modifying groups.

59. A method of Claim 58, wherein the modifying groups increase the lipophilicity of the 2-deoxystreptamine aminoglycoside.

60. A method of Claim 55, wherein the amino functionalities of the 2-deoxystreptamine aminoglycoside are independently methyl substituted or are primary amines.

61. A method of Claim 60, wherein no hydroxyl moieties of the 2-deoxystreptamine aminoglycoside are functionalized.

62. A method of Claim 54, wherein the 2-deoxystreptamine aminoglycoside contains one, two or four sugar moieties.

63. A method of Claim 62, wherein one or more amino groups of the 2-deoxystreptamine aminoglycoside are functionalized with modifying groups.

64. A method of Claim 63, wherein the modifying groups increase the lipophilicity of the 2-deoxystreptamine aminoglycoside.

65. A method of Claim 62, wherein one or more of the hydroxyl groups of the 2-deoxystreptamine aminoglycoside are functionalized with modifying groups.

66. A method of Claim 65, wherein the modifying groups increase the lipophilicity of the 2-deoxystreptamine aminoglycoside.

67. A method of Claim 62, wherein the amino functionalities of the 2-deoxystreptamine aminoglycoside are independently methyl substituted or are primary amines.

68. A method of Claim 67, wherein no hydroxyl moieties are functionalized.

69. A method of Claim 54, wherein the 2-deoxystreptamine aminoglycoside is tobramycin.

70. A method of Claim 54, wherein the 2-deoxystreptamine aminoglycoside is neomycin B.

71. A method of Claim 54, wherein the 2-deoxystreptamine aminoglycoside is neamine.

72. A method of Claim 54, wherein the 2-deoxystreptamine aminoglycoside is ribostamycin.

73. A method of Claim 54, wherein the 2-deoxystreptamine aminoglycoside is lividomycin A.

74. A method of Claim 54, wherein the 2-deoxystreptamine aminoglycoside is kanamycin B.

75. A method of Claim 54, wherein the 2-deoxystreptamine aminoglycoside is amikacin.

76. A method of Claim 54, wherein the 2-deoxystreptamine aminoglycoside is gentamicin C.



FIGURE 1A

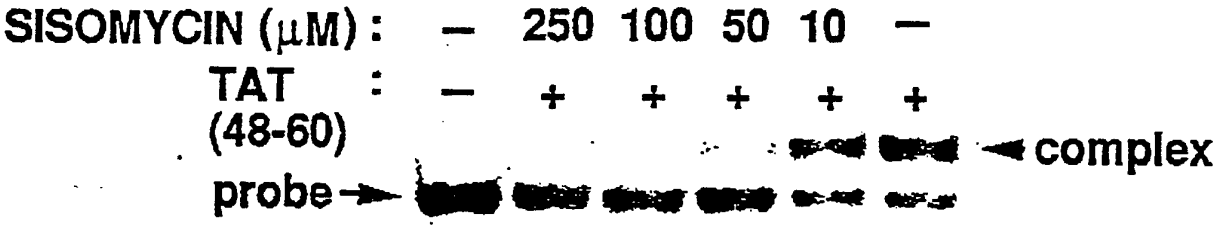


FIGURE 1B

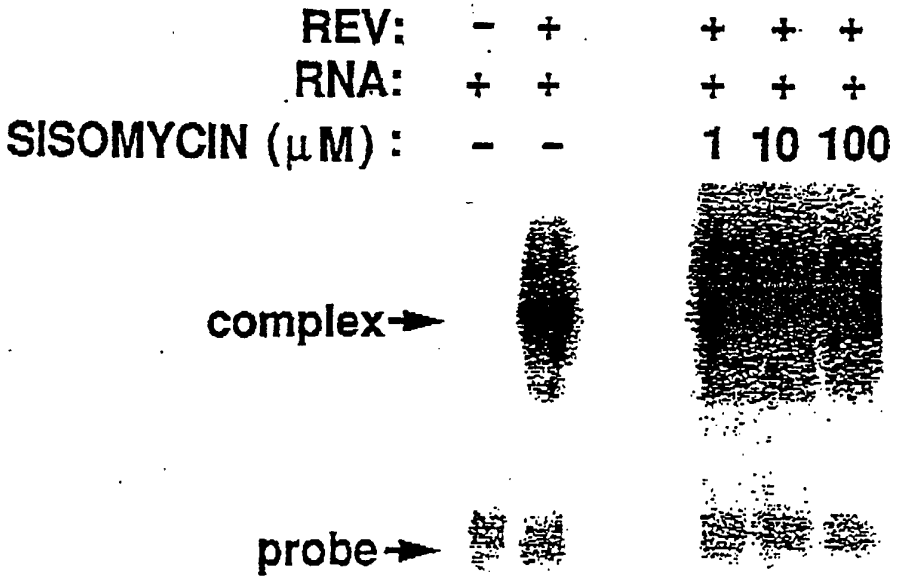


FIGURE 2A

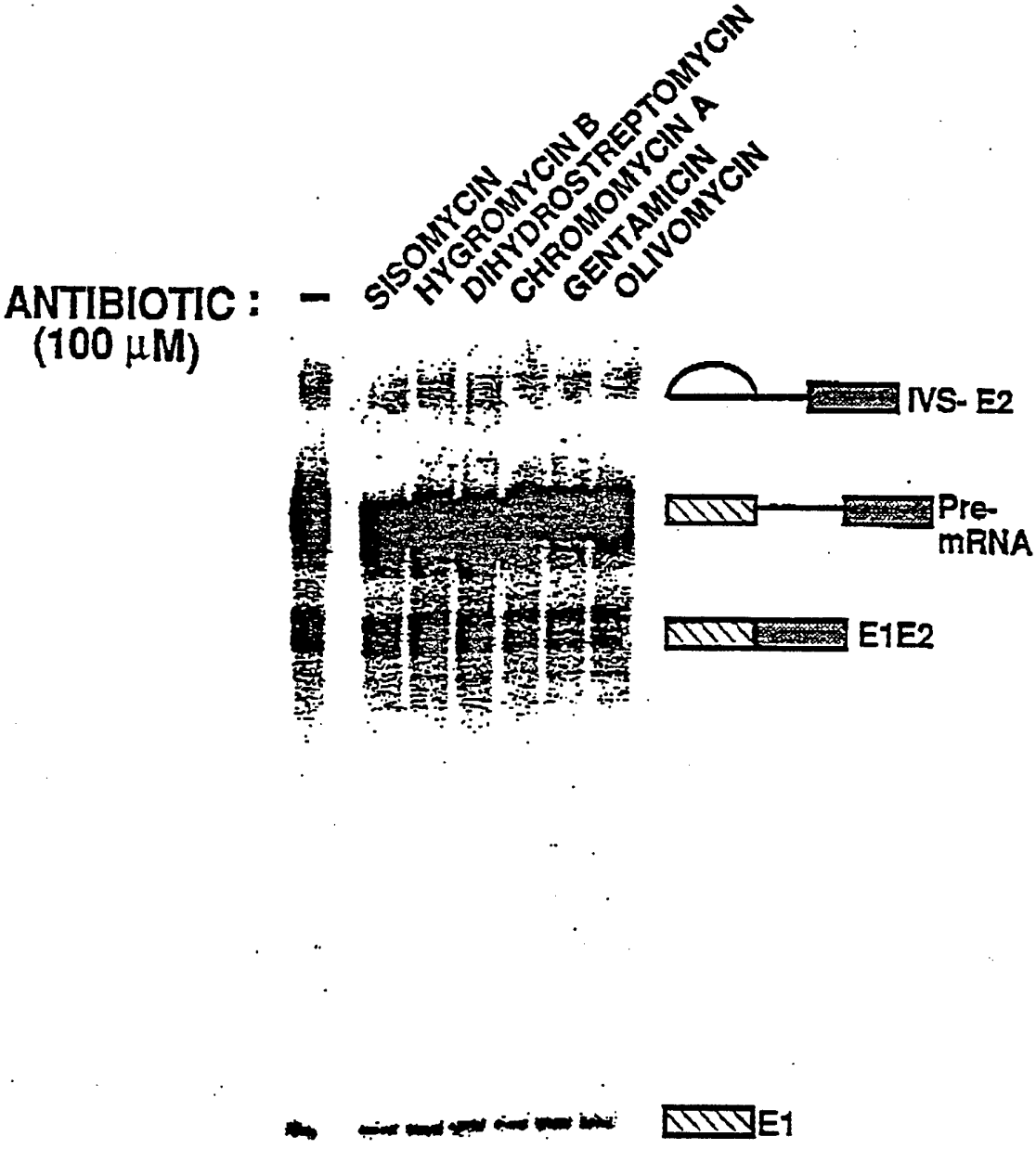

ANTIBIOTIC :
(100 μM)
—
SISOMYCIN
HYGROMYCIN B
DIHYDROSTREPTOMYCIN
CHROMOMYCIN A
GENTAMICIN
OLIVOMYCIN
IVS- E2
Pre-
mRNA
E1E2
E1

FIGURE 2B

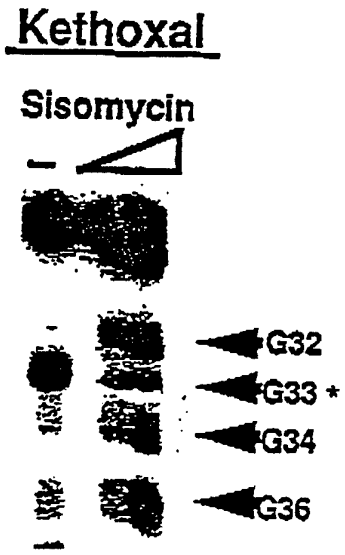


FIGURE 3

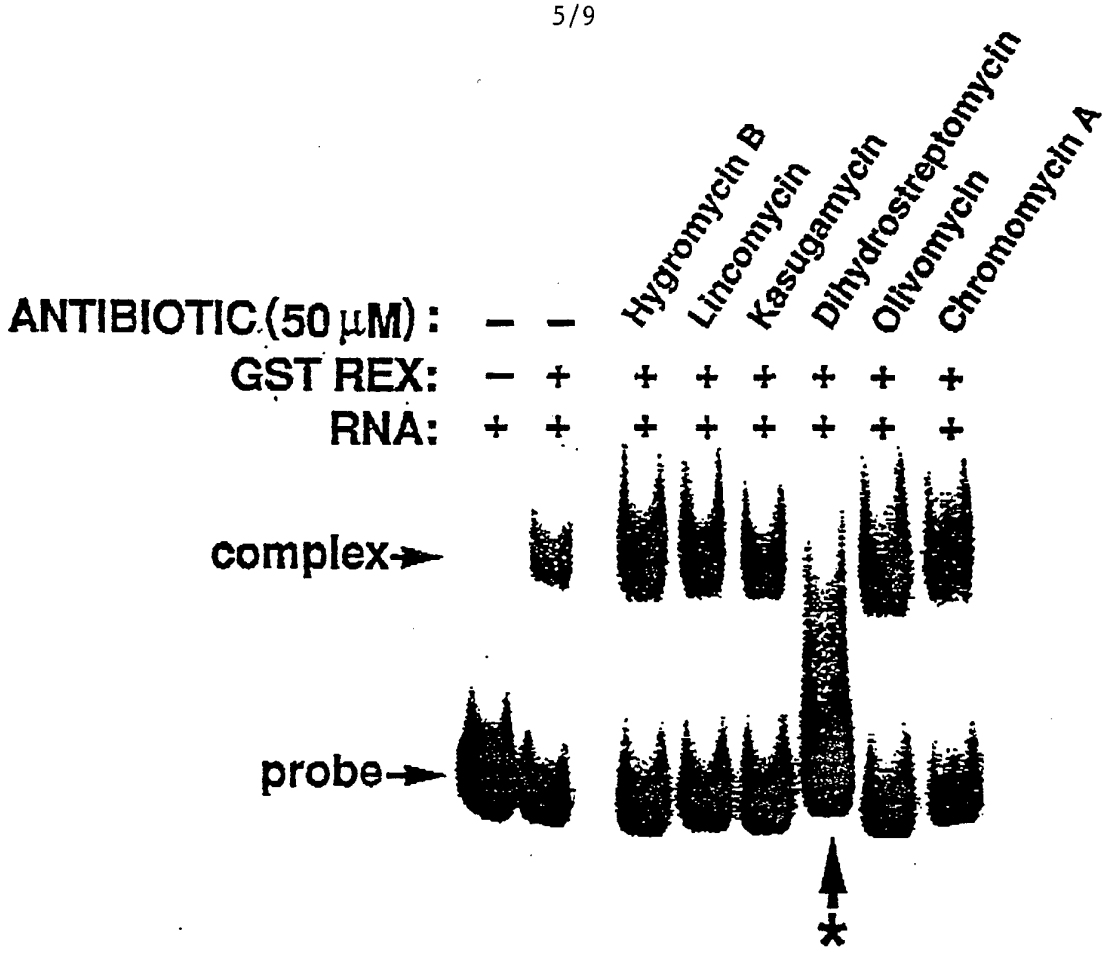

Hygromycin B
Lincomycin
Kasugamycin
Dihydrostreptomycin
Olivomycin
Chromomycin A
ANTIBIOTIC (50 μM) : – – + + + + + +
GST REX: – + + + + + + +
RNA: + + + + + + + +
complex
probe
*

FIGURE 4

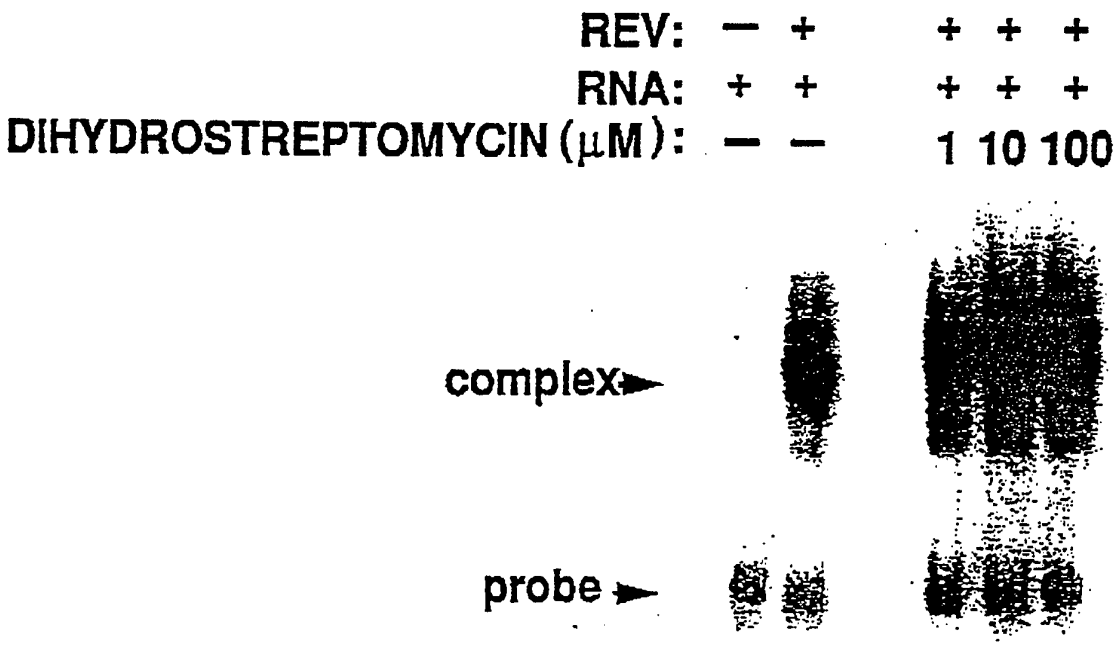


FIGURE 5

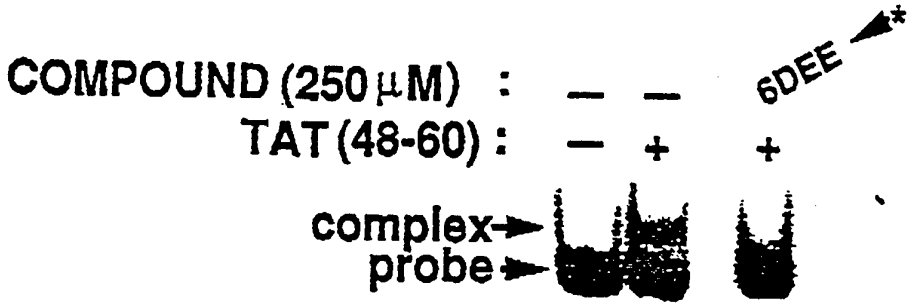


FIGURE 6A

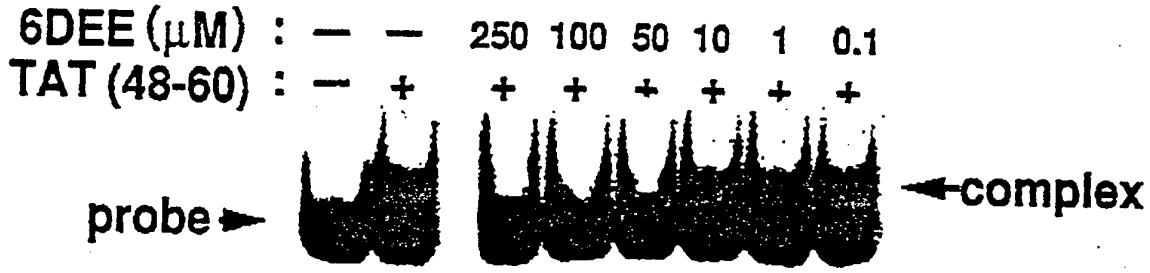


FIGURE 6B

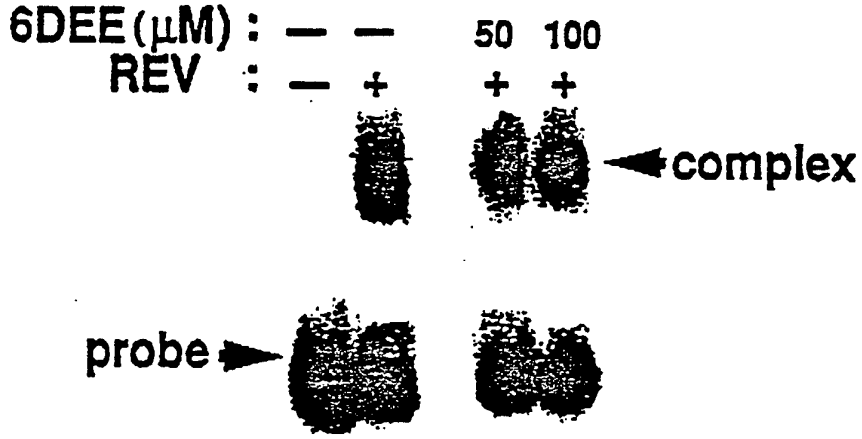


FIGURE 7



FIGURE 8

INTERNATIONAL SEARCH REPORT

Inten. ..nal Application No
PCT/US 93/10183

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K31/70 A61K31/71 A61K31/475

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 475 231 (HOFFMANN-LA ROCHE) 18 March 1992 *see claims 1,4,7,8; and page 6 line 20 - page 7 line 20* ---	1,2, 5-10,12
X	EP,A,0 462 522 (HOFFMANN-LA ROCHE) 27 December 1991 * see claims 1,5,7,8, and page 4 line 24 - page 5 line 20* ---	1,2, 5-10,12
X	WO,A,92 02530 (UPJOHN) 20 February 1992 * see claims 1-7, 10, and page 5 line 12 - page 9 * ---	1-10,12, 15,17,53
P,Y	WO,A,93 00907 (VON AHSEN) 21 January 1993 * see the whole document* --- -/--	53

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 March 1994	29. 03. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Isert, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,90 08780 (UNITED STATES OF AMERICA) 9 August 1990	53
A	* see the whole document *	1-52, 54-76

P,X	CELL vol. 74, no. 3 , 24 September 1993 pages 969 - 978 ZAPP ET AL. 'Small molecules that selectively block RNA binding of HIV-1 Rev protein inhibit rev function and viral production' * see the whole document *	1-76

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INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Internr al Application No
PCT/US 93/10183

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0475231	18-03-92	AU-A-	8374091	12-03-92
		CA-A-	2050858	11-03-92
		JP-A-	4305578	28-10-92
EP-A-0462522	27-12-91	US-A-	5141735	25-08-92
		AU-A-	7846991	02-01-92
		CA-A-	2044694	19-12-91
		CN-A-	1057462	01-01-92
		JP-A-	4230280	19-08-92
WO-A-9202530	20-02-92	AU-B-	640511	26-08-93
		AU-A-	8230091	02-03-92
		EP-A-	0541597	19-05-93
		JP-T-	5509306	22-12-93
WO-A-9300907	21-01-93	AT-B-	397102	25-02-94
		AU-A-	2272192	11-02-93
		CA-A-	2091264	10-01-93
		EP-A-	0548314	30-06-93
WO-A-9008780	09-08-90	US-A-	5278042	11-01-94
		AU-B-	629310	01-10-92
		AU-A-	5109090	24-08-90
		EP-A-	0455741	13-11-91
		JP-T-	4501803	02-04-92