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(54) Title: NOVEL ANTI-VIRAL AGENTS BASED UPON DERIVATIVES OF THE AROMATIC HETEROCYCLE PHENANTHRIDINE

(57) Abstract: A series of substituted phenanthridine derivatives has been synthesized by converting the amines at the 3- and 8-positions of ethidium bromide into guanidine, pyrrole, urea, and various substituted ureas. The resulting derivatives exhibit unique spectral properties that change upon binding nucleic acids. The compounds have an enhanced affinity and specificity for HIV-1 Rev Response Element as compared to ethidium bromide.



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**Novel Anti-Viral Agents Based Upon Derivatives
of the Aromatic Heterocycle Phenanthridine**

CROSS REFERENCE TO RELATED APPLICATION

[0001] This disclosure claims priority under 35 U.S.C. §119 to provisional application serial no. 60/495,445, filed August 11, 2003, the disclosure of which is incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] The invention was funded in part by Grant No. AI 47673 awarded by the National Institutes of Health. The government may have certain rights in the invention.

TECHNICAL FIELD

[0003] This invention relates to phenanthridine derivatives and methods of treating infections such as viral infections, using such compounds and formulations.

BACKGROUND

[0004] Ethidium bromide is the common name for 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide. Ethidium is a common laboratory stain for double-stranded DNA and RNA, but it is also known to possess significant anti-cancer, and anti-viral activities (Nishiwaki *et al.*, *Cancer Res.* 1974, 34, 2699-2703; Balda *et al.*, *Yale J. Biol. Med.* 1973, 46, 464-470; Vilagines, *Archiv her die Gesamte Virusforschung*, 1970, 30, 59-66; Simmons *et al.*, *Am. J. Vet. Res.*, 1976, 37, 69-73; Guntaka *et al.*, *Nature*, 1975, 253, 507-51). Ethidium's potential applications in human treatment have been prevented, however, due to its mutagenic activities in model systems. Despite this, ethidium is still marketed by Laprovect as a safe and inexpensive treatment for cattle suffering from trypanosome infections. A possible relationship between ethidium's nucleic acid binding and its biological activities

have been examined by a number of groups both *in vivo* and *in vitro*. Sea urchin eggs exposed to water containing 50 μM or more of ethidium develop chromosomal abnormalities and fail to divide normally. Experiments reported by Nass indicated that the growth of both mouse fibroblasts and hamster kidney cells are inhibited by 0.3 - 13 μM of ethidium, and that mitochondrial, not nuclear, DNA synthesis was inhibited by ethidium (Nass, M. M. K. *Experimental Cell Research*, 1972, 72,211-222). A separate study showed that ethidium accumulates in isolated rat mitochondrion and interferes with the metabolic activities related to respiration. (Pena et al., *Biochem. Biophys.* 1977, 180, 522-529). Ethidium is, however, only moderately toxic to mammals, with an LD_{50} in mice of $\sim 300 \mu\text{M}$ (100 mg/kg, subcutaneous), and is an effective trypanocide in cattle at $\sim 3 \mu\text{M}$ (1 mg/kg, intravenous).

[0005] The *in vitro* study of ethidium-nucleic acid binding can be conducted by monitoring the photophysical changes of ethidium upon addition of nucleic acids. Ethidium binds to DNA and RNA duplexes, depending on the sequence, with good to moderate affinities ($K_d = 1 - 500 \mu\text{M}$) and with variable stoichiometry (2 - 5 equivalents of ethidium per helical repeat). LePecq and Paoletti first proposed that ethidium binds to nucleic acids via two distinct modes: (i) at low ionic strengths it binds to the surface of nucleic acids through electrostatic interactions, and at (ii) higher, physiologically relevant ionic strengths it intercalates between base pairs (LePecq, J. B.; Paoletti, C. *J. Mol. Biol.* 1967, 27, 87-106). Crystallographic and NMR studies have subsequently confirmed ethidium's ability to bind to nucleic acids through these two distinct modes.

[0006] To date, a relatively small number of ethidium derivatives has been reported in the literature. Early modifications included variation of the alkyl chain with groups other than ethyl (methyl, propyl, and the like).

Compared to ethidium, these derivatives have approximately the same DNA affinity, but are significantly more toxic. The 6-position of ethidium has been substituted with various groups (4-amino phenyl, 4-nitro phenyl, methyl, and naphthyl). Again, similar affinities were measured for these derivative. Ethidium's exocyclic amines have been converted to azido (N_3), leading to highly reactive photo-crosslinking agents. These compounds also have a similar DNA affinity as ethidium, and are highly mutagenic. Amino acids have been conjugated to ethidium through its exocyclic amines and through its phenyl ring, but the DNA affinities of these derivatives have not yet been reported.

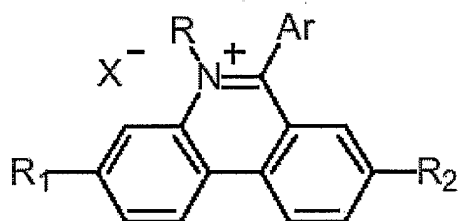
[0007] The rapid evolution of viruses demands the development of new and improved anti-viral agents. Currently there are no useful anti-viral agents that exert their activity by binding to nucleic acids. One reason for this is that compounds that bind to DNA are often highly toxic and/or mutagenic. Accordingly, there is a need for new classes of anti-viral agents that can be used to treated viral infections.

SUMMARY

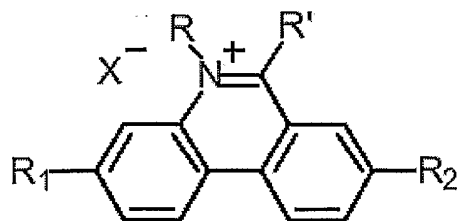
[0008] The invention provides compositions and method for the synthesis of a series of phenanthridine derivatives that have relatively low affinities to DNA, but maintain the anti-viral activity found in more toxic compounds.

[0009] The invention provides a compound comprising a substituted phenanthridine derivatives wherein the amines at the 3- and 8- positions of ethidium bromide are substituted with a guanidine, a diBoc-guanidine, a pyrrole, a urea, a substituted urea, conjugated amino acids and/or carbohydrates.

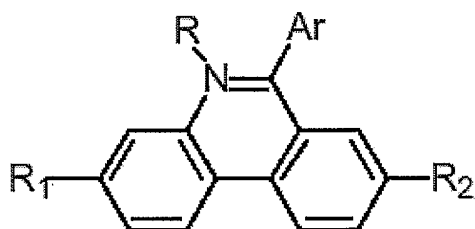
[0010] The invention provides a compound comprising a member selected from the group consisting of:



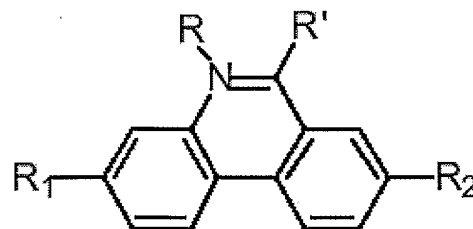
Formula 1A,



Formula 1B

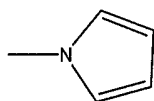


Formula 2A,

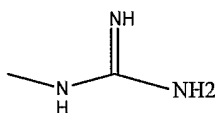


Formula 2B,

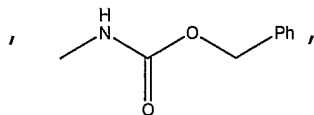
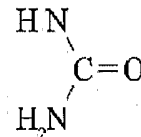
wherein R and R' can be any functionalized or unfunctionalized alkyl, alkenyl, alkynyl, aryl, alkaryl, heteroaryl, or alkheteroaryl, and Ar can be phenyl or any aromatic residue (substituted or not) and wherein R₁ and R₂ are each independently selected from the group consisting of:

NH₂,

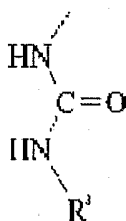
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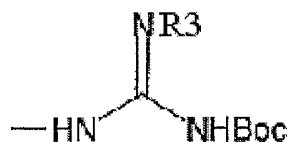
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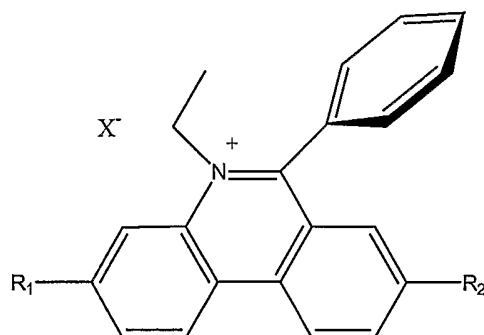
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[0011] The invention also provides a compound having the following formula: (Formula 3)



[0012] The invention also provides a composition comprising a compound as set forth by the invention (e.g., formulas 1-3) and a pharmaceutically acceptable carrier.

[0013] The invention further provides a method of treating a subject having or at risk of having a bacterial or viral infection, comprising, contacting the subject with a compound or composition of the invention in an amount sufficient to inhibit or prevent the bacterial or viral infection.

[0014] The details of one or more embodiments of the invention are set forth in the accompanying figures and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

[0015] Figure 1A shows a secondary structure of the 66 nucleotide Rev Response Element "RRE66" from the HIV-1 isolate HXB3. The high affinity Rev binding site is shown in bold.

[0016] Figure 1B shows the RRE binding domain from the Rev protein "Rev₃₄₋₅₀". RevF1 is used for fluorescence anisotropy displacement experiments and has a RRE affinity similar to the Rev protein.

[0017] Figure 2 shows the protection of ethidium bromide with benzyl chloroformate. Reagents and conditions: (a) Benzyl chloroformate, acetone, aqueous buffer pH 6.6. (b) AG1-X4 (Cl⁻) ion exchange resin.

[0018] Figure 3 shows a synthesis of guanidino derivatives of ethidium. Reagents and conditions: (a) N,N'-diBoc-N''-triflylguanidine, (b) N,N'-di-tert-Butoxycarbonyl-5-chloro-1H-benzotriazole-1-carboxamide, (c) N,N'-bis-Boc-S-methyl-isothioureia, mercury dichloride, and 2,4,6-collidine, (d) 6 M HCl/methanol, 100°C.

[0019] Figure 4 shows a synthesis of unsubstituted urea derivatives of ethidium. Reagents and conditions: (a) Phenyl

chloroformate, acetone, aqueous buffer pH 6.6. (b) Methanolic ammonia, 76 °C (c) 6 M HCl/methanol, 100 °C.

[0020] Figure 5 shows examples of ethidium-urea conjugates. Reagents and conditions: (a) Pyrrolidine, DMSO, 90 °C. (b) L-Arg, water/DMSO, 2,4,6-collidine, 90 °C. (c) Ethylene diamine, DMSO, 85 °C. (d) 2-deoxystreptamine, water/DMSO, phenol, Na₂CO₃, 90 °C.

[0021] Figure 6 shows the synthesis of pyrrole derivatives of ethidium. Reagents and conditions: (a) 2, 5 dimethoxy tetrahydrofuran, acetic acid, 120 °C. (b) H₂/Pd.

[0022] Figure 7 shows examples of RevFI-RRE association and inhibition. (a) The fluorescence anisotropy of 10 nM solution of RevFl is monitored as the RRE66 is titrated. (b) Upon mixing 10 nM each of RevFl and RRE66, ethidium bromide (1) is titrated while monitoring the fluorescence anisotropy of RevFl (b).

[0023] Figure 8 shows the binding of ethidium bromide (1) by calf thymus (CT) DNA. The fluorescence intensity of a 1 μM solution of ethidium in aqueous buffer increases upon addition of CT DNA (excitation 480 nm). By assuming a linear relationship between fluorescence intensity (606 nm) and the fraction bound, a simple binding isotherm is revealed (inset).

[0024] Figure 9 is a schematic of an assay for measuring biological effects of the compounds.

DETAILED DESCRIPTION

[0025] Many viruses, including HIV, use RNA for their genetic material. Small organic molecules that selectively bind to viral RNA and/or DNA sites have potential as anti-viral agents. Many of these compounds also bind to the host cell's DNA and thus exhibit toxic and/or mutagenic side effects as well. The invention provides a library of compounds that maximize the binding affinity of phenanthridine to viral RNA and DNA sites, while minimizing the binding to

host-cell DNA. The antiviral activity of the compounds can thus be maximized, while toxic and/or mutagenic side effects are minimized. The potential of these compounds has been measured by studying the ability of these compounds to bind to HIV-1 viral RNA versus Calf Thymus DNA. In addition, the compounds were evaluated by measuring anti-HIV activity of the compounds in cell cultures.

[0026] Recent studies have shown that ethidium bromide has the ability to bind to the HIV-1 Rev Response Element (RRE) with high affinity. Subsequent evaluation for its ability to inhibit HIV-1 gene expression indicates that ethidium is a potent inhibitor of HIV replication, with an IC_{50} of approximately $0.2 \mu M$ ($8 \times 10^{-5} g/L$). The Rev-RRE interaction is a protein-RNA interaction essential for the replication of HIV. The Rev protein binds to the RRE and facilitates the export of the viral transcript from the nucleus, while protecting it from the cell's splicing machinery. Without Rev-RRE binding, the proteins needed for viral production are never translated. The Rev binding site on the RRE is found to be highly conserved even between different groups of HIV isolates (bold bases, Figure 1(SEQ ID NO:1)). Compounds that inhibit HIV replication by binding to the RRE and displacing Rev are expected, therefore, to retain activity across genetically diverse HIV. The potent anti-HIV activity of ethidium can, in principle, be related to the inhibition of DNA integration, RNA synthesis, protein synthesis, viral packaging, and the like.

[0027] Many natural compounds that bear a guanidine function have biological activity that make them useful as pharmaceuticals. Among these compounds are antimicrobials, antifungals, antivirals, neurotoxins, hormones, and agents that act as agonists or antagonists to biological signals. A review of these natural products is presented in *Progress in the Chemistry of Organic Natural Products* (1995) 66:119 and

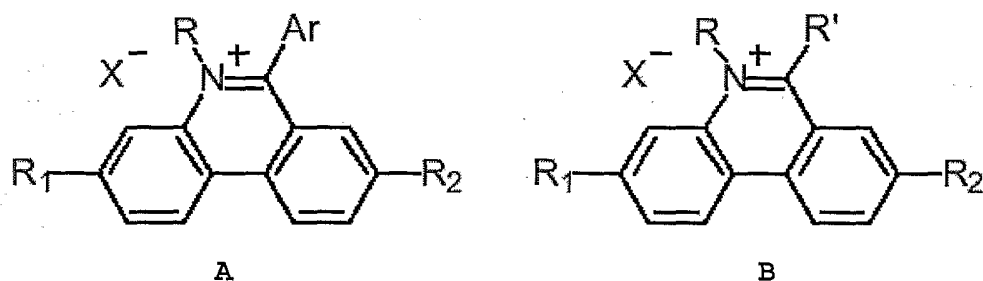
Berlinck, R. G. S. (1996) *Nat. Prod. Reports* 13(5):377409. Much effort has been directed to developing routes for preparing these compounds or their analogues synthetically.

[0028] To date, a relatively small number of ethidium derivatives have been reported in the literature. Surprisingly, the exocyclic amines of ethidium have not, until now, been systematically substituted with other functional groups. Modification of one, or both, of these amines provides a "modular" approach for introducing new chemical diversity onto the phenanthridinium core of ethidium. These modifications dramatically affect the electronic structure of ethidium, as well as the nucleic acid affinity and specificity of the resulting derivatives.

[0029] In an attempt to decrease its DNA affinity (and hopefully its toxic and mutagenic activities as well) a library of phenanthridinium derivatives were synthesized by modifying the exocyclic amines of ethidium bromide. The synthesis, characterization, and spectroscopic properties of these new derivatives are further described herein. The nucleic acid specificity of these compounds was conducted by measuring the apparent affinity of each compound to calf thymus DNA as compared to the Rev binding site on the RRE. By decreasing the DNA affinity of ethidium, the derivatives will be more selective for viral RNA sites and will, consequently, have better anti-viral potency and decreased mutagenic activities.

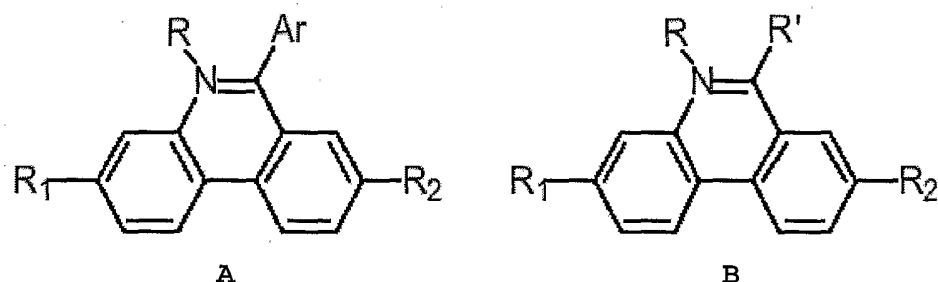
[0030] The invention provides a library of compounds based upon phenanthridine (see Table 1). These compounds have been characterized for their binding ability to DNA as well as viral-RNA (e.g. the HIV-1 RRE). Of particular interest are the derivatives 3-urea ethidium, 8-urea ethidium, and 3,8-bis urea ethidium, which exhibit potent anti-HIV activities, but have a much lower affinity to DNA and do not exhibit signs of toxicity to HeLA cell cultures at 10 μ M (Table 1).

[0031] The invention provides a compound comprising a charged phenanthridinium derivative having the general formulas:



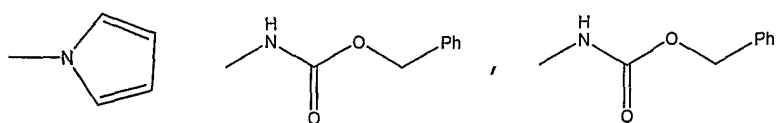
Formula 1

In another aspect, the invention provides a compound comprising an uncharged phenanthridine derivative having the general formulas:



Formula 2

Wherein R and R' can be any functionalized or unfunctionalized alkyl, alkenyl, alkynyl, aryl, alkaryl, heteroaryl, or alkheteroaryl, and Ar can be phenyl or any aromatic residue (substituted or not). Wherein R₁ and R₂ are each independently selected from the group consisting of a urea, a substituted urea, a diboc-guanidine, conjugated amino acids, carbohydrates, -NH₂,



[0032] "Alkyl" refers to a saturated branched, straight chain or cyclic hydrocarbon radical. Typical alkyl groups include, but are not limited to, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, isopentyl, hexyl, and the like.

[0033] "Alkenyl" refers to an unsaturated branched, straight chain or cyclic hydrocarbon radical having at least one carbon-carbon double bond. The radical may be in either the cis or trans conformation about the double bond(s). Typical alkenyl groups include, but are not limited to, ethenyl, propenyl, isopropenyl, butenyl, isobutenyl, tert-butenyl, pentenyl, hexenyl and the like.

[0034] "Alkynyl" refers to an unsaturated branched, straight chain or cyclic hydrocarbon radical having at least one carbon-carbon triple bond. Typical alkynyl groups include, but are not limited to, ethynyl, propynyl, butynyl, isobutynyl, pentynyl, hexynyl and the like.

[0035] "Aryl" refers to an unsaturated cyclic hydrocarbon radical having a conjugated π electron system. Typical aryl groups include, but are not limited to, penta-2,4-diene, phenyl, naphthyl, anthracyl, azulenyl, chrysenyl, coronenyl, fluoranthenyl, indacenyl, idenyl, ovalenyl, perylenyl, phenalenyl, phenanthrenyl, picenyl, pleiadenyl, pyrenyl, pyranthrenyl, rubicenyl, and the like.

[0036] "Alkaryl" refers to a straight-chain alkyl, alkenyl or alkynyl group wherein one of the hydrogen atoms bonded to a terminal carbon is replaced with an aryl moiety. Typical alkaryl groups include, but are not limited to, benzyl, benzylidene, benzylidyne, benzenobenzyl, naphthenobenzyl and the like. For example, the alkaryl group can be (C_6 - C_{26}) alkaryl, e.g., the alkyl, alkenyl or alkynyl moiety of the alkaryl group is (C_1 - C_6) and the aryl moiety is (C_5 - C_{20}).

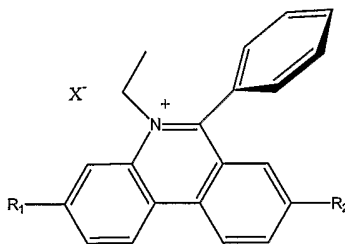
[0037] "Heteroaryl" refers to an aryl moiety wherein one or more carbon atoms is replaced with another atom, such as N, P,

O, S, As, Se, Si, Te, and the like. Typical heteroaryl groups include, but are not limited to, acridarsine, acridine, arsanthridine, arsindeole, arsinoline, carbazole, β -carboline, chromene, cinnoline, furan, imidazole, indazole, indole, indolizine, isoarsindeole, isoarsinoline, isobenzofuran, isochromene, isoindole, isophosphoindole, isophosphinoline, isoquinoline, isothiazole, isoxazole, naphthyridine, perimidine, phenanthridine, phenanthroline, phenazine, phosphoindole, phosphinoline, phthalazine, pteridine, purine, pyran, pyrazine, pyrazole, pyridazine, pyridine, pyrimidine, pyrrole, pyrrolizine, quinazoline, quinoline, quinolizine, quinoxaline, selenophene, tellurophene, thiophene and xanthene.

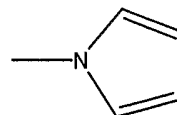
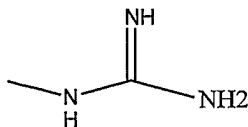
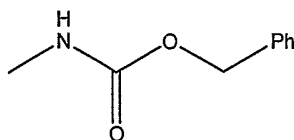
[0038] "Alkheteroaryl" refers to a straight-chain alkyl, alkenyl or alkynyl group where one of the hydrogen atoms bonded to a terminal carbon atom is replaced with a heteroaryl moiety.

[0039] "Substituted Alkyl, Alkenyl, Alkynyl, Aryl, Alkaryl, Heteroaryl or Alkheteroaryl" refers to an alkyl, alkenyl, alkynyl, aryl, alkaryl, heteroaryl or alkheteroaryl group in which one or more hydrogen atoms is replaced with another substituent.

[0040] In one aspect of the invention, the compound has the formula: (Formula 3)

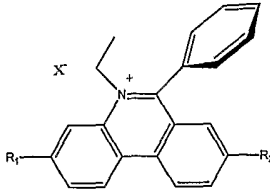


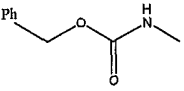
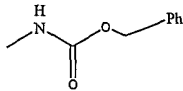
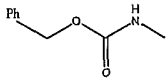
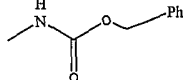
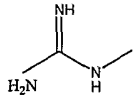
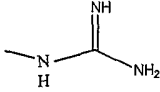
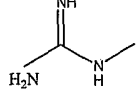
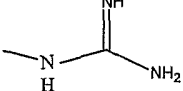
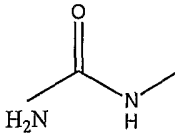
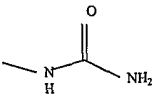
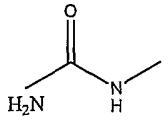
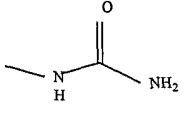
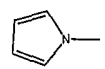
Wherein R_1 and R_2 are each independently selected from the group consisting of: a urea, a substituted urea, a diboc-guanidine, conjugated amino acids, carbohydrates, $-NH_2$,



[0041] The invention also provides compounds based upon Formula 1A and/or B, having the respective R groups and properties as set forth in Table 1:

Table 1: Phenanthridine derivatives and some biological activities.



Compound	R ₁	R ₂	DNA Affinity K _d (μM)	Rev-RRE IC ₅₀ (μM)	HIV-1 IC ₅₀ (μM)	Toxic HeLa at 10 μM
3,8-diamino-5-ethyl. 6-phenyl-phenanthridinium (ethidium)	H ₂ N-	-NH ₂	3.1	0.2	0.2	YES
3-cbz ethidium		-NH ₂	N.D.	N.D.	N.D.	N.D.
8-cbz-ethidium	H ₂ N-		N.D.	N.D.	N.D.	N.D.
3,8-bis-coz-ethidium			N.D.	N.D.	N.D.	N.D.
3-guanidino-ethidium		-NH ₂	8.6	4.1	N.D.	N.D.
8-guanidino ethidium	H ₂ N-		36	8.1	N.D.	N.D.
3,8-bis guanidine ethidium			21	11	N.D.	N.D.
3-urea-ethidium		-NH ₂	36	0.4	15.	NO
8-urea-ethidium	H ₂ N-		18	4.0	3.0	NO
3,8-bis urea ethidium			106	>1.0	1.5	NO
3-pyrole ethidium		-NH ₂	12	0.6	N.D.	N.D.
8-pyrole ethidium	H ₂ N-		5.6	0.4	1.5	YES
3,8-bis pyrole ethidium			N.D.	>4.0	N.D.	N.D.

[0042] In one embodiment, the invention provides a method for treating a subject having a bacterial or viral infection or treating a subject susceptible to infection with a bacteria or virus. The method includes administering a compound, an analogue, derivative, or salt thereof of the invention or a pharmaceutical composition comprising a compound, an analogue, derivative, or salt thereof of the invention, prior to, simultaneously with, or subsequent to infection by a bacteria or viral organism.

[0043] In another embodiment, the invention provides a method of inhibiting or modulating the progression of viral infections (e.g., retroviral infections associated with HIV, HBV, and the like), bacterial infections, and disorders associated with, for example, inappropriate mitogenic signaling, non-insulin-dependent diabetes, and inhibition of disorders associated with thrombin, glycosidases, and nitric oxide synthases.

[0044] The compounds of the invention, as well as analogues, derivatives, or salts thereof, are useful in the treatment of bacterial or viral infections in general, either separately or in combination with other antibiotic or antiviral agents. These compounds may be administered orally, topically or parenterally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes subcutaneous injections, aerosol, intravenous, intramuscular, intrathecal, intracranial, intrastemal injection or infusion techniques.

[0045] The invention also has the objective of providing suitable topical, oral, and parenteral pharmaceutical preparations for use in the treatment of bacterial and viral infections. The compounds of the invention may be administered orally as tablets, aqueous or oily suspensions, lozenges, troches, powders, granules, emulsions, capsules,

syrups or elixirs. The composition for oral use may contain one or more agents selected from the group consisting of sweetening agents, flavouring agents, colouring agents and preserving agents in order to produce pharmaceutically palatable preparations. The tablets contain the active ingredient (a compound of the invention) in admixture with non-toxic pharmaceutically acceptable excipients which are suitable for the manufacture of tablets. These excipients may be, for example, (1) inert diluents, such as calcium carbonate, lactose, calcium phosphate or sodium phosphate; (2) granulating and disintegrating agents, such as corn starch or alginic acid; (3) binding agents, such as starch, gelatin or acacia; and (4) lubricating agents, such as magnesium stearate, stearic acid or talc. These tablets may be uncoated or coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed. Coating may also be performed using techniques described in the U.S. Pat. Nos. 4,256,108; 4,160,452; and 4,265,874 to form osmotic therapeutic tablets for control release. In one aspect, the invention provides pharmaceutical or therapeutic preparation comprising a compound of the invention for delivery to the gastrointestinal tract, which tends to be the site of viral loads of HIV.

[0046] The phenanthridine derivatives of the invention (including analogues or salts thereof) can be administered, for *in vivo* application, parenterally by injection or by gradual perfusion over time independently or together. Administration may be intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally. For *in vitro* studies the agents may be added or dissolved in an appropriate biologically acceptable buffer and added to a cell or tissue.

[0047] Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, growth factors and inert gases and the like.

[0048] The invention relates to a method of administering to a subject an amount of the composition of the invention, which is effective to inhibit the spread of viral particle in a subject, to maintain or decrease viral load in a subject having a viral infection, and/or to inhibit activation of viral particles in a subject, including reverse transcription and transcription of viral nucleic acids.

[0049] The effective amount of the composition will vary depending on such factors as the subject being treated, the particular mode of administration, the activity of the particular active ingredients employed, the age, bodyweight, general health, sex and diet of the subject, time of administration, rate of excretion, the particular combination of ingredients employed, the viral load of a subject infected with a virus, and the total content of the main ingredients of the composition. It is within the skill of the person of ordinary skill in the art to account for these factors.

[0050] As noted above, the particular route of administration can influence the effective amount and duration

of treatment with the composition of the invention as well as the frequency of administration. For example, orally administered agents may require higher concentrations to deliver an effective amount to a target area or tissue than administration to a mucus membrane or intravenous or intraperitoneal routes.

[0051] The compositions and formulations of the invention can be used to treat viral infections as discussed herein. For example, the methods, compositions, and formulations can be used to treat retroviral infections as well as non-retroviral infections.

[0052] The methods of the invention can be used to treat viral infections by RNA retroviruses. When a host cell is infected with a retrovirus, the genomic RNA is reverse transcribed into a DNA intermediate that is integrated very efficiently into the chromosomal DNA of infected cells. The integrated DNA intermediate is referred to as a provirus. The family Retroviridae are enveloped single-stranded RNA viruses typically infect mammals, such as, for example, bovines, monkeys, sheep, and humans. Retroviruses are unique among RNA viruses in that their multiplication involves the synthesis of a DNA copy of the RNA, which is then integrated into the genome of the infected cell.

[0053] The Retroviridae family consists of three groups: the spumaviruses (or foamy viruses) such as the human foamy virus (HFV); the lentiviruses, as well as visna virus of sheep; and the oncoviruses (although not all viruses within this group are oncogenic). The term "lentivirus" is used in its conventional sense to describe a genus of viruses containing reverse transcriptase. The lentiviruses include the "immunodeficiency viruses" which include human immunodeficiency virus (HIV) type 1 and type 2 (HIV-1 and HIV-2) and simian immunodeficiency virus (SIV). In the absence of effective therapy, most individuals infected with a human

immunodeficiency virus develop acquired immune deficiency syndrome (AIDS) and succumb to either opportunistic infections and malignancies resulting from either the deterioration of the immune system or the direct effects of the virus. The oncoviruses are further subdivided into groups A, B, C and D on the basis of particle morphology, as seen under the electron microscope during viral maturation. The prototype B-type virus is mouse mammary tumor virus (MMTV). The C-type viruses are the most commonly studied and include many of the avian and murine leukemia viruses. Bovine leukemia virus (BLV), and the human T-cell leukemia viruses types I and II (HTLV-I/II) are similarly classified as C-type particles because of the morphology of their budding from the cell surface. Mason Pfizer monkey virus (MPMV) is the prototype D-type virus.

[0054] Retroviruses are defined by the way in which they replicate their genetic material. During replication the RNA is converted into DNA. Following infection of the cell a double-stranded molecule of DNA is generated from the two molecules of RNA, which are carried in the viral particle by the molecular process known as reverse transcription. The DNA form becomes covalently integrated in the host cell genome as a provirus, from which viral RNAs are expressed with the aid of cellular and/or viral factors. The expressed viral RNAs are packaged into particles and released as infectious virions.

[0055] The formulations, compositions and methods of the invention are useful to treat viral infection. It is contemplated that the formulations and compositions may include or be used with additional anti-viral agents, including anti-inflammatory agents (antibodies, peptides, peptidomimetics, chemical compositions, and the like) alone, or in combination with antivirals (antibodies, peptides, virus protein/enzyme inhibitors, and the like). These formulations,

compositions and methods are all useful for treating subjects either having or at risk of having an immunodeficiency virus (e.g., HIV) related disorder, or having or at risk of transmitting an HIV related disorder. AIDS and ARC are particular examples of such disorders. HIV-associated disorders have been recognized primarily in "at risk" groups, including homosexually active males, intravenous drug users, recipients of blood or blood products, and certain populations from Central Africa and the Caribbean. The syndrome has also been recognized in heterosexual partners of individuals in all "at risk" groups and in infants of affected mothers.

[0056] Retroviruses have been linked to a wide range of diseases, including anemia, neurological disorders, immune suppression, and malignancy. HTLV-I, for example, is associated with tropical spastic paraparesis, a condition similar in some respects to multiple sclerosis.

[0057] The compositions and formulations of the invention can be used to treat such viral infections as well as the underlying diseases or pathologies associated with infection.

[0058] In another aspect, the invention provides methods of making the phenanthridine derivatives of the disclosure. The exocyclic amines of ethidium are poor nucleophiles and only weakly basic ($\text{pK}_{\text{a}1} = 0.8$, $\text{pK}_{\text{a}2} = 2$). The electron withdrawing effect of the phenanthridinium core requires the use of highly reactive electrophiles to modify ethidium's exocyclic amines. Until now, no general method for the systematic protection and "modular" modification of ethidium's exocyclic amines has been reported. Each of ethidium's exocyclic amines can be protected in a one-pot reaction with benzyl chloroformate (cbz-chloride). By using one equivalent of cbz-chloride, a mixture of products is obtained that can be purified using standard silica gel chromatography (Figure 2). The main products are 8-cbz-ethidium chloride (50%) and 3-cbz-ethidium chloride (10%). The remainder is 3,8-bis-cbz ethidium chloride (4%) and

unreacted starting material (Figure 2). The assignment of each mono-protected cbz compound (2) and (3) has been confirmed using X-ray Crystallography. Other research groups have also reported a greater reactivity of the exocyclic amine at the 8 position (relative to the 3 position) and have attributed the difference to steric constraints imposed by dimerization of ethidium in solution. It is more likely, however, that the inherent electronic characteristics of ethidium are responsible for the differences in reactivity of its exocyclic amines. Guanidinylation of ethidium was conducted using three different methods (Figure 3). The best reagent for guanidinylation of ethidium proved to be N,N'-bis-Boc-S-methyl-isothiourea (activated with (II) mercury chloride) which afforded a 70% isolated yield of the protected product (3-diBoc-guanidino-8-cbz ethidium chloride) (Figure 3). Removal of both the cbz and Boc groups was performed simultaneously by refluxing 3-diBoc-guanidino-8-cbz-ethidium chloride in 6M HCl/MeOH for 1 hr. Reversed-phase chromatography was used to purify the desired product 6 that was obtained in 70% yield. The other guanidino ethidium derivatives, 8 and 9, were synthesized in similar yields using the same method (Figure 3).

[0059] Urea is a non-charged isostructural analog of guanidine, and provides an important comparison for guanidinium-containing ethidium derivatives. Once again, the limited reactivity of ethidium's electron-poor exocyclic amines rendered some urea-forming reagents ineffective. By using phenyl chloroformate, however, ethidium's exocyclic amines can be activated for subsequent urea formation. This two-step approach allows for facile synthesis of substituted and unsubstituted ureas. Displacement of phenol by ammonia produces the unsubstituted urea derivatives 11, 13, and 15 (Figure 4). Displacement of phenol by other amines yields the substituted ureas 16 - 19 (Figure 5).

[0060] The pyrrole-containing ethidium derivatives **20**, **21**, and **22** have been prepared using 2,5-dimethoxytetrahydrofuran (Figure 6). The yields obtained for the mono-substituted derivatives **20** and **21** are associated with the removal of cbz using H₂/Pd. The phenanthridinium core of ethidium is susceptible to reduction and degradation under these conditions. Better yields can be obtained by refluxing the protected products in 6M HCl/methanol to remove cbz (step b, Figure 6).

[0061] Fluorescence anisotropy was used to monitor the formation and subsequent inhibition of a Rev-RRE complex. The anisotropy of a fluorescent Rev peptide "RevFI" increases upon titration of the RRE66 (Figure 7A). Analysis of this isotherm yields a dissociation constant of 5 ± 1 nM. This is similar to the affinity reported for Rev protein itself. Upon titration of an inhibitory ligand, RevFI is displaced from the RRE into solution and the anisotropy value decreases back to the value of the free peptide (Figure 7B). The concentration of each inhibitor needed to displace 50% of RevFI from the RRE (the IC₅₀ value) is given in Table 2. From the IC₅₀ value, the apparent binding affinity (K_i) for each compound at or near the Rev binding site can be calculated. This value assumes a single binding site for the small molecule is responsible for displacing Rev, hence the term *apparent* affinity is used (Table 2). Most derivatives tested have a significantly lower RRE lower affinity at or near the Rev binding site of the RRE as compared to ethidium bromide (Table 2). One exception is the substituted urea derivative 3,8-bis-urea-ethylenediamine-5-ethyl-6-phenylphenanthridinium trifluoroacetate (**18**). This compound has a modest (2-fold) higher apparent RRE affinity as compared to ethidium bromide (Table 2). A much larger difference in RRE specificity is, however, observed for **18**.

Table 2: Summary of RevF1 displacement experiments (by anisotropy) and of direct binding experiments with calf thymus (CT) DNA

Compound (Trivial names)	Rev-RRE IC ₅₀ (μM) ^I	Apparent RRE K _i (μM) ^{II}	CT DNA C ₅₀ (μM) ^{III}	Apparent CT DNA K _d (μM) ^{IV}	RRE Selectivity Ratio ^V
Ethidium (1)	0.2	0.05	14	2.3	46
3-guanidino-ethidium (6)	4.1	1.0	30	5.5	5.5
8-guanidino-ethidium (8)	8.1	2.0	120	24	12
3,8-bis-guanidino-ethidium (9)	11	2.8	70	14	5
3-urea-ethidium (11)	0.4	0.10	120	24	240
8-urea-ethidium (13)	4.0	1.0	60	12	12
3,8-bisurea-ethidium (15)	>1 ^{VI}	>0.25 ^{VI}	350	70	<280 ^{VI}
3,8-bis-urea-ethylenediamine-ethidium (18)	0.1	0.02	200	40	2,000
3,8-bisurea-2-DOS-ethidium (19)	0.2	0.05	20	3.5	70
3-pyrole-ethidium (20)	0.6	0.15	40	7.5	50
8-pyrole-ethidium (21)	0.4	0.10	20	3.5	35
3,8-diamino-6-phenyl-phenanthridine	>4 ^{VI}	>1 ^{VI}	120	24	<24 ^{VI}

^I 10 nM each RevF1 and RRE66, approximate error +/- 30% of the reported value.

^{II} Apparent K_i = ((IC₅₀ - 0.007)/4).

^{III} Concentration of CT DNA (inb.p.) needed to bind 1/2 of a 1 μM solution of each ligand.

^{IV} Apparent K_d = ((C₅₀ ° 2) - 0.5).

^V Ratio of (CT DNA K_d/Rev-RRE K_i)

^{VI} Fluorescence interference with RevF1 allows only a limit to be reported.

[0062] To evaluate the RRE specificity and mutagenic potential of each compound the binding affinity to calf thymus (CT) DNA has been determined. The fluorescence emission spectrum of each derivative shows unique changes upon binding CT DNA (see Figure 8 for a representative titration, and Table 3 for a summary of the spectral changes for each compound). The concentration of DNA (in base pairs) needed to bind 50% of each compound (the C₅₀ value) is measured by assuming that the change in fluorescence intensity of each compound is proportional to the fraction of the compound bound by DNA (see inset of Figure 8). From each C₅₀ value, the apparent binding affinity (K_d) is calculated by assuming that the binding stoichiometry established for ethidium bromide and CT DNA (0.2 ethidium molecules per base pair), holds for all compounds tested (Table 2). By taking the apparent affinity of each compound to CT DNA divided by its apparent affinity to the Rev binding site, an RRE selectivity ratio is calculated (Table

2). The higher this ratio is, the more selective each compound is for the RRE (relative to CT DNA). Interestingly, all the compounds evaluated exhibit a higher affinity to the RRE as compared to CT DNA (Table 2). This is consistent with the observation that intercalating agents have a higher affinity to duplex regions that contain bulged bases and other imperfections as compared to unperturbed duplexes.

[0063] 3,8-diamino-6-phenylphenanthridine, an uncharged analog of ethidium has at least a 10-fold lower affinity to both the RRE and CT DNA (Table 2). This suggests that positive charge afforded by ethidium's quarternary amine is important for its high-affinity binding of DNA and the RRE. It was hypothesized that the conversion of the amino groups on ethidium into guanidinium would increase its total charge and, therefore, increase its affinity to the RRE. Indeed, at pH 7.5, the guanidino derivatives 6, 8, and 9 each have a 2⁺ charge, while ethidium has a charge of 1⁺. According to RevFl displacement experiments, the three guanidino derivatives 6, 8, and 9 have a 20 - 60 fold lower RRE affinity as compared to ethidium (Table 2). The urea derivatives 11, 13, and 15 were evaluated for RRE affinity (Table 2). Interestingly, the urea derivatives have much better RRE affinities as compared to the corresponding guanidino derivatives (Table 2). It appears, therefore, that the additional positive charge of the guanidino derivatives actually decreases their RRE affinity. This is the opposite trend as observed for compounds that bind to the surfaces of RNA and DNA. It is possible that the introduction of an additional charged group disrupts the charge distribution on the core of ethidium, resulting in less favorable base stacking interaction. Alternatively, the desolvation of the guanidinium group upon intercalation may impose a significant energetic penalty for binding. Interestingly, the urea derivatives 11 and 15 have lower affinities to CT DNA compared to the corresponding guanidino

derivatives 6 and 9 (Table 2). This is the opposite trend as observed for the RRE. Despite their differences in affinity, the changes in spectral properties of 3,8-bis-guanidino ethidium (9) (upon saturation with CT DNA) are very similar to the changes observed for bis-urea-ethidium (15), suggesting a common binding mode for these derivatives (Table 3). It is possible that the higher positive charge afforded by 9 gives it a higher affinity to the surface of CT DNA when compared to 15.

Table 3: Summary of the maximum wavelength of absorbance ($\lambda_{\max}$), maximum wavelength of emission (λ_{em}), and change in emission intensity upon saturation with CT DNA.

Compound	$\lambda_{\max}(\text{nm})$	Δ in $\lambda_{\max}$ (nm) with DNA ^{II}	$\lambda_{\text{em}}(\text{nm})$	Δ in λ_{em} (nm) with DNA ^{II}	Change in emission intensity ^{III}
Ethidium (1)	480	+40	606	-7	+520%
3-guanidino-ethidium (6)	444	+31	590	-4	+40%
8-guanidino-ethidium (8)	454	+36	605	+7	+150%
3,8-bis-guanidino-ethidium (9)	397	+24	500	0	-50%
3-urea-ethidium (11)	458	+34	587	-17	+300%
8-urea-ethidium (13)	463	+31	601	-13	+570%
3,8-bisurea-ethidium (15)	434	+21	520	0	-63%
3,8-bis-urea-ethylenediamine- ethidium (18)	438	+22	522	-2	-11%
3,8-bis-urea-2DOS-ethidium (19)	438	+22	522	-4	+30%
3-pyrole-ethidium (20)	454	+39	592	-18	+300%
8-pyrole-ethidium (21)	462	+36	603	-10	+580%
3,8-bis-pyrole-ethidium (22)	429	+18	502	0	0%
3,8-diamino-6-phenyl- phenanthridine ^{IV}	402	+111	533	0	-75%

^I 10 μM of each compound in aqueous buffer pH 7.5 (see experimental).

^{II} Difference upon saturation with calf thymus DNA

^{III} Percent change in total emission intensity upon saturation with CT DNA, relative to the intensity of the compound in buffer only (excitation at $\lambda_{\max}$).

^{IV} Becomes protonated upon binding DNA, see reference 58.

[0064] Ethidium bromide is very selective for the RRE, exhibiting almost a 50-fold higher affinity to the RRE as compared to CT DNA. The unsubstituted urea derivative, 3-urea ethidium (II), is even more selective, exhibiting a 240-fold higher affinity to the RRE as compared to CT DNA (Table 2). Compound 11 has a modestly lower RRE affinity when compared to ethidium, but it has a much lower affinity to CT DNA,

resulting in a 5-fold higher RRE selectivity ratio than ethidium (Table 2). A number of other compounds, including **8**, **15**, and **18** also have substantially lower affinities to CT DNA as compared to ethidium (Table 2). This should, in theory, decrease the mutagenic potential of these compounds. One compound, 3,8-bis-urea-ethylene-diamine ethidium (**18**) has both a higher RRE affinity and a lower DNA affinity as compared to ethidium. Compound **18** has about a 2-fold higher RRE affinity and a 20-fold lower DNA affinity as compared to **1** (Table 2). It is possible that the guanidinylation of **18** will improve its RRE affinity and specificity. Compounds **18** and **19** show different spectral changes upon saturation with CT DNA (Table 3). The fluorescence intensity of **19** increases upon binding CT DNA, while the intensity of **18** decreases (Table 3). This may indicate different binding modes (surface binding versus intercalation) of these compounds. The discovery of new ligands that possess both high affinity and high specificity for a therapeutically important RNA site is a challenging modification of ethidium's exocyclic amines. Interestingly, the metabolic activation of ethidium's exocyclic amines by at least three separate enzymes is known to be important for its mutagenic activities *in vivo*. Most of the novel derivatives presented herein (including **15** - **19**) should not be recognized by enzymes that modify aromatic amines. In addition, most of these novel derivatives possess significantly lower DNA affinities when compared to ethidium bromide. Taken together, this suggests that these new derivatives will have significantly lower mutagenetic activities than ethidium. These properties, along with the anti-HIV activities and other potential therapeutic applications are encompassed by the invention.

[0065] The biological activity of a phenanthridine derivatives of the invention can be measured using an assay system comprising HIV-1 response elements (see, e.g., U.S.

Patent No. 6,525,182). For example, RRE-specific binders as well as TAR specific binders can be identified using the methods described herein. The assay is presented with reference to FIG. 9. The Rev-RRE assay relies on the release of a fluorescently tagged Rev fragment from an immobilized RRE-Rev complex and can be performed under various stringency levels. The assay can be conducted in the presence of competing RNA molecules, other potential cellular targets, or cellular extracts. Only high affinity ligands can effectively compete with the bound Rev₃₄₋₅₀ peptide (K_d is approximately 1 nM), thus releasing the highly fluorescent molecules into solution. Non-selective binders will be scavenged by competitor molecules present in solution. Only when a highly selective RRE binder is present will a positive fluorescent signal be elicited in solution.

[0066] The assay is useful for identifying a phenanthridine derivative which binds to RRE. The assay includes an immobilized RRE, a detectably labeled Rev, and a test compound. A test compound that liberates the labeled Rev due to competitive binding to the RRE results in an increase in the labeled Rev in the supernatant of the assay. Accordingly, following incubation of immobilized RRE, a labeled Rev, and a test compound, the supernatant is measured and the amount of label in the supernatant is determined. The amount of label in the supernatant is indicative of the degree or ability of the test compound to bind to the RRE or REV polypeptide. By comparing the amount of label in the supernatant with the amount in a control or standard sample, one can identify a test compound which effectively competes with the Rev for binding to RRE. In the assay depicted in FIG. 9, the ternary complex containing a fluorescently tagged Rev peptide bound to an immobilized RRE is incubated with the test compound (a phenanthridine derivative) under conditions and in the presence of competing RNA molecules in a microcentrifuge

filter tube. After incubation, the mixture is filtered and the filtrate is illuminated. In the presence of a strong and selective RRE binder, the fluorescently labeled peptide will be released from the beads into solution and the filtrate will become fluorescent.

[0067] The RRE can be effectively immobilized to a solid support. For example, a tag is covalently linked to the RRE and the tag is then bound to a tag-binding molecule attached to a solid support, thereby effectively binding the RRE to the solid support. The tag can be any of a variety of components. In general, a molecule which binds the tag (a tag binder) is fixed to a solid support, and the tagged nucleic acid (e.g., the RRE) is attached to the solid support by interaction of the tag and the tag binder. A number of tags and tag binders can be used, based upon known molecular interactions well described in the literature. For example, where a tag has a natural binder, for example, biotin, protein A, or protein G, it can be used in conjunction with appropriate tag binders (avidin, streptavidin, neutravidin, the Fe region of an immunoglobulin, and the like).

[0068] The solid support may be any material known to those of ordinary skill in the art to which a tag-binder may be attached, such as a test well in a microtiter plate, a nitrocellulose filter or another suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex, polymers, metals, metalloids, ceramics, organics, or a plastic such as polystyrene or polyvinylchloride.

[0069] The Rev may be labeled with any number of detectable molecules. The labels can be primary labels (where the label comprises an element which is detected directly) or secondary labels (where the detected label binds to a primary label, e.g., as is common in immunological labeling). Useful primary and secondary labels include spectral labels such as

fluorescent dyes (e.g., fluorescein and derivatives such as fluorescein isothiocyanate (FITC) and Oregon Green™), rhodamine and derivatives (e.g., Texas red, tetrarhodintine isothiocyanate (TRITC), and the like), dioxigenin, biotin, phycoerythrin, AMCA, CyDyes™, and the like), radiolabels (e.g., ^3H , ^{125}I , ^{35}S , ^{14}C , ^{32}P , ^{33}P , and the like), enzymes (e.g., horse-radish peroxidase, alkaline phosphatase, and the like), and spectral calorimetric labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, and the like) beads. The label may be coupled directly or indirectly to Rev according to methods known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions. In general, a detector is adapted to the particular label which is used. Typical detectors include spectrophotometers, phototubes and photodiodes, microscopes, scintillation counters, cameras, film and the like, as well as combinations thereof. Examples of suitable detectors are widely available from a variety of commercial sources known to persons of skill in the art.

[0070] Commonly used labels include those which utilize 1) chemiluminescence (using horseradish peroxidase and/or alkaline phosphatase with substrates that produce photons as breakdown products) with kits being available, e.g., from Molecular Probes, Amersham, Boehringer-Mannheim and Life Technologies/Gibco BRL; 2) color production (using both horseradish peroxidase and/or alkaline phosphatase with substrates that produce a colored precipitate; kits available from Life Technologies/Gibco BRL, and Boehringer-Mannheim); 3) hemifluorescence using, e.g., alkaline phosphatase and the substrate AttoPhos (Amersham) or other substrates that produce fluorescent products, 4) Fluorescence (e.g., using Cy-5

(Amersham), fluorescein, and other fluorescent tags); and 5) radioactivity. Other methods for labeling and detection will be readily apparent to one skilled in the art.

[0071] The basic principle of the assay systems used to identify compounds that inhibit binding of Rev to RRE or compounds that interact with RRE involves preparing a reaction mixture containing the target (e.g., RRE) and the binding partner (e.g., labeled-Rev) under conditions and for a time sufficient to allow the two to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound can be initially included in the reaction mixture, or can be added at a time subsequent to the addition of the RRE and its binding partner, Rev. Control reaction mixtures are incubated without the test compound or with a placebo. The presence of labeled-Rev that is not bound to RRE over that amount found in a control or standard sample is indicative of a test compound that competes with Rev for binding to RRE.

[0072] In a modification of the above assay, the solid support aspect of the above embodiment is removed. The biophysical characteristics of potential inhibitory test compounds can be detected by fluorescence anisotropy. The RRE-bound fluorescent peptide has a slower Brownian tumbling motion relative to the free peptide in solution. Upon displacement of the fluorescently labeled Rev from the RRE by a competing inhibitor, a decrease in the anisotropy value is observed. The displacement of the labeled-Rev can be followed by monitoring the anisotropy at the emission wavelength of the Rev₃₄₋₅₀-bound fluorophore.

EXAMPLES

[0073] A solution of sonicated CT DNA was purchased from Gibco BRL and quantified using a molecular extinction coefficient of 13,100 cm⁻¹M⁻¹ per base pair. All titrations and

photophysical properties were measured at 22 °C in a buffer containing 30 mM HEPES (pH 7.5, KCl (100 mM), sodium phosphate (10 mM), NH₄OAc (20 mM), guanidinium HCl (20 mM), MgCl₂ (2 mM), NaCl (20 mM), EDTA (0.5 mM), and Nonidet P-40 (0.001 %). Fluorescence anisotropy experiments were conducted and K_i values calculated. CT DNA affinities were measured by exciting a 1 µM solution of each compound at the appropriate wavelength (λ_{max}) then adding small aliquots of concentrated CT DNA and monitoring the emission intensity of the compound at the appropriate wavelength (λ_{em}). K_d values are calculated from C₅₀ values based upon the definition of K_d: $K_d = ([\text{dye}] * [\text{nucleic acid}]) / [\text{complex}]$. Once the C₅₀ value is reached, the concentration of free dye [dye] is equal to the concentration of bound dye [complex], so that the K_d = the concentration of free nucleic acid in available binding sites [nucleic acid]. This value is equal to the total concentration of nucleic acid (in base pairs) multiplied by the binding stoichiometry (0.2 equivalents of compound per base pair), minus the concentration of complex (0.5 µM). Hence, under these conditions, the $K_d = ((C_{50} * 0.2) - 0.5)$.

[0074] 3-Cbz-ethidium . Cl (2), 8-cbz-ethidium . Cl (3), and 3,8-bis-cbz ethidium - Cl (4). Ethidium bromide / 8% water (4.13 g, 9.64 mmol) was dissolved in 0.2 M sodium phosphate pH 6.6 (100 mL), acetone (80 mL) and warmed to 32 °C. To this, a solution of benzyl chloroformate (1.43 mL, 10 mmol, 1 equiv) in acetone (20 mL) was slowly added and the reaction warmed to 40 °C for 20 min. AGI-X4 (Cl⁻) ion exchange resin (20 g, 70 mmol, 7 mequiv) was then added and stirred 5 min, 40 °C. The slurry was loaded onto a column containing another 20 g (7 mequiv) of AGI-X4 (Cl⁻) ion exchange resin and the eluent collected. The resin was washed with 30 mL of 1:1 water:acetone, and the eluents were combined and reduced to a solid under reduced pressure. The products were separated on silica gel using three consecutive columns (8 - 10% MeOH /

CH₂Cl₂, 5 - 12% MeOH / CH₂Cl₂, and 10% MeOH / CH₂Cl₂). The pure fractions from each column were combined to yield : 0.48 g of the orange-red solid, **3-cbz-ethidium - Cl (2)** (10%). R_f = 0.5 (20% MeOH / CHCl₃). ¹H NMR (400 MHz, d₆-DMSO, 25 °C): δ 10.61 (s, 1H), δ 8.92 (d, J=9.2Hz, 1H), δ 8.76 (d, J=9.2Hz, 1H), δ 8.67 (s, 1H), δ 7.97 (d, J = 9.2 Hz, 1H), δ 7.72-7.79 (m, 5H), δ 7.58 (dd J₁ = 9.2 Hz, J₂ = 2.2 Hz, 1H), δ 7.36-7.48 (m, 5H), δ 6.38 (d, J = 2.2 Hz, 1H), δ 6.22 (s, 2H), δ 5.24 (s, 2H), δ 4.54 (q, J = 7.0 Hz, 2H), δ 1.45 (t, J = 7.0 Hz, 3H). ESI MS calculated for C₂₉H₂₆N₃O₂: 448.2, found 448.3 [M]⁺. UV-vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm⁻¹ M⁻¹): 212 (4.11 x 10⁴), 284 (5.6 x 10⁴), 454 (4.9 x 10³). 2.3 g of the purple solid, **8-cbz-ethidium Cl (3)** (50%). R_f = 0.38 (20% MeOH/CHCl₃). ¹H NMR (400 MHz, d₆-DMSO, 25 °C): δ 10.31 (s, 1H), δ 8.83 (d, J = 9.6 Hz, 1H), δ 8.78 (d, J = 9.2 Hz, 1H), δ 8.11 (dd J_f = 9.6 Hz, J₂ = 1.6 Hz, 1H), δ 7.71-7.77 (m, 5H), δ 7.64 (d, J = 1.6 Hz, 1H), 7.44 (s, 1H), δ 7.36-7.42 (m, 6H), 6.66 (s, 2H), 5.08 (s, 2H), δ 4.49 (q, J = 7.2 Hz, 2H), δ 1.42 (t, J = 7.2 Hz, 3H). ESI MS calculated for C₂₉H₂₆N₃O₂: 448.2, found 448.3 [M]⁺. UV-vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm⁻¹ M⁻¹): 214 (3.4x10⁴), 286 (4.4 x 10⁴), 460 (4.5 x 10³). 0.23 g of the yellow solid, **3,8-bis-cbz-ethidium - Cl (4)** (4%). R_f = 0.67 (20% MeOH/CHCl₃). ¹H NMR (400 MHz, d₆-DMSO, 25 °C): δ 10.76 (s, 1H), δ 10.45 (s, 1H), δ 8.85 (d, J = 9.2 Hz, 1 H), δ 9.03 (d, J = 9.2 Hz, 1H), δ 8.78 (s, 1H), δ 8.28 (dd J₁ = 9.2 Hz, J₂ = 2.0 Hz, 1H), δ 8.10 (d, J = 9.2 Hz, 1H), 6 7.74-7.81 (m, 6H), δ 7.33-7.49 (m, 10H), δ 5.26 (s, 2H), 5.10 (s, 2H), δ 4.62 (q, J= 7.0 Hz, 2H), δ 1.49 (t, J = 7.2 Hz, 3H). ESI MS calculated for C₃₇H₃₂N₃O₄: 582, found 582 [M]⁺.

[0075] **3-diBoc-guanidino-8-cbz-ethidium - Cl (5)**. 8-cbz ethidium - Cl (3) (48 mg, 100 μmoles, DMF (4 mL), N,N'-bis-Boc-S-methyl-isothioureia (145 mg, 500 μmoles, 5 equiv), and mercury (11) chloride (227 mg, 837 μmoles, 8.4 equiv) were combined and sonicated. 2,4,6 collidine (177 μL, 1.34 mmoles,

13.3 equiv) was added dropwise and the reaction was stirred at RT for 15 min with occasional sonication. The reaction mixture was then dissolved in CHCl_3 (150 mL) and washed with 0.1 M citric acid (4x40 mL), brine (40 mL), dried over sodium sulfate and concentrated to a solid under reduced pressure. The product was purified on a short (2 in) silica gel column (20 mL) using a gradient (0-5% MeOH/ CHCl_3) to yield 47 mg of a solid yellow product (70%). ^1H NMR (400 MHz, d_6 -DMSO, 25 °C): δ 11.32 (s, 1H), δ 10.51 (s, 1H), δ 10.44 (s, 1H), δ 9.23 (s, 1H), δ 9.06-9.09 (m, 2H), δ 8.26 (d, J = 9.2 Hz, 1H), δ 8.17 (d, J = 8.4 Hz, 1H), δ 7.77-7.80 (m, 7H), δ 7.35-7.38 (m, 5H), δ 5.10 (s, 2H), δ 4.68 (q, J = 7.2 Hz, 2H), δ 1.33-1.56 (m, 21H).

[0076] 3-Guanidino-ethidium . 2HCl (6). 3-diBoc-guanidino-8-cbz-ethidium - Cl (5) (20 mg, 29 μmoles) was dissolved in methanol (2 mL) and saturated HCl (2 mL), and heated at 120 °C for 40 min. The reaction flask was then cooled on ice and 2 M NaOH was added dropwise until the yellow solution started to turn orange. The solution was loaded directly onto an activated Water's "Sep-pack" C-18 reversed phase column (activated with 10 mL acetonitrile, 10 mL water) and washed with water (5 mL). The product was eluted with 20-30% acetonitrile/water (0.01 M HCl) and lyophilized to yield 13 mg (99%) of an orange solid. ^1H NMR (300 MHz, d_6 -DMSO, 25 °C): δ 10.61 (s, 1H), δ 9.01 (d, J = 9.0 Hz, 1H), δ 8.85 (d, J = 9.3 Hz, 1H), δ 8.34 (s, 1H), δ 7.74-7.91 (m, 10H), δ 7.62 (dd J , = 9.0 Hz, J_2 = 2.1 Hz, 1H), δ 6.43 (d, J = 2.1 Hz, 1H), δ 6.35 (br s, 2H), δ 4.66 (q, J = 6.9 Hz, 2H), δ 1.43 (t, J = 7.1 Hz, 3H). FAB MS calculated for $\text{C}_{22}\text{H}_{22}\text{N}_5$: 356.1876, found 356.1892 $[\text{M}]^+$. UV-vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ϵ ($\text{cm}^{-1}\text{M}^{-1}$): 214 (3.3×10^4), 284 (4.8×10^4), 444 (4.1×10^3).

[0077] 3-Cbz-8-diBoc-guanidino-ethidium . Cl (7). 3-cbz-ethidium - Cl (2) (37 mg, 76 μmoles , DMF (4 mL), N,N' -bis-Boc-S-methyl-isothiourea (112 mg, 290 μmoles , 3.8 equiv), and

mercury dichloride (175 mg, 645 μ moles, 8.5 equiv) were combined, sonicated, and 2,4,6-collidine (136 μ L, 1.03 mmoles, 13.5 equiv) was added dropwise. The reaction was stirred at RT for 30 min with occasional sonication. The reaction was then diluted into CHCl_3 (200 mL) and washed with 0.1 M citric acid (3 x 50 mL), brine (50 mL), dried over sodium sulfate and concentrated to a solid under reduced pressure. The product was purified on a short (2 in) silica gel column (20 mL) using a gradient (0-5% MeOH/ CHCl_3) to yield 40 mg of a solid yellow product (76%). ^1H NMR (300 MHz, d_6 -DMSO, 25 $^\circ\text{C}$): δ 10.92 (s, 1H), δ 10.74 (s, 1H), δ 10.14 (s, 1H), δ 9.13 (d, J = 9.3 Hz, 1H), δ 9.04 (d, J = 9.3 Hz, 1H), δ 8.78 (s, 1H), δ 8.31 (dd, J_1 = 9.0 Hz, J_2 = 2.1 Hz, 1H), δ 8.08 (d, J = 8.7 Hz, 1H), δ 7.85 (d, J = 2.1 Hz, 1H), δ 7.75-7.77 (m, 5H), δ 7.37-7.49 (m, 5H), δ 5.26 (s, 2H), δ 4.59 (q, J = 6.3 Hz, 2H), δ 1.31-1.51 (m, 21H).

[0078] **8-Guanidino-ethidium - 2HCl (8).** 3-Cbz-8-diBoc-guanidino-ethidium - Cl (7) (6 mg, 8.7 μ moles) was dissolved in 6M HCl (2 mL) and heated to 100 $^\circ\text{C}$ for 1 hr. The reaction flask was then cooled on ice and NaHCO_3 was added until the yellow solution turned orange. The solution was loaded directly onto an activated Water's "Sep-pack" C-18 reversed phase column (activated with 10 mL acetonitrile, 10 mL water). The column was washed with 1M NaCl (5 mL), water (5 mL) and the product eluted with 25% acetonitrile/water (0.01M HCl) and lyophilized to 3.5 mg (95%) of an orange solid. ^1H NMR (300 MHz, d_6 -DMSO, 20 $^\circ\text{C}$): δ 10.28 (s, 1H), δ 8.91 (d, J = 9.6 Hz, 1H), δ 8.88 (d, J = 9.3 Hz, 1H), δ 8.02 (dd, J = 9.3 Hz, J_2 = 2.1 Hz, 1H), δ 7.72-7.85 (m, 9H), δ 7.47 (s, 1H), δ 7.43 (d, J = 8.7 Hz, 1H), δ 7.02 (d, J = 2.1 Hz, 1H), δ 6.78 (br s, 2H), δ 4.52 (q, J = 6.9 Hz, 2H), δ 1.43 (t, J = 6.9 Hz, 3H). FAB MS calculated for $\text{C}_{22}\text{H}_{22}\text{N}_5$: 356.1876, found 356.1862 $[\text{M}]^+$. UV-vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ϵ ($\text{cm}^{-1}\text{M}^{-1}$): 213 (2.8×10^4), 242 (1.5×10^4), 288 (3.6×10^4), 453 (4.4×10^3).

[0079] **3,8-Bis-guanidino-ethidium - 3HCl (9).** Ethidium bromide/8% water (1) (30 mg, 70 μ moles) was dissolved in DMF (3 mL), brought to 0 °C, and N,N'-bis-Boc-S-methyl-isothioureia (180 mg, 620 μ moles, 4.4 mequiv), mercury dichloride (282 mg, 1.04 μ moles, 7.4 mequiv) and 2,4,6-collidine (220 μ L, 1.67 μ moles, 12 mequiv) were added. The reaction was slowly warmed to RT, stirred for an additional 12 h, then diluted into 150 mL CHCl_3 and washed with 0.1 M citric acid (3x50 mL), 50 g/L of EDTA (40 mL), brine (40 mL) dried over sodium sulfate and concentrated to a solid under reduced pressure. Silica gel (40 mL) was used to purify the BOC-protected product (1.5 - 5% MeOH/ CHCl_3) to yield 40 mg of a yellow solid. The product was then deprotected by adding TFA (4 mL, containing 2.5 % (v/v) of triisopropylsilane) and mixing for 1 hr at RT, it was then diluted into 150 mL water and washed with diethyl ether (3x40 mL) and CHCl_3 (3x40 mL). The aqueous phase was concentrated to a solid, then dissolved in water (5 mL) and treated with AGI-X4 (Cl^-) ion exchange resin (1.5 g, 5.2 μ moles, 74 mequiv) for 5 min at RT. It was then filtered over an activated Water's "Sep-pack" C-18 reversed phase column (activated with 10 mL acetonitrile, 10 mL water), the remainder of the product eluted from the column using 20% acetonitrile / water and lyophilized to 25 mg of a yellow solid (76%). ^1H NMR (300 MHz, D_2O , 25 °C): δ 8.90 (d, J = 9.0 Hz, 1H), δ 8.83 (d, J = 9.3 Hz, 1H), δ 8.22 (d, J = 1.8 Hz, 1H), δ 7.96 (dd J_1 = 8.7 Hz, J_2 = 2.1 Hz, 1H), δ 7.84 (dd, J = 9.0 Hz, J_2 = 1.8 Hz, 1H), δ 7.57-7.65 (m, 3H), δ 7.42-7.46 (m, 2H), δ 7.32 (d, J = 2.4 Hz, 1H), δ 4.73 (q, J = 7.2 Hz, 2H), δ 1.39 (t, J = 7.2 Hz, 3H). ESI MS calculated for $\text{C}_{23}\text{H}_{24}\text{N}_7$: 398.2, found 398.3 $[\text{M}]^+$. UV-vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ϵ ($\text{cm}^{-1}\text{M}^{-1}$): 213 (3.6×10^4), 278 (5.4×10^4) ~ 398 (5.0×10^3).

[0080] **3-Phenoxycarbamate-8-cbz ethidium . H_2PO_4 (10).** 8-cbz-ethidium . Cl (3) (180 mg, 372 μ moles), acetone (12 mL), and 500 mM sodium phosphate pH 6.6 (4 mL) were combined and

phenyl chloroformate (200 μ L, 1.58 mmol, 4.2 mequiv, diluted into 2 mL of acetone) was added dropwise and stirred for 2 h at RT. Water (5 mL) was then added dropwise and the precipitate was collected by vacuum filtration. The precipitate was then washed with water (10 mL), 3:1 water/acetone (10 mL) and eluted from the filter with methanol (200 mL). The methanolic fraction was then concentrated to a solid under reduced pressure to give 211 mg of a yellow solid (90%). ^1H NMR (400 MHz, d_6 -DMSO, 20 $^\circ\text{C}$): δ 11.20 (s, 1H), δ 10.46 (s, 1H), δ 9.13 (d, J = 8.8 Hz, 1H), δ 9.05 (d, J = 9.2 Hz, 1H), δ 8.82 (d, J = 1.2 Hz, 1H), δ 8.29 (dd, J = 9.2 Hz, J_2 = 2.0 Hz, 1H), δ 8.14 (dd J_Y = 8.8 Hz, J_2 = 1.2 Hz, 1H), δ 7.76-7.81 (m, 6H), δ 7.45-7.49 (m, 2H), δ 7.30-7.39 (m, 8H), δ 5.10 (s, 2H), δ 4.62 (q, J = 7.2 Hz, 2H), 1.49 (t, J = 7.6 Hz, 3H). ESI MS calculated for $\text{C}_{36}\text{H}_{30}\text{N}_3\text{O}_4$: 568.2, found 568.3 $[\text{M}]^+$.

[0081] 3-Urea-ethidium - Cl (11). In a 15 mL pressure tube, 3-phenoxy carbamate-8-cbz-ethidium H_2PO_4 (**10**) (40 mg, 60 μ moles) and methanol (8 mL) were mixed and brought to -78 $^\circ\text{C}$ whereupon approximately 3 mL of liquid ammonia was added (by bubbling in ammonia gas). The pressure tube was sealed and allowed to warm to RT. The reaction was then heated at 76 $^\circ\text{C}$ for 2 hr, cooled back to -78 $^\circ\text{C}$, the tube opened and the ammonia was out-gassed by passing argon into the solution as it slowly warmed to RT. All volatiles were then removed under reduced pressure. The solid yellow product was then dissolved in 8 mL of 1:1 mixture of methanol and saturated HCl (in water) and heated at 96 $^\circ\text{C}$ for 1 hr. The reaction was then concentrated to a solid under reduced pressure and purified by reversed-phase chromatography (C-18 silica gel 60). The column was conditioned with methanol, water, and the crude product was then loaded in water (0.01M HCl) and a methanol gradient (0-10% methanol/water (0.01M HCl)) was used to separate ethidium chloride (elutes first) from the desired product (elutes second) to yield 16.5 mg of an orange solid (70%). ^1H NMR (400

MHz, d_6 -DMSO, 20 °C): δ 9.58 (s, 1H), δ 8.83 (d, J = 9.2 Hz, 1H), δ 8.75 (d, J = 1.2 Hz, 1H), δ 8.73 (d, J = 9.2 Hz, 1H), δ 7.85 (dd J_1 = 9.2 Hz, J_2 = 2.0 Hz, 1H), δ 7.73-7.78 (m, 5H), δ 7.57 (dd J_1 = 8.8 Hz, J_2 = 2.4 Hz, 1H), δ 6.35 (d, J = 2.0 Hz, 1H), 6.29 (br s, 2H), 6.04 (br s, 2H), δ 4.53 (4, J = 7.6 Hz, 2H), δ 1.45 (t, J = 7.0 Hz, 3H). ESI MS calculated for $C_{22}H_{21}N_4O$: 357.2, found 357.3 $[M]^+$. UV-vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ϵ ($cm^{-1}M^{-1}$): 214 (2.4×10^4), 284 (3.5×10^4), 458 (3.1×10^3).

[0082] 3-Cbz-8-phenoxy carbamate-ethidium . H_2PO_4 (12). 3-cbz-ethidium - Cl (2) (180 mg, 372 μ moles), acetone (10 mL), and 500 mM sodium phosphate pH 6.6 (4 mL) were combined and phenyl chloroformate (200 μ L, 1.58 mmoles, 4.2 mequiv, diluted into 2 mL of acetone) was added dropwise and stirred for 30 min at RT. Water was then added (6 mL) dropwise and the precipitate collected by vacuum filtration and washed with water (20 mL). The precipitate was dried under reduced pressure to yield 195 mg (79%) of a yellow solid. ^1H NMR (300 MHz, d_6 -DMSO, 20 °C): δ 10.88 (s, 1H), δ 10.74 (s, 1H), δ 9.11 (d, J = 9.3 Hz, 1H), δ 9.07 (d, J = 9.0 Hz, 1H), δ 8.29 (dd J_1 = 9.0 Hz, J_2 = 1.8 Hz, 1H), δ 8.09 (d, J = 8.4 Hz, 1H), δ 7.74-7.76 (m, 5H), δ 7.37-7.49 (m, 7H), δ 7.16-7.29 (m, 3H), δ 5.26 (s, 2H), δ 4.62 (4, J = 7.5 Hz, 2H), 1.49 (t, J = 7.2 Hz, 3H). ESI MS calculated for $C_{36}H_{30}N_3O_4$: 568.2, found 568.3 $[M]^+$.

[0083] 8-Urea-ethidium - Cl (13). In a 15 mL pressure tube, 3-Cbz-8-phenoxy carbamate-ethidium . H_2PO_4 (12) (45 mg, 67 μ moles) and methanol (8 mL) were mixed and brought to -78 °C whereupon approximately 2 mL of ammonia was added (by bubbling in ammonia gas). The pressure tube was sealed and allowed to warm to RT. The reaction was then heated at 80 °C for 1 hr, cooled back to -78 °C, the tube opened and the ammonia was out-gassed by passing argon into the solution as it slowly warmed to RT. All volatiles were then removed under reduced pressure. The solid yellow product was then dissolved in 10 mL of 1:1

mixture of methanol and saturated HCl (in water) and heated at 96 °C for 1 hr. The reaction was then concentrated to a solid under reduced pressure and purified by reversed-phase chromatography (C-18 silica gel 60). The column was conditioned with methanol, water, and the crude product was loaded in 2% methanol / water (0.01M HCl) and a methanol gradient (2-10% methanol/water (0.01M HCl)) was used to separate ethidium chloride (elutes first) from the desired product (elutes second), to afford 21 mg of an orange solid (80%). ¹H NMR (300 MHz, d₆-DMSO, 20 °C): δ 9.12 (s, 1H), δ 8.76 (d, *J* = 9.0 Hz, 2H), δ 8.22 (dd *J*₁ = 9.0 Hz, *J*₂ = 2.1 Hz, 1H), δ 7.71-7.77 (m, 5H), δ 7.33-7.39 (m, 5H), δ 6.55 (br s, 2H), 5.97 (br s, 2H), δ 4.48 (q, *J* = 7.2 Hz, 2H), δ 1.41 (t, *J* = 6.9 Hz, 3H). ESI MS calculated for C₂₂H₂₁N₄O: 357.2, found 357.3 [M]⁺. UV-vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm⁻¹M⁻¹): 286 (4.9 x 10⁴), 464 (4.7 x 10³).

[0084] 3,8-Bis-phenoxy-carbamate-ethidium H₂PO₄ (14).

Ethidium bromide/8% water (1) (200 mg, 466 umoles), 500 mM sodium phosphate pH 6.6 (5 mL), and acetone (8 mL) were combined, and phenyl chloroformate (587 μL, 4.66 mmoles, 5 mequiv, pre-dissolved in 2.5 mL acetone) was added dropwise. After 10 min at RT the reaction was cooled to -80 °C and vacuum filtered. The precipitate was washed with 20% acetone/water (10 mL), 100% acetone (-80 °C, 10 mL), and dried under reduced pressure to yield 300 mg (98%) of a yellow solid. ¹H NMR (400 MHz, d₆-DMSO 20 °C): δ 11.36 (s, 1H), δ 10.98 (s, 1H), δ 9.17 (d, *J* = 9.2 Hz, 1H), δ 9.12 (d, *J* = 8.8 Hz, 1H), δ 8.87 (s, 1H), δ 8.36 (dd *J* = 9.2 Hz, *J*₂ = 2.0 Hz, 1H), δ 8.22 (d, *J* = 9.2 Hz, 1H), δ 7.85 (d, *J* = 2.4 Hz, 1H), δ 7.77 (s, 5H), δ 7.38-7.50 (m, 4H), δ 7.24-7.43 (m, 4H), δ 7.15-7.19 (m, 2H), δ 4.64 (q, *J* = 7.6 Hz, 2H), 1.48 (t, *J* = 7.2 Hz, 3H). ESI MS calculated for C₃₆H₃₀N₃O₄: 554.2, found 554.3 [M]⁺.

[0085] 3,8-Bis-urea-ethidium - Cl(15). In a 15 mL pressure tube, 3,8-bis-phenoxy-carbamate-ethidium - H₂PO₄ (14) (48 mg, 74

umoles) and methanol (10 mL) were mixed and brought to -78 °C whereupon approximately 2 mL of ammonia was added (by bubbling in ammonia gas). The pressure tube was sealed and allowed to warm to RT. The reaction was then heated at 80 °C for 1 hr and cooled back to -78 °C. The tube was opened and the ammonia was out-gassed by passing argon into the solution as it slowly warmed to RT. All volatiles were then removed under reduced pressure. The solid product was washed with diethyl ether (2x20 mL) then dissolved in 20% acetonitrile/water and treated with AGI-X4 (Cl⁻) exchange resin (1 g, 3.5 mmoles, 47 mequiv) for 5 min at RT. The resin was removed by filtration, and the solution lyophilized to yield 32 mg (99%) of a yellow solid. ¹H NMR (300 MHz, d₆-DMSO, 20 °C): δ 9.76 (s, 1H), δ 9.34 (s, 1H), δ 8.97 (d, *J* = 9.3 Hz, 1H), δ 8.92 (d, *J* = 9.3 Hz, 1H), δ 8.85 (d, *J* = 1.2 Hz, 1H), δ 8.30 (dd *J*₇ = 9.3 Hz, *J*_p = 2.4 Hz, 1H), δ 7.93 (dd *J*₇ = 9.3 Hz, *J*_p = 1.2 Hz, 1H), δ 7.74-7.78 (m, 5H), δ 7.55 (d, *J* = 2.1 Hz, 1H), δ 6.36 (s, 2H), 6.05 (s, 2H), δ 4.58 (q, *J* = 7.8 Hz, 2H), δ 1.48 (t, *J* = 6.9 Hz, 3H). ESI MS calculated for C₂₃H₂₂N₅O₂: 400.2, found 400.3 [M]⁺. UV-vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm⁻¹M⁻¹): 280 (4.3x10⁴), 434 (3.6x10³).

[0086] 3,8-Bis-urea-pyrrolidine-ethidium - TFAc (16). 3,8-Bis-phenoxy carbamate-ethidium - H₂PO₄ (14) (12 mg, 19 μmoles), DMSO (1 mL) and pyrrolidine (40 μL, 460 μmoles, 27 equiv) were combined and heated for 5 min (90 °C). The reaction was then diluted into water (9 mL, 0.1% TFA) and loaded onto an activated Water's "Sep-pack" C-18 reversed phase column (activated with 10 mL acetonitrile, 10 mL water). The column was washed with water (10 mL, 0.1% TFA), then 10% acetonitrile (10 mL, in water with 0.1% TFA). The product was then eluted with 35% acetonitrile (10 mL, in water with 0.1% TFA) and lyophilized to yield a yellow solid (12 mg, 100%). ¹H NMR (400 MHz, d₆-DMSO, 21 °C): δ 9.01 (d, *J* = 9.6 Hz, 1H), δ 8.98 (s, 1H), δ 8.94 (d, *J* = 9.6 Hz, 1H), δ 8.89 (d, *J* = 2.0 Hz, 1H), δ

8.72 (s, 1H), δ 8.40 (dd $J = 9.2$ Hz, $J_p = 2.4$ Hz, 1H), δ 8.26 (dd $J = 9.2$ Hz, $J_2 = 1.6$ Hz, 1H), δ 7.34-7.80 (m, 6H), δ 4.56 (4, $J = 6.4$ Hz, 2H), δ 3.46 (t, $J = 6.4$ Hz, 2H), δ 3.15 (t, $J = 6.6$ Hz, 2H), δ 1.91 (t, $J = 6.4$ Hz, 2H), δ 2.81 (t, $J = 6.6$ Hz, 2H), δ 1.48 (t, $J = 7.2$ Hz, 3H). ESI MS calculated for $C_{31}H_{34}N_5O_2$: 508, found 508 $[M]^+$. UV-vis (50 mM sodium phosphate pH 7.5): $\lambda_{\max}$ (nm) and ϵ ($\text{cm}^{-1}\text{M}^{-1}$): 288 (4.7×10^4), 438 (4.5×10^3).

[0087] 3,8-Bis-urea-arginine-ethidium - TFAc3 (17). 3,8-Bis-phenoxy carbamate-ethidium. H_2PO_4 (14) (10 mg, 15.3 μmoles), DMSO (400 μL), water (100 μL), L-Arg - HCl (50 mg, 237 μmoles , 8.6 mequiv) and 2,4,6-collidine (84 μL , 711 μmoles , 26 mequiv) were heated for at 90 $^\circ\text{C}$ for 1hr then cooled to RT and quenched with 500 mM sodium phosphate pH 6.5 (0.6 mL). The reaction was then diluted into 5 mL water (0.1 % TFA) and loaded onto an activated Waters' "Sep-pack" C-18 reversed phase column (activated with 10 mL acetonitrile, 10 mL water). The column was washed with water / 0.1% TFA (5 mL), the product eluted with 25% acetonitrile/water (0.1 % TFA) and was lyophilized. The product was further purified using a reversed phase C-18 semi-prep HPLC column using 15% acetonitrile / water (0.1% TFA) (RT = 6.3 min) to yield 4.5 mg (31%) of a yellow solid. ^1H NMR (400 MHz, D_2O , 20 $^\circ\text{C}$): δ 8.37 (d, $J = 8.8$ Hz, 1H), δ 8.31 (d, $J = 8.8$ Hz, 1H), δ 8.30 (d, $J = 8.8$ Hz, 1H), δ 7.67-7.76 (m, 4H), δ 7.53 (d, $J = 9.2$ Hz, 1H), δ 7.39-7.45 (m, 3H), δ 4.59 (q, $J = 6.8$ Hz, 2H), δ 4.20 (t, $J = 5.6$ Hz, 1H), δ 4.08 (t, $J = 5.2$ Hz, 1H), δ 3.15 (t, $J = 6.6$ Hz, 2H), δ 3.10 (t, $J = 6.8$ Hz, 2H), δ 1.52-1.83 (m, 8H), δ 1.33 (t, $J = 7.0$ Hz, 3H). ESI MS calculated for $C_{35}H_{44}N_{11}O_6$: 714, found 715 $[M+H]^+$, UV-vis (50 mM sodium phosphate pH 7.5): $\lambda_{\max}$ (nm) and ϵ ($\text{cm}^{-1}\text{M}^{-1}$): 216 (3.8×10^4), 288 (6.8×10^4), 444 (5.7×10^3).

[0088] 3,8-Bis-urea-ethylenediamine-ethidium . TFAs (18). 3,8-Bis-phenoxy carbamate ethidium - H_2PO_4 (14) (9 mg, 13.8

μmoles), DMSO (300 μL), and ethylene diamine (100 μL) were heated at 85 °C for 30 min then cooled to RT. 500 mM sodium phosphate pH 6.5 (0.6 mL) was added and the reaction was diluted into water (5 mL, 0.1% TFA) and loaded onto an activated Water's "Sep-pack" C-18 reversed phase column (activated with 10 mL acetonitrile, 10 mL water). The column was washed with water / 0.1% TFA (5 mL), the product eluted with 25% acetonitrile/water (0.1% TFA) and lyophilized to yield 10.5 mg (91%) of a yellow solid. ¹H NMR (300 MHz, D₂O, 20 °C): δ 8.58 (d, *J* = 8.4 Hz, 1H), δ 8.50 (d, *J* = 8.4 Hz, 1H), δ 8.43 (s, 1H), δ 7.76 (d, *J* = 9.0 Hz, 1H), δ 7.60-7.69 (m, 4H), δ 7.50 (s, 1H), δ 7.40-7.43 (m, 2H), δ 4.60 (q, *J* = 7.5 Hz, 2H), δ 3.40 (t, *J* = 6.0 Hz, 2H), δ 3.26 (t, *J* = 5.7 Hz, 2H), δ 3.03 (t, *J* = 5.7 Hz, 2H), δ 2.92 (t, *J* = 5.7 Hz, 2H), δ 1.37 (t, *J* = 7.2 Hz, 3H). ESI MS calculated for C₂₇H₃₂N₇O₂: 486, found 486 [M]⁺. UV-vis (50 mM sodium phosphate pH 7.5): λ_{max} (nm) and ε (cm⁻¹M⁻¹): 216 (3.8 x 10⁴), 286 (6.6 x 10⁴) ~ 444 (6.5 x 10³).

[0089] **3,8-Bis-urea-2-DOS ethidium . TFA₃ (19).** 3,8-Bis-phenoxy carbamate-ethidium - H₂PO₄ (14) (20 mg, 31 μmoles), DMSO (1.5 mL), phenol (1.5 g), Na₂CO₃ (50 mg, 472 μmoles, 15 equiv), 2-deoxy streptamine . 2HCl (110 mg, 277 μmoles, 8.9 equiv) pre-dissolved in water (0.7 mL), were heated at 85 °C for 45 min. The reaction was diluted into water (80 mL) and washed with CH₂Cl₂ (2x40 mL), CHCl₃ (2x40 mL), and ethyl acetate (40 mL). The aqueous phase was then concentrated to a solid and purified by reversed-phase chromatography (C-18 silica gel 60). The column was conditioned with pure acetonitrile, pure water, and the crude product was loaded in water (0.1 % TFA) and an acetonitrile gradient (0-8% acetonitrile / water (0.1% TFA) was used to elute the product. Fractions were collected and lyophilized to yield 8 mg of a yellow solid (25%). ¹H NMR (400 MHz, D₂O, 20 °C): δ 8.55 (d, *J* = 8.4 Hz, 1H), δ 8.47 (d, *J* = 8.4 Hz, 1H), δ 8.44 (s, 1H), δ 7.62-7.74 (m, 6H), δ 7.50 (s,

1H), δ 7.39 (s, 1H), δ 7.38 (d, J = 8.4 Hz, 1H), δ 4.59 (q, J = 6.4 Hz, 2H), δ 3.70 (m, 1H), δ 3.52 (m, 1H), δ 3.08-3.38 (m, 8H), δ 2.21 (td J_7 = 12.4 Hz, J_2 = 4.0 Hz, 1H), δ 2.08 (td J_7 = 12.4 Hz, J_2 = 4.0 Hz, 1H), δ 1.56 (q, J = 12.4 Hz, 1H), δ 1.44 (q, J = 12.4 Hz, 1H), δ 1.37 (t, J = 6.4 Hz, 3H). ESI MS calculated for $C_{35}H_{44}N_7O_8$: 690, found 690 $[M]^+$, UV-vis (50 mM sodium phosphate pH 7.5): λ_{max} , (nm) and ϵ ($cm^{-1}M^{-1}$): 216 (3.8×10^4), 288 (6.8×10^4), 444 (5.7×10^3).

[0090] 3-Pyrrole-ethidium TFA (20), and 8-pyrrole-ethidium - TFA (21). A mixture (5:1 respectively) of 8-cbz-ethidium - Cl (3) and 3-cbz-ethidium - Cl (2) (90 mg, 186 μ moles) was added to glacial acetic acid (4 mL), heated to 120 $^{\circ}C$, and 3 portions (15 minutes apart) of dimethoxytetrahydrofuran (3 x 15 μ L, 348 μ moles total, 1.87 mequiv) were added over 30 min. The reaction was kept at 120 $^{\circ}C$ for an additional 45 min then cooled to RT, diluted into $CHCl_3$ (100 mL), and washed with saturated sodium bicarbonate (3x50 mL), brine (50 mL), dried over sodium sulfate, and concentrated to a solid under reduced pressure. The mixture of cbz-protected products was purified using a neutral alumina column using pure acetone as an eluent and concentrated to a yellow solid. This mixture was carried over, directly to the next step. Deprotection was conducted in a 3:1 mix of methanollacetic acid (4 mL), with Pd black (30 mg), and rigorously stirring under 1 atm of H_2 for 3 hr at RT. The catalyst was removed by centrifugation, and the solution concentrated to an orange solid under reduced pressure. The products separated using a reversed phase C-18 semi-prep column using 38% acetonitrile / water (0.1% TFA) to yield 14.6 mg (18%) of **3-pyrrole-ethidium TFA (20)** (R_t = 13.8 min). ^1H NMR (400 MHz, D_2O , 20 $^{\circ}C$): δ 8.43 (d, J = 9.2 Hz, 1H), δ 8.31 (d, J = 9.2 Hz, 1H), δ 7.87 (d, J = 1.6 Hz, 1H), δ 7.70 (dd J_1 = 9.2 Hz, J_2 = 1.6 Hz, 1H), δ 7.60-7.67 (m, 3H), δ 7.42 (dd J_7 = 9.2 Hz, J_2 = 2.4 Hz, 1H), δ 7.30-7.32 (m, 2H), δ 7.19 (dd, J_1 = J_2 = 2.0 Hz, 2H), δ 6.52 (d, J = 2.4 Hz, 1H), δ 6.29 (dd, J_7 = J_2

= 2.0 Hz, 2H), δ 4.61 (4, J = 7.2 Hz, 2H), δ 1.30 (t, J = 7.0 Hz, 3H). FAB MS calculated for $C_{25}H_{22}N_3$: 364.1814, found 364.1823 $[M]^+$. UV-vis (50 mM sodium phosphate pH 7.5): $\lambda_{\max}$ (nm) and ϵ ($\text{cm}^{-1}\text{M}^{-1}$): 223 (2.8×10^4), 287 (4.7×10^4), 453 (4.0×10^3).

8-pyrrole-ethidium . TFA (21) (3.3 mg (4%)), (RT = 18.1 min). ^1H NMR (400 MHz, D_2O , 20 °C): δ 8.42 (d, J = 9.2 Hz, 1H), δ 8.40 (d, J = 8.8 Hz, 1H), δ 7.86 (dd J = 9.2 Hz, J_2 = 2.0 Hz, 1H), δ 7.61-7.71 (m, 3H), δ 7.36-7.37 (m, 2H), δ 7.29 (d, J = 1.2 Hz, 1H), δ 7.24 (dd J_7 = 8.8 Hz, J_2 = 1.2 Hz, 1H), δ 6.99 (d, J = 2.4 Hz, 1H), δ 6.84 (dd, J_7 = J_2 = 2.0 Hz, 2H), δ 6.13 (dd, J_7 = J_2 = 2.0 Hz, 2H), δ 4.49 (q, J = 7.6 Hz, 2H), δ 1-3.0 (t, J = 7.6 Hz, 3H). FAB MS calculated for $C_{25}H_{22}N_3$: 364.1814, found 364.1822 $[M]^+$. UV-vis (50 mM sodium phosphate pH 7.5): $\lambda_{\max}$ (nm) and ϵ ($\text{cm}^{-1}\text{M}^{-1}$): 223 (2.1×10^4), 239 (1.4×10^4), 289 (4.8×10^4), 466 (4.1×10^3).

[0091] **3,8-Bis-pyrrole-ethidium OAc (22)**. Ethidium bromide/4% water (264 mg, 670 μmoles) was dissolved in glacial acetic acid (10 mL) (by sonication) and brought to 130 °C. Two portions of dimethoxytetrahydrofuran (2x110 μL , 1.65 mmol total, 2.5 equiv) were added 15 min apart. The reaction was kept under reflux (at 130 °C) for an additional 1h and cooled to RT. All volatiles were then removed under reduced pressure, and the solid was dissolved in methanol (~40 mL) and filtered over a plug of silica gel (~30 mL). The gel was washed with methanol (~60 mL), and the methanolic fractions combined and concentrated to 280 mg (90%) of a yellow solid under reduced pressure. The product can be further purified using a reversed phase C-18 semi-prep column with a 50 - 80% acetonitrile / water (0.1% TFA) gradient over 20 min. ^1H NMR (400 MHz, d_6 -acetone, 20 °C): δ 9.38 (d, J = 9.0 Hz, 1H), δ 9.32 (d, J = 9.0 Hz, 1H), δ 8.73 (d, J = 2.1 Hz, 1H), δ 8.64 (dd J_7 = 9.0 Hz, J_z = 2.4 Hz, 1H), δ 8.49 (dd J_7 = 9.0 Hz, J_2 = 2.1 Hz, 1H), δ 7.91-8.01 (m, 5H), δ 7.72 (dd, J_1 = J_z = 2.1 Hz, 2H), δ 7.54 (d, J = 2.4 Hz, 1H), δ 7.24 (dd, J_7 = J_z = 2.1 Hz, 2H), δ 6.46 (dd,

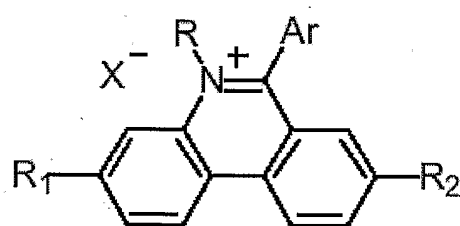
$J = J_2 = 2.1$ Hz, 2H), δ 6.34 (dd, $J_y = J_2 = 2.1$ Hz, 2H), δ 5.24 (q, $J = 7.2$ Hz, 2H), δ 1.58 (t, $J = 7.2$ Hz, 3H). FAB MS calculated for $C_{29}H_{24}N_3$: 414.1970, found 414.1951 $[M]^+$ UV-vis (50 mM sodium phosphate pH 7.5): $\lambda_{\max}$ (nm) and ϵ ($\text{cm}^{-1}\text{M}^{-1}$): 302 (4.6×10^4) $\sim$ 428 (5.2×10^3).

[0092] A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

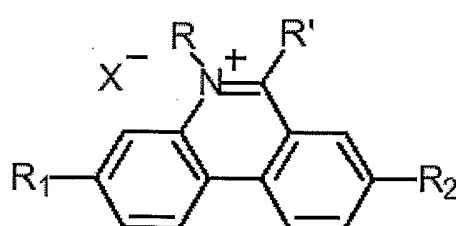
WHAT IS CLAIMED IS:

1. A compound comprising a substituted phenanthridine derivatives wherein the amines at the 3- and 8- positions of ethidium bromide are substituted with a guanidine, a pyrrole, a urea, and/or a substituted urea.

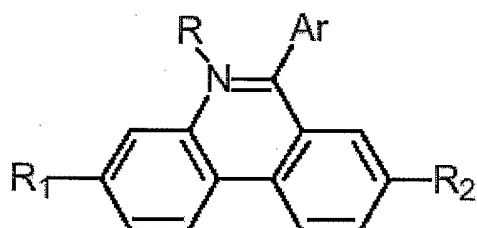
2. The compound of claim 1, wherein the compound comprising a member selected from the group consisting of:



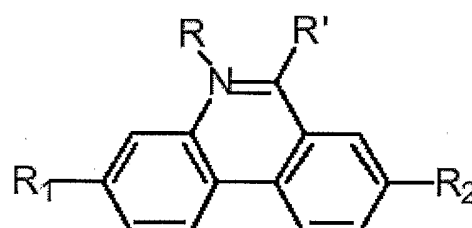
Formula 1A,



Formula 1B



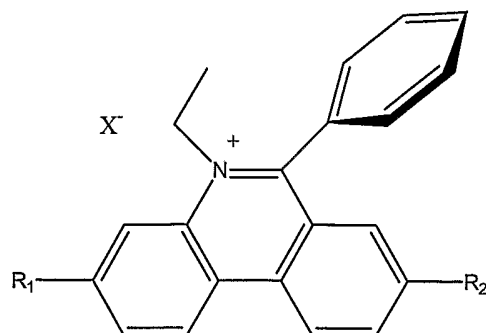
Formula 2A, and



Formula 2B,

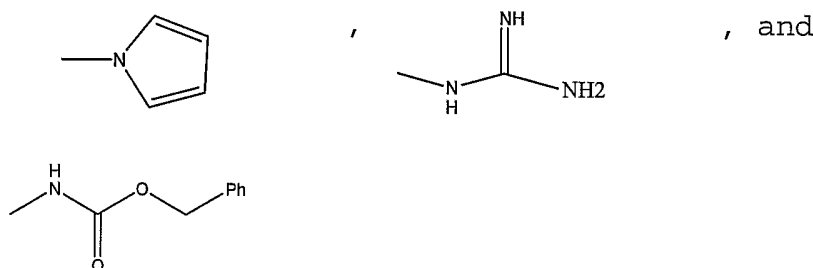
wherein R and R' can be any functionalized or unfunctionalized alkyl, alkenyl, alkynyl, aryl, alkaryl, heteroaryl, or alkheteroaryl, and Ar can be phenyl or any aromatic residue (substituted or not).

3. The compound of claim 2, comprising the general formula:



Formula 3

4. The compound of claim 2 or 3, wherein R_1 and R_2 are each independently selected from the group consisting of: a urea, a substituted urea, a diboc-guanidine, conjugated amino acids, carbohydrates, $-NH_2$,



5. The compound of claim 2, wherein the alkyl is selected from the group consisting of methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, pentyl, isopentyl, and hexyl.

6. The compound of claim 2, wherein the alkenyl is selected from the group consisting of ethenyl, propenyl, isopropenyl, butenyl, isobutenyl, tert-butenyl, pentenyl, and hexenyl.

7. The compound of claim 2, wherein the alkynyl is selected from the group consisting of ethynyl, propynyl, butynyl, isobutynyl, pentynyl, and hexynyl.

8. The compound of claim 2, wherein the aryl is selected from the group consisting of penta-2,4-diene, phenyl, naphthyl, anthracyl, azulenyl, chrysenyl, coronenyl, fluoranthenyl, indacenyl, idenyl, ovalenyl, perylenyl, phenalenyl, phenanthrenyl, picenyl, pleiadenyl, pyrenyl, pyranthrenyl, and rubicenyl.
9. The compound of claim 2, wherein the alkaryl is selected from the group consisting of benzyl, benzylidene, benzylidyne, benzenobenzyl, and naphthenobenzyl and the like.
10. The compound of claim 2, wherein the alkaryl is a C₆-C₂₆ alkaryl.
11. The compound of claim 2, wherein the heteroaryl is selected from the group consisting of acridarsine, acridine, arsanthridine, arsindole, arsindoline, carbazole, .beta.-carboline, chromene, cinnoline, furan, imidazole, indazole, indole, indolizine, isoarsindole, isoarsinoline, isobenzofuran, isochromene, isoindole, isophosphoindole, isophosphinoline, isoquinoline, isothiazole, isoxazole, naphthyridine, perimidine, phenanthridine, phenanthroline, phenazine, phosphoindole, phosphinoline, phthalazine, pteridine, purine, pyran, pyrazine, pyrazole, pyridazine, pyridine, pyrimidine, pyrrole, pyrrolizine, quinazoline, quinoline, quinolizine, quinoxaline, selenophene, tellurophene, thiophene and xanthene.
12. The compound of claim 2, wherein the alkyl, alkenyl, alkynyl, aryl, alkaryl, heteroaryl and alkheteroaryl comprise substituted alkyl, alkenyl, alkynyl, aryl, alkaryl, heteroaryl and alkheteroaryl.

13. A composition comprising the compound of any one of claims 2-4 and a pharmaceutically acceptable carrier.

14. A method of treating a subject having or at risk of having a bacterial or viral infection, comprising, contacting the subject with a compound of any one of claims 2-4 in an amount sufficient to inhibit or prevent the bacterial or viral infection.

15. The method of claim 14, wherein the subject is a mammal.

16. The method of claim 15, wherein the mammal is a human.

17. The method of claim 14, wherein the contacting is *ex vivo*.

18. The method of claim 15, wherein the contacting is *in vivo*.

19. The method of claim 15, wherein the contacting is by intravenous, intraperitoneal, intramuscular, subcutaneous, intracavity, or transdermal administration.

20. The method of claim 14, wherein the contacting is by oral or topical administration.

21. A method of treating a viral infection, comprising administering the composition of claim 13 to a subject infected with a virus in an amount sufficient to treat the infection.

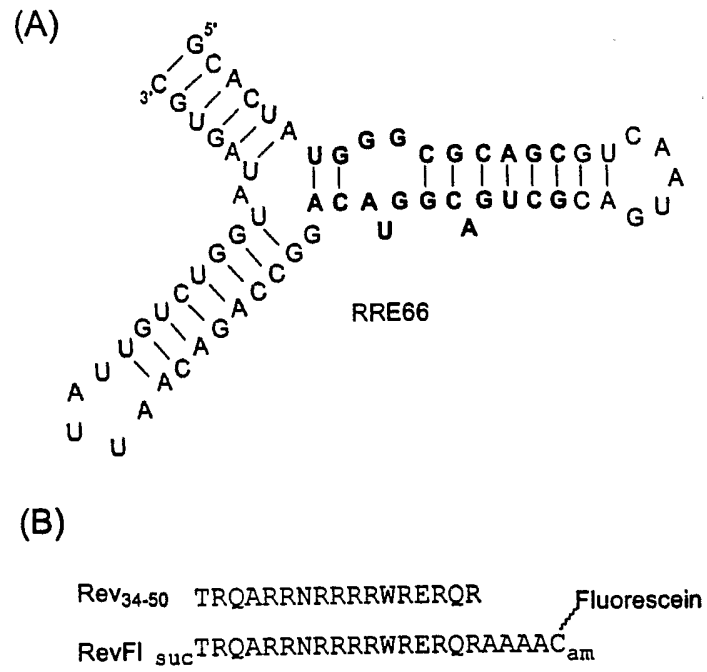
Figure 1:

Figure 2

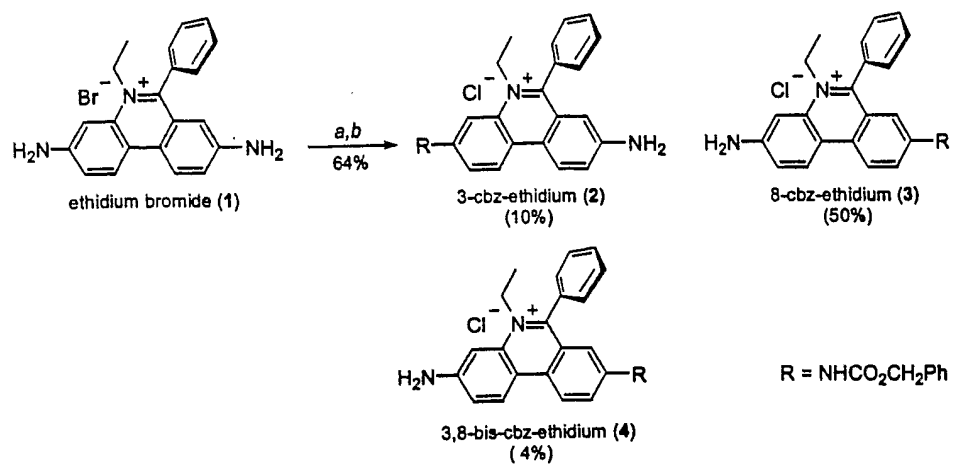


Figure 3:

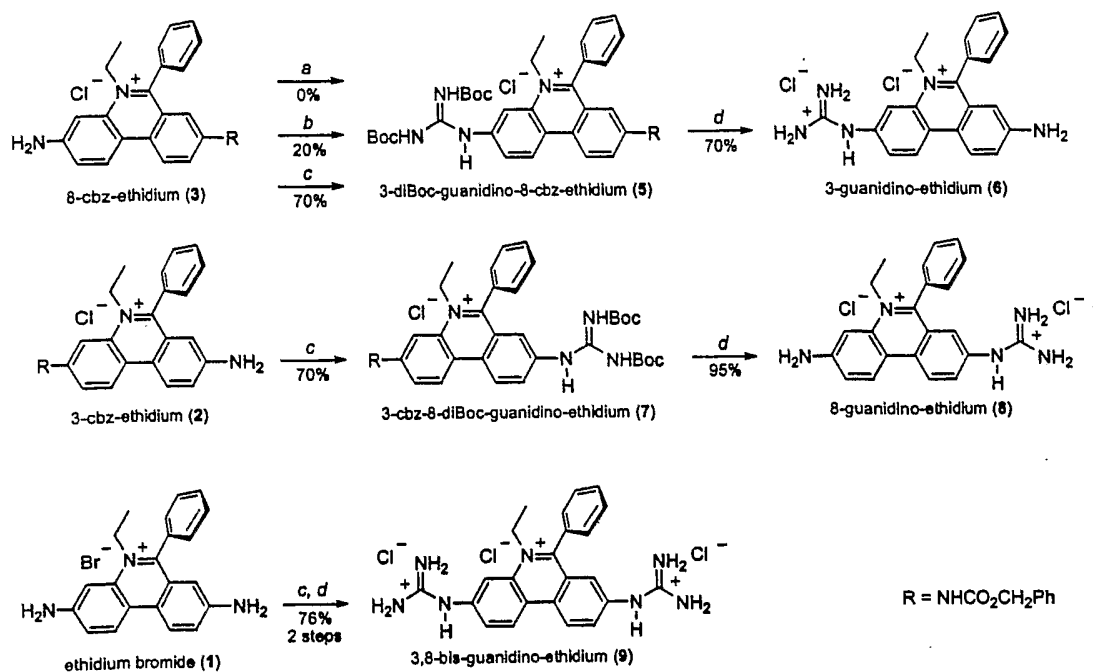


Figure 4

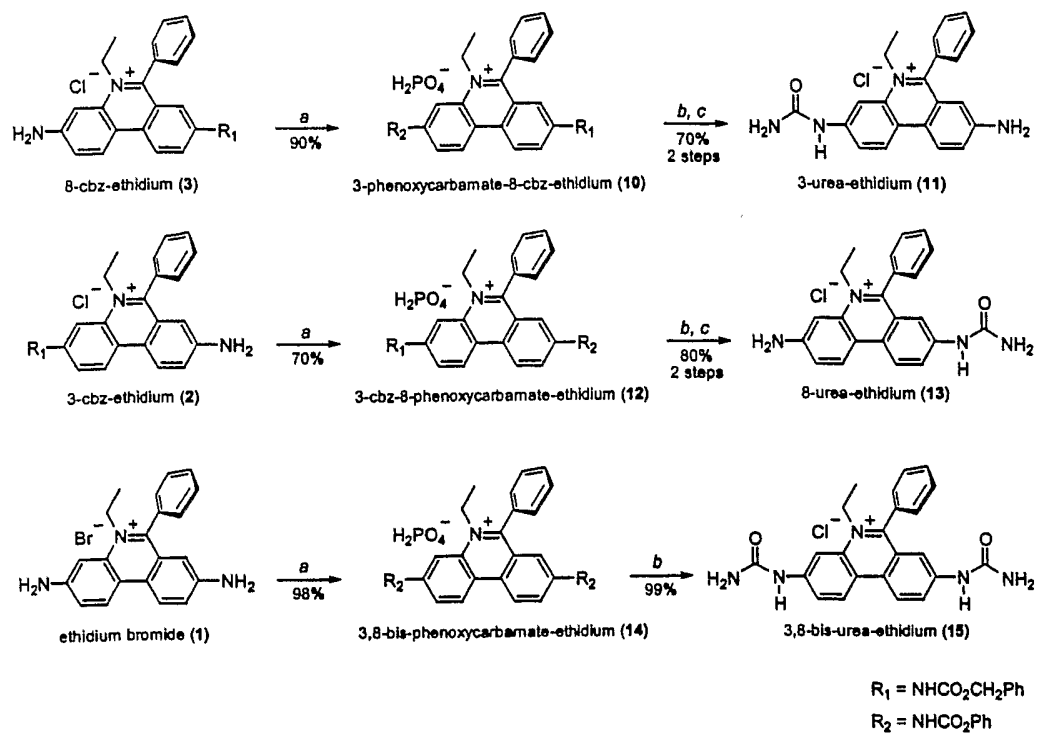


Figure 5.

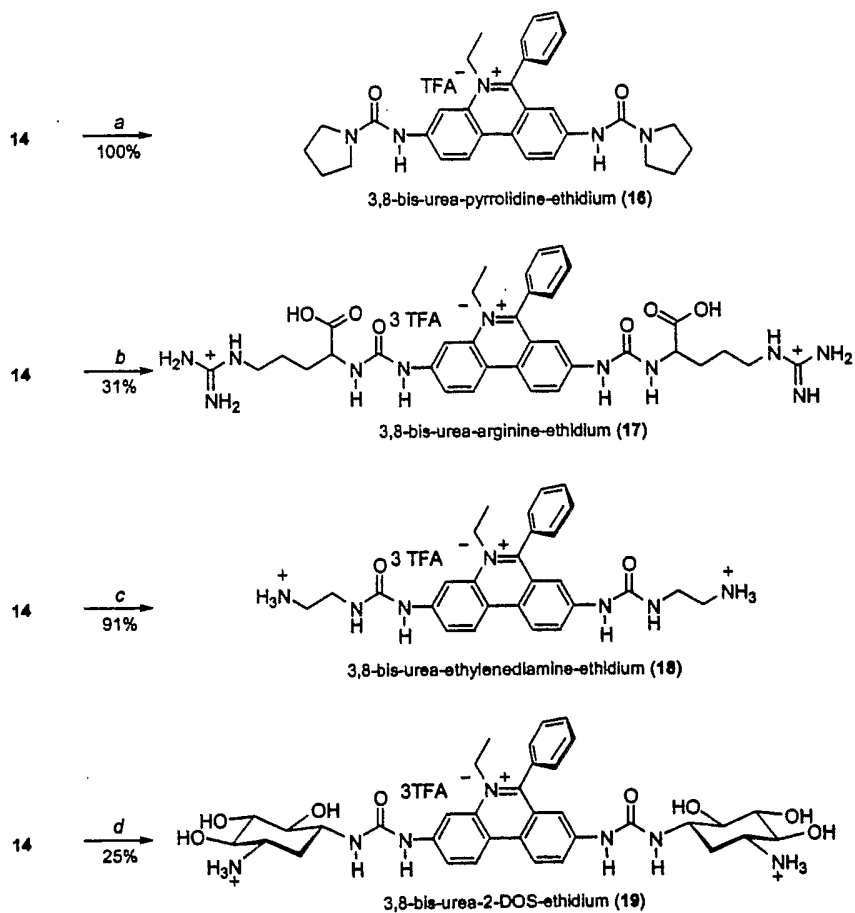


Figure 6.

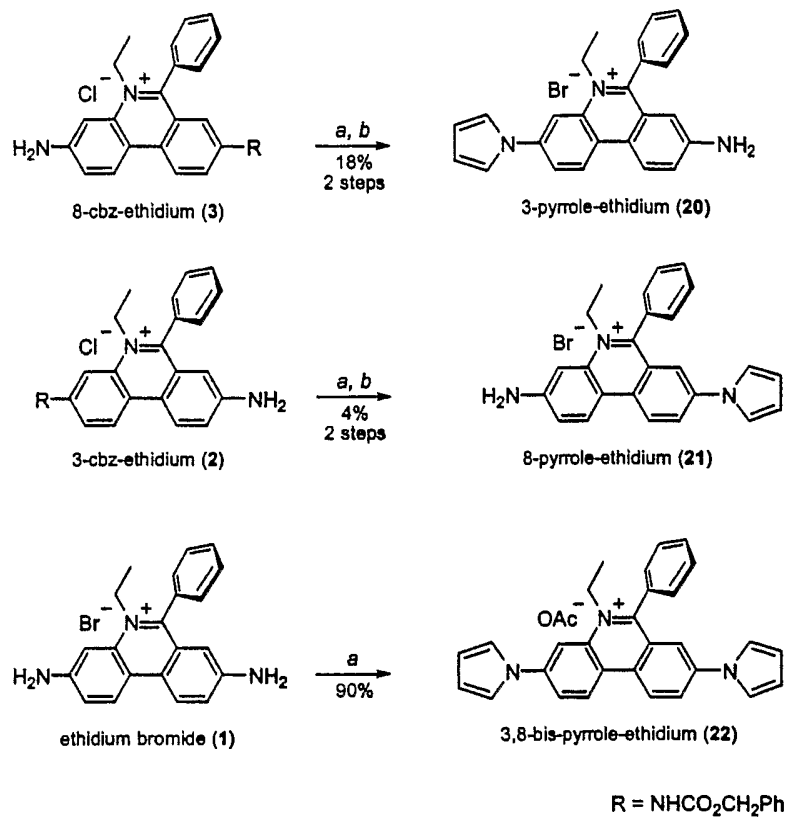


Figure 7:

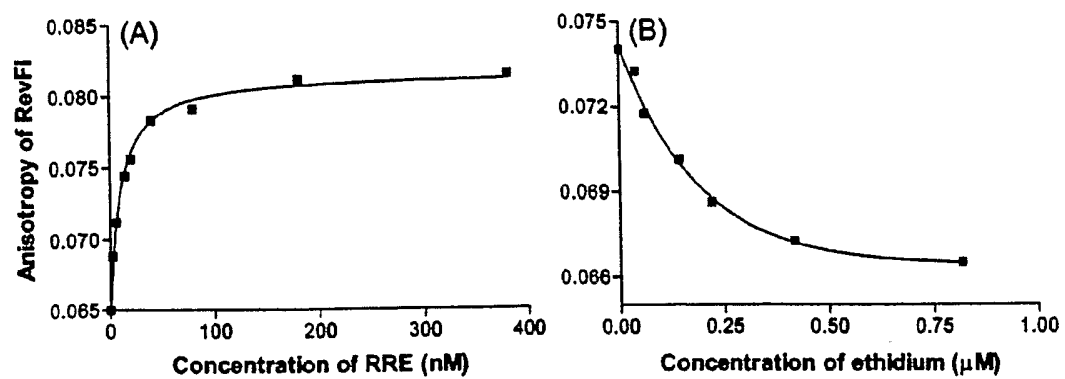
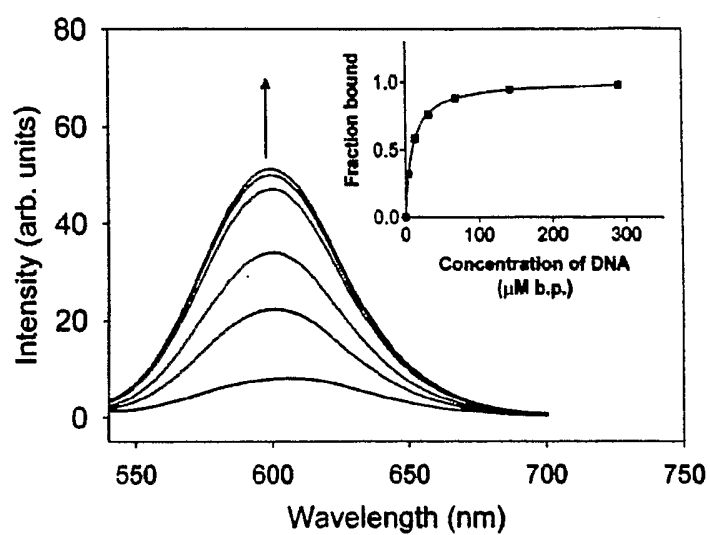


Figure 8



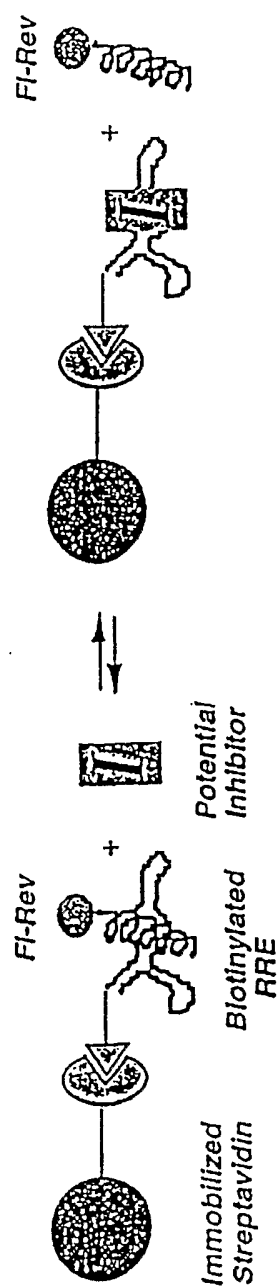


Figure 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/26188

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/473 ; C07D 221/12

US CL : 514/298 ; 546/109, 108

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)

U.S. : 514/298 ; 546/109, 108

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 practicable, search terms used)
STN STRUCTURE SEARCH, FILE CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	LUEDTKE, N. et al.: Synthesis, Photophysical properties, and nucleic acid binding of Phenanthridinium derivatives based on Ethidium. Bioorg. & Medicin. Chem. November 2003, Vol. 11, pages 5235-5247, especially compounds in table 1 on page 5238.	1-21
A	LECOINTE, P. et al. : The hepatic metabolism of Ethidium bromide to reactive mutagenic species: biochemical and structural requirements. Biochem. Pharmacol. 1981, Vol. 30, pages 601-609, especially compounds I-XI in table 1 on page 602.	1-21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

09 December 2004 (09.12.2004)

Date of mailing of the international search report

07 JAN 2005

Name and mailing address of the ISA/US

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