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(54) Title: BISANTHRAPYRAZOLES AS ANTI-CANCER AGENTS

(57) Abstract: Disclosed are novel bisanthrapyrazoles that likely act as bisintercalators and topoisomerase IIα inhibitors. These compounds show potent inhibition of cancer cell growth, and certain bisanthrapyrazoles bind to DNA more tightly than doxorubicin, a known intercalating anti-cancer drug. Molecular modeling techniques are also disclosed that allow one to predict the DNA binding strength of various intercalators.

DESCRIPTION

BISANTHRAPYRAZOLES AS ANTI-CANCER AGENTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application Serial No. 60/911,783 filed April 13, 2007, the entire contents of which are expressly incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the field of cancer treatment. More particularly, it concerns novel compounds useful for chemotherapy, methods of synthesizing these compounds and methods of treatment employing these compounds. The novel compounds are bisanthrapyrazoles that are thought to exert their anti-cancer activity, at least in part, by behaving as DNA intercalators and topoisomerase IIα inhibitors. The present invention also relates to molecular modeling methods for modeling compounds with DNA and estimating their DNA binding strength.

2. Description of Related Art

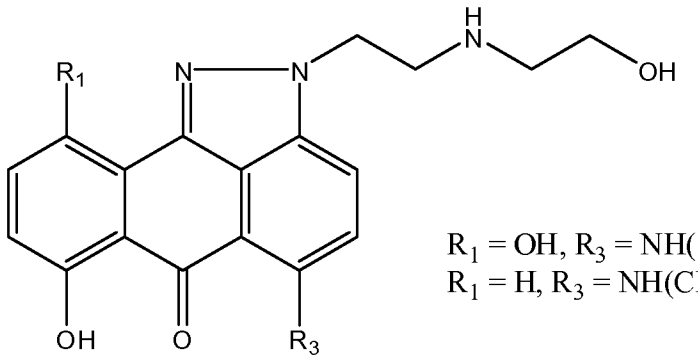
A variety of anti-cancer agents interfere with cancerous activity at the level of DNA, or upon enzymes that process DNA. Some chemotherapeutics, for example, act as DNA intercalators, while others act as inhibitors or poisons of topoisomerase, an enzyme that acts on the topology of DNA. Certain drugs may act *via* one or more of these methods.

DNA intercalating agents are one of the most widely used classes of cancer chemotherapeutic agents currently employed for the management of human cancers. These agents, which are typically polycyclic, aromatic and planar, stack between base pairs of DNA and induce local structural changes, such as the unwinding of the double helix and lengthening of the DNA strand. These structure modifications lead to functional changes, often the inhibition of transcription and replication processes. As such, DNA intercalators are typically mutagens and are often carcinogenic (*e.g.,* benzopyrene diol epoxide, bisbenzimide, aflatoxin and ethidium bromide). Bisintercalating agents are also known, wherein two parts of the same molecule

intercalate DNA. See, *e.g.*, Wakelin, 1986; Phillips *et al.*, 1992; Skorobogaty *et al.*, 1988; and U.S. Patent No. 4,112,217.

Anthrapyrazoles are a family of compounds that include certain compounds thought to behave as intercalators (Begleiter *et al.*, 2006; Liang *et al.*, 2006). Because the anthrapyrazoles do not contain a quinone group as do the anthracyclines, they are unable to be reductively activated like doxorubicin and other anthracycline: thus, it is thought that this family of compounds may exhibit less cardiotoxicity, and they were designed with this benefit in mind (Begleiter *et al.*, 2006; Leteurtre *et al.*, 1994; Gogas and Mansi, 1995). Examples of anthrapyrazoles include teloxantrone, losoxantrone and piroxantrone (Begleiter *et al.*, 2006; Hartley *et al.*, 1998; Showalter *et al.*, 1987). Structure-activity relationship studies have been performed for anthrapyrazole cytotoxicity and DNA binding as well as analyses of their effects on different types of cancer, including breast, prostate, head and neck cancer and leukemia (Gogas and Mansi, 1995; Begleiter *et al.*, 2006; Liang *et al.*, 2006; Ingle *et al.*, 1994; Talbot *et al.*, 1991; Huan *et al.*, 2000). Aza-bioisosteres of anthrapyrazoles have also been studied in clinical trials (Sissi and Palumbo, 2004; Sissi *et al.*, 2004; *see also* U.S. Patent No. 6,747,039).

Piroxantrone, shown below, is an anthrapyrazole with broad antitumor activity *in vitro* (Berg *et al.*, 1993). This synthetic compound, which has undergone clinical trials for anti-tumor activity, intercalates into DNA and inhibits topoisomerase II, thereby inhibiting DNA replication and repair (Ingle *et al.*, 1994). Although less cardiotoxic than doxorubicin, this agent exhibits a narrow spectrum of antineoplastic activity. Losoxantrone, also shown below, is an anti-cancer anthrapyrazole that has also undergone clinical trials for breast cancer treatment (Joshi *et al.*, 2001; Talbot *et al.*, 1991). This synthetic compound is a topoisomerase II inhibitor (Leteurtre *et al.*, 1994).



$R_1 = OH$, $R_3 = NH(CH_2)_3NH_2$; piroxantrone
$R_1 = H$, $R_3 = NH(CH_2)_3NH(CH_2)_2OH$; losoxantrone

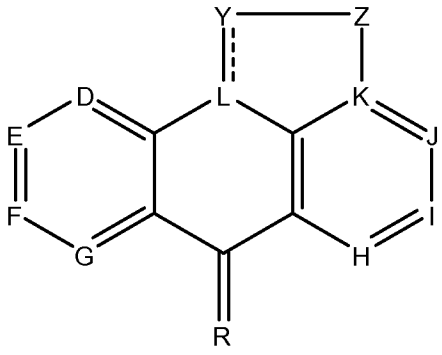
While certain anthrapyrazoles have shown less cardiotoxicity than the anthracyclines, piroxantrone has been shown to produce cardiotoxicity at high cumulative doses (Gogas and Mansi, 1995). Further, certain anthrapyrazoles have been shown to cause myelosuppression (Gogas and Mansi, 1995). Thus, preparation of other anthrapyrazole derivatives may offer improved chemotherapeutic effects over known anthrapyrazoles as well as other DNA intercalating compounds.

SUMMARY OF THE INVENTION

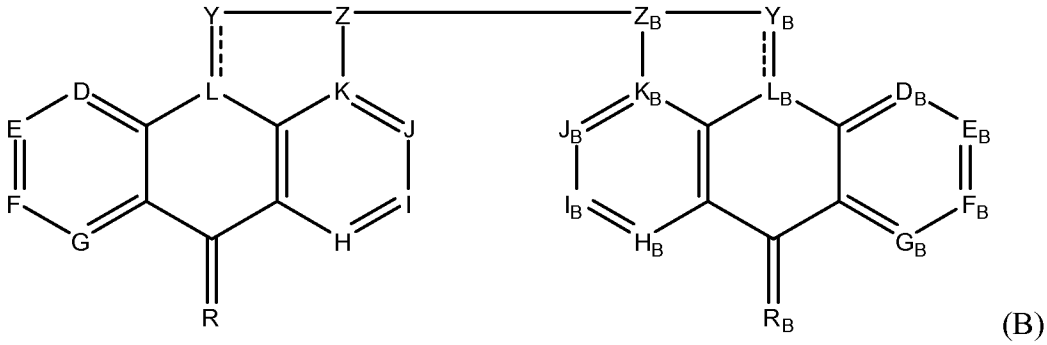
The present invention provides for novel bisanthrapyrazoles as anti-cancer agents. In general, these bisanthrapyrazoles are based on aspects of the structures of losoxantrone and piraxantrone. The present inventors have found that these bisanthrapyrazoles are likely DNA intercalators, some of which surprisingly bind to DNA more tightly than doxorubicin. Certain bisanthrapyrazoles of the present invention are topoisomerase IIα inhibitors, and have shown *in vitro* cancer cell growth inhibitory effects. Accordingly, the present invention also involves novel compounds that have utility as anti-tumor and/or chemotherapeutic drugs, methods of synthesizing these compounds and methods of using these compounds to treat patients with cancer. Non-limiting types of cancer that may be treated using the methods and compounds of the present invention include, for example, breast, prostate, head and neck cancer and leukemia.

The present invention, then, generally contemplates a compound comprising a first anthrapyrazole operatively linked to a second anthrapyrazole. By "operatively linked," it is meant that the two anthrapyrazoles are joined *via* any chemical means known in the art, such as covalent, ionic, or dative means, for example. In particular embodiments, two anthrapyrazoles are joined *via* an operative linkage comprising, or

consisting of, one or more covalent bonds. In certain embodiments, the first anthrapyrazole is operatively linked to a second anthrapyrazole *via* a linker. The linker may join the two anthrapyrazoles at any two or more atoms of either anthrapyrazole. In certain embodiments, if the core skeleton of each of a first and second anthrapyrazole is represented by structure (A), a linker may link each Z atom of the first and second anthrapyrazoles to form a bisanthrapyrazole having the core structure of formula (B):



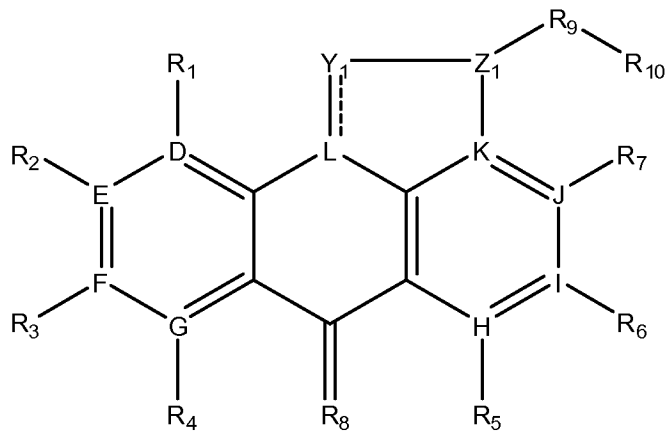
(A)



(B)

wherein: each of ring atoms D, E, F, G, H, I, J, K, L, Y, Z, D_B, E_B, F_B, G_B, H_B, I_B, J_B, K_B, L_B, Y_B and Z_B are each independently carbon or nitrogen; the bond between L and Y and the bond between L_B and Y_B may each independently be a double or single bond; and R and R_B are each independently oxygen or sulfur; and wherein the first and second anthrapyrazoles have the same or different core skeleton. In certain embodiments, a linker linking the two Z groups (Z and Z_B) comprises at least 7 linear atoms (that is, 7 atoms or higher, including subranges such as 7-10, 7-15, 7-20 linear atoms, *etc.*), which may be substituted or unsubstituted. The linear atoms may each be any atom known to those of skill in the art. In certain embodiments, the linear atoms are selected from the group consisting of carbon, nitrogen, oxygen, sulfur and phosphorus.

In certain embodiments, the first and second anthrapyrazoles are each independently selected from the compound of formula (I):



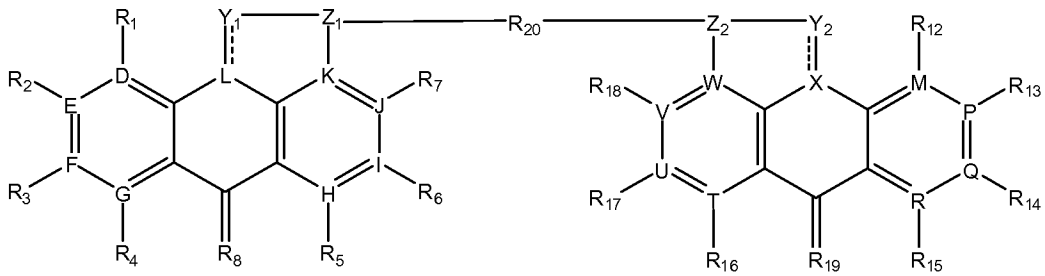
(I)

wherein: R_1-R_7 are each independently hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl, phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group, or any one or more of Y_1-R_1, R_1-R_2, R_2-R_3, R_3-R_4, R_5-R_6, R_6-R_7 or R_7-Z_1 together forms a cyclic group, or any combination of one or more of these groups; R_8 is either oxygen or sulfur; R_9 is either not present or is a linker; and R_{10} is either hydrogen, alkyl, a nucleophile or a leaving group; D, E, F, G, H, I, J, K and L are each independently carbon, -CH or nitrogen; Y_1 is selected from the group consisting of -CH, -CH_2, oxygen, nitrogen, -NH and sulfur; Z_1 is selected from the group consisting of –CH and nitrogen; and the bond between L and Y_1 is either a single or a double bond.

Regarding the compound of formula (I), R_1-R_{10} may each individually comprise any functional group, as well as H and/or alkyl. In certain embodiments, R_1 is either H, alkyl, substituted alkyl, or OH. In certain embodiments, R_4 is hydrogen, halogen, hydroxyl, or substituted alkyl. The substituted alkyl may be, for example, an aminoalkyl. The aminoalkyl group may be, for example, -$NH(CH_2)_2N(CH_3)_2$. The substituted alkyl may be a haloalkyl. In certain embodiments, R_5 is hydrogen, halogen, or substituted alkyl. The substituted alkyl may be, for example, aminoalkyl, wherein the aminoalkyl group may be, for example, –$NH(CH_2)_2N(CH_3)_2$ or –$NH(CH_2)_3NH_2$. The substituted alkyl may be hydroxyalkylamino, such as –$NH(CH_2)_3NH(CH_2)_2OH$. The substituted alkyl may be a haloalkyl. In certain embodiments, R_2, R_3, R_6 and R_7 are each hydrogen. R_8 may be oxygen, in certain

embodiments. In certain embodiments, R_9 is not present and R_{10} is hydroxyl. R_9 may be, in certain embodiments, $-N(R_{11})(CH_2)_2-$, wherein R_{11} may be any functional group, including H and alkyl. In certain embodiments, R_{11} is selected from the group consisting of H, alkyl and substituted alkyl. In certain embodiments, such as when R_9 is $-N(R_{11})(CH_2)_2-$, R_1, R_2, R_3, R_5, R_6 and R_7 is H, R_4 is Cl, R_8 is O, R_{10} is hydroxyl and R_{11} is CH_3. In certain embodiments, R_{10} is hydroxyl, azido or amino. In certain embodiments regarding the compound of formula (I), the number of linear atoms linking the Z_1 groups of the first and second anthrapyrazoles is 7 or higher (*e.g.*, 7 or more, including subranges such as 7-10, 7-15, 7-20 linear atoms, *etc.*). The linear atoms may each be any atom known to those of skill in the art. In certain embodiments, the linear atoms are selected from the group consisting of carbon, nitrogen, oxygen, sulfur and phosphorus. In certain embodiments, R_9 comprises an alkyl group, a substituted alkyl group, a heterocyclyl group, an oxo group, a sulfo group, a thioether group, an aryl group, an amide group, a sulfonamide group, a carbonyl group, a thiocarbonyl group, a secondary amine group, a tertiary amine group, an ester group, a thioester group, a sulfonyl group, or any combination of one or more of these groups. The linker may comprise an alkyl group, an ester, and/or amide, in certain embodiments. In certain embodiments, the linker may be further defined as $-(CH_2)_2N(CH_3)(CH_2)_2OC(O)(CH_2)_nC(O)O(CH_2)_2N(CH_3)(CH_2)_2-$, wherein n = 1-10, such as 1-5. In certain embodiments, the linker may be further defined as $-(CH_2)_2N(CH_3)(CH_2)_2NHC(O)(CH_2)_nC(O)NH(CH_2)_2N(CH_3)(CH_2)_2-$, wherein n = 1-10, such as 1-5. Other non-limiting examples of linkers are described herein, and are also known to those of skill in the art.

Other general aspects of the present invention contemplate a compound of formula (II):

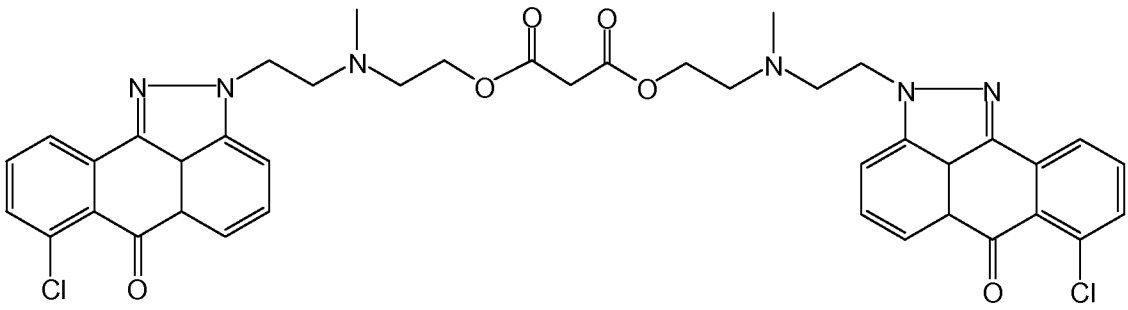


(II)

wherein: R_1-R_7 and R_{12}-R_{18} are each independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl,

phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group, or any one or more of Y_1-R_1, R_1-R_2, R_2-R_3, R_3-R_4, R_5-R_6, R_6-R_7, R_7-Z_1, Y_2-R_{12}, R_{12}-R_{13}, R_{13}-R_{14}, R_{14}-R_{15}, R_{16}-R_{17}, R_{17}-R_{18} or R_{18}-Z_2 together forms a cyclic group, or any combination of one or more of these groups; R_8 and R_{19} are each independently oxygen or sulfur; D, E, F, G, H, I, J, K, L, M, P, Q, R, T, U, V, W and X are each independently selected from the group consisting of carbon, -CH and nitrogen; Y_1 and Y_2 are each independently selected from the group consisting of -CH, $-CH_2$, oxygen, nitrogen, -NH or sulfur; Z_1 and Z_2 are each independently selected from the group consisting of -CH and nitrogen; the L-Y_1 bond and the X-Y_2 bond are each independently a single or a double bond; and R_{20} is a linker.

Regarding the compound of formula (II), R_1-R_7 and R_{12}-R_{18} may each, in certain embodiments, be independently H or halogen. In particular embodiments, the halogen is chlorine. In certain embodiments, R_8 and R_{19} are each oxygen. In certain embodiments, D, E, F, G, H, I, J, K, L, M, P, Q, R, T, U, V, W and X are each carbon. In certain embodiments, Y_1, Y_2, Z_1 and Z_2 are each carbon. In certain embodiments, the L-Y_1 bond and the X-Y_2 bond are each double bonds. The linker of R_{20} may be that of any known to those of skill in the art. In certain embodiments, the linker is selected from the group consisting of an alkyl group, a substituted alkyl group, a heterocyclyl group, an oxo group, a sulfo group, a thioether group, an aryl group, an amide group, a sulfonamide group, a carbonyl group, a thiocarbonyl group, a secondary amine group, a tertiary amine group, an ester group, a thioester group, a sulfonyl group, or any combination of one or more of these groups. In particular embodiments, the linker may comprise an alkyl, at least one ester, and/or at least one amide. In certain embodiments, R_{20} is further defined as $-(CH_2)_2NHC(O)(CH_2)_mC(O)NH(CH_2)_2-$, wherein m = 1-10, such as 1-5; or $-(CH_2)_2N(CH_3)(CH_2)_2NHC(O)(CH_2)_nC(O)NH(CH_2)_2N(CH_3)(CH_2)_2-$, wherein n = 1-10, such as 1-5. In certain embodiments, the compound of formula (II) may be further defined as a prodrug, as defined herein. In particular embodiments, the compound of formula (II) is further defined as:



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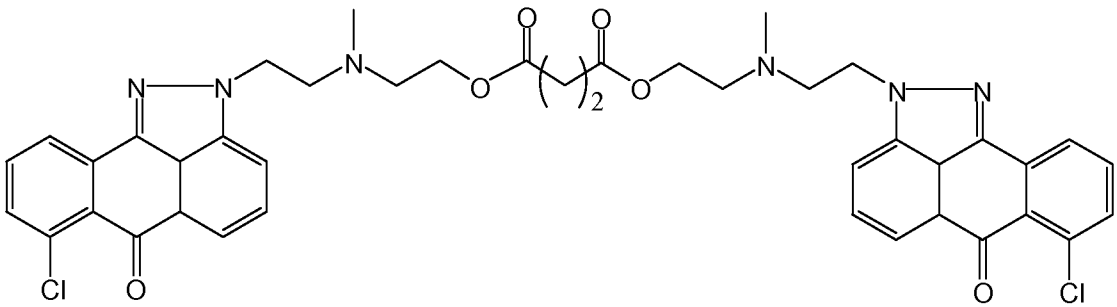
In particular embodiments, the compound of formula (II) is further defined as:



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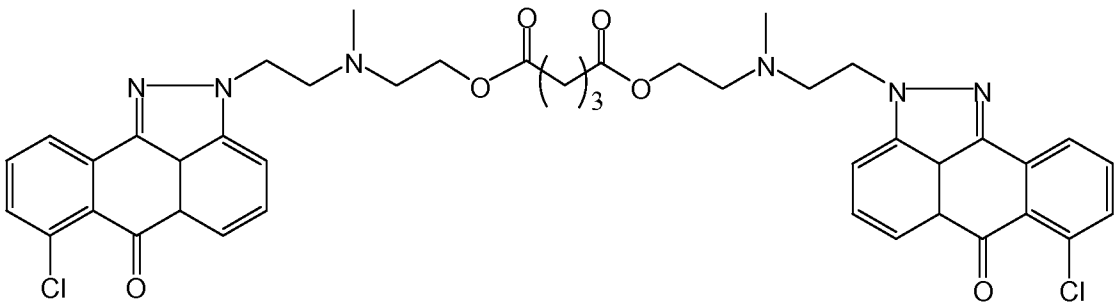
In particular embodiments, the compound of formula (II) is further defined as:



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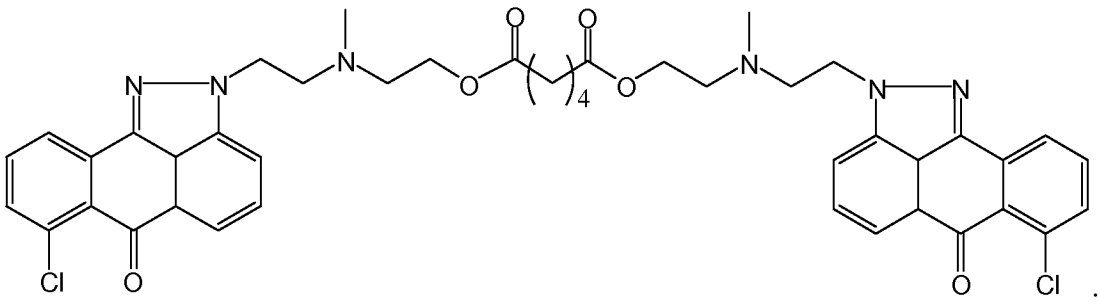
In particular embodiments, the compound of formula (II) is further defined as:



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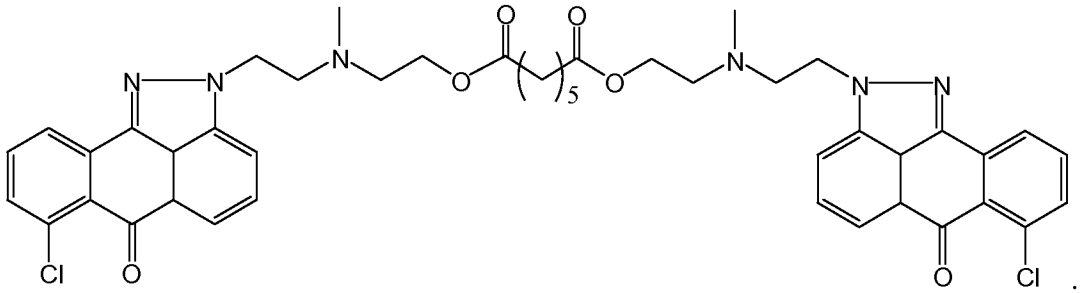
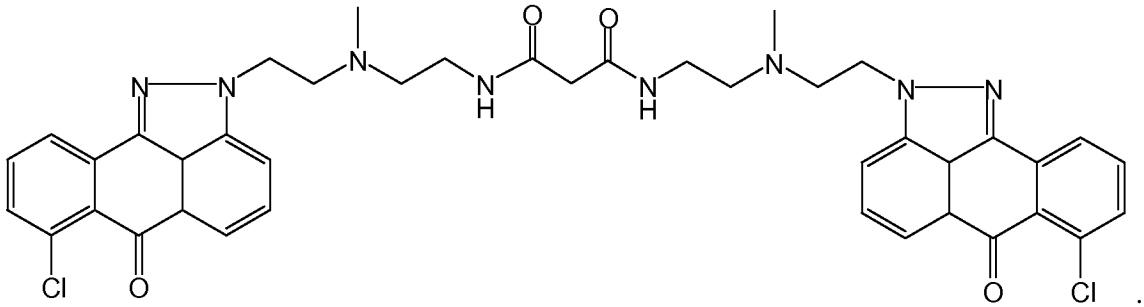
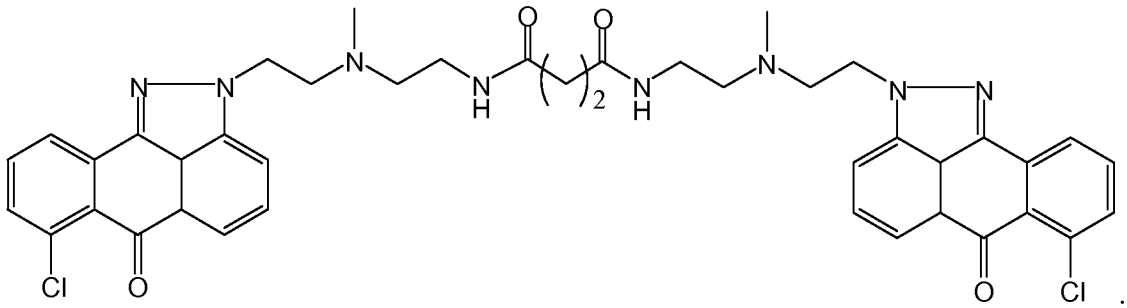
In particular embodiments, the compound of formula (II) is further defined as:



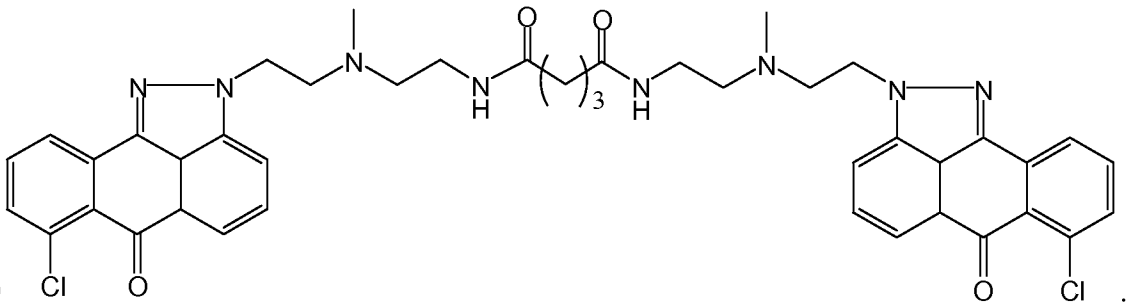
In particular embodiments, the compound of formula (II) is further defined as:



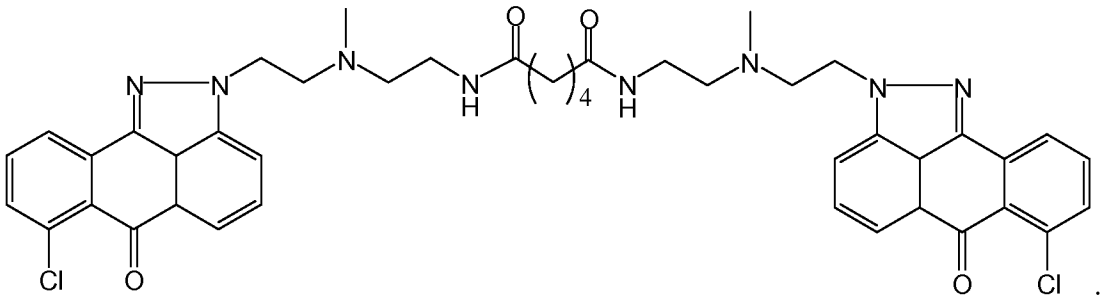
In particular embodiments, the compound of formula (II) is further defined as:



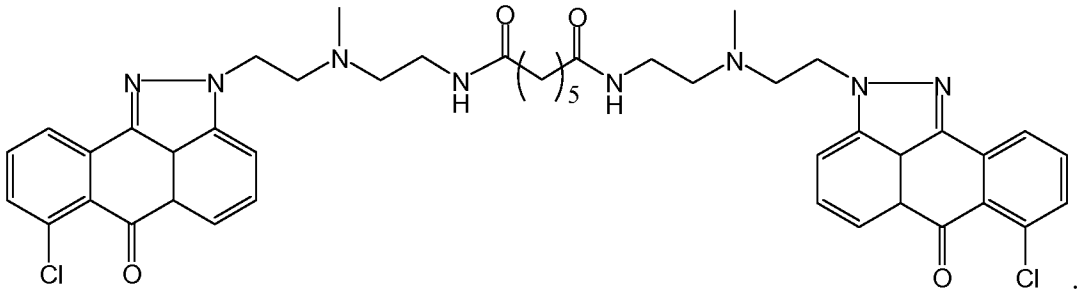
In particular embodiments, the compound of formula (II) is further defined as:



In particular embodiments, the compound of formula (II) is further defined as:

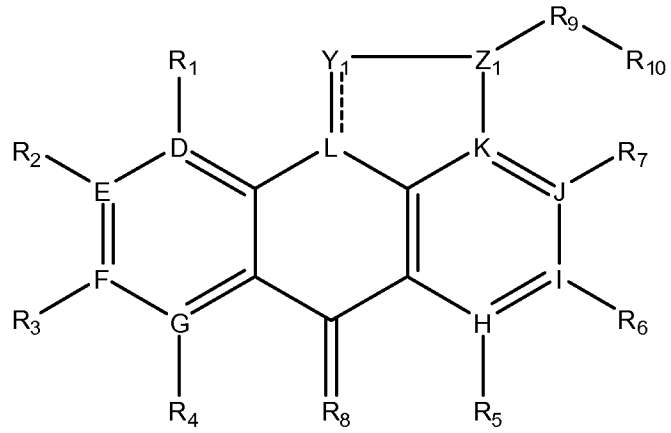


In particular embodiments, the compound of formula (II) is further defined as:



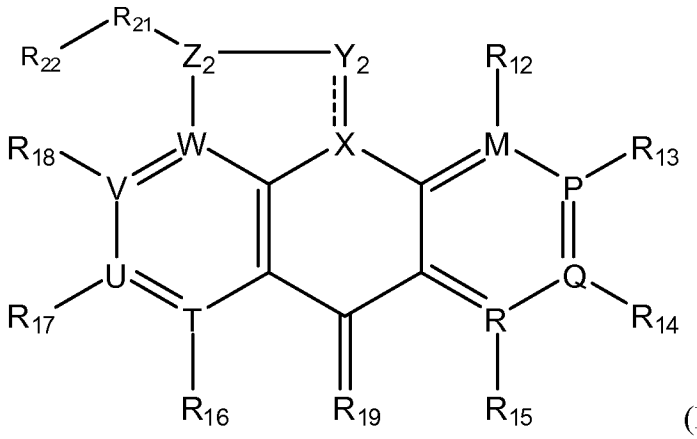
Another aspect of the present invention contemplates a method of preparing a bisanthrapyrazole comprising preparing a first anthrapyrazole, preparing a second anthrapyrazole, and conjugating the first anthrapyrazole to the second anthrapyrazole either directly or through a linker. The linker may join the two anthrapyrazoles at any two or more atoms of either anthrapyrazole. The linker may be any type of linker described herein and as known to those of skill in the art. The joined atoms of the first and second anthrapyrazoles may be any two atoms as described herein (*e.g.*, the Z atoms of the compounds of core skeleton formula (A), or the Z_1 atoms of the anthrapyrazoles of the compound of formula (I)). Typically, one of the two anthrapyrazoles comprises a nucleophile and the other comprises a leaving group, and the two anthrapyrazoles are linked together *via* reaction of the nucleophile and the atom to which the leaving group is attached.

Also contemplated by the present invention is a method of preparing a compound of formula (II) comprising: preparing a first anthrapyrazole of formula (I):



(I)

wherein: R_1-R_7 are each independently hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl, phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group, or any one or more of Y_1-R_1, R_1-R_2, R_2-R_3, R_3-R_4, R_5-R_6, R_6-R_7 or R_7-Z_1 together forms a cyclic group, or any combination of one or more of these groups; R_8 is either oxygen or sulfur; R_9 is either not present or is a linker; and R_{10} is either hydrogen, alkyl, a nucleophile or a leaving group; D, E, F, G, H, I, J, K and L are each independently carbon, -CH or nitrogen; Y_1 is selected from the group consisting of -CH, -CH_2, oxygen, nitrogen, -NH and sulfur; Z_1 is selected from the group consisting of –CH and nitrogen; and the bond between L and Y_1 is either a single or a double bond; preparing a second anthrapyrazole of formula (III):



(III)

wherein R_{12}-R_{18} are each independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl, phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group, or any one or more of Y_2-R_{12}, R_{12}-R_{13}, R_{13}-R_{14}, R_{14}-R_{15}, R_{16}-R_{17}, R_{17}-R_{18} or

R_{18}-Z_2 together forms a cyclic group, or any combination of one or more of these groups; R_{19} is either oxygen or sulfur; R_{21} is either not present or is a linker; and R_{22} is either H, alkyl, a nucleophile or a leaving group; M, P, Q, R, T, U V, W and X are each independently selected from the group consisting of carbon, -CH and nitrogen; Y_2 is selected from the group consisting of -CH, -CH_2, oxygen, nitrogen, -NH and sulfur; Z_2 is selected from the group consisting of –CH and nitrogen; and the bond between X and Y_2 is either a single or a double bond; and conjugating said first anthrapyrazole to said second anthrapyrazole either directly or through a linker. The conjugation may take place *via* any method known to those of skill in the art, and may take place between two or more atoms on either anthrapyrazole. In certain embodiments, the conjugation step comprises conjugating said first anthrapyrazole to said second anthrapyrazole through a linker that joins the Z_1 and Z_2 positions of each anthrapyrazole. In certain embodiments of this method, the linker comprises an alkyl group, a substituted alkyl group, a heterocyclyl group, an oxo group, a sulfo group, a thioether group, an aryl group, an amide group, a sulfonamide group, a carbonyl group, a thiocarbonyl group, a secondary amine group, a tertiary amine group, an ester group, a thioester group, a sulfonyl group, or any combination of one or more of these groups. In more particular embodiments, the linker may comprise an alkyl group, at least one ester, and/or at least one amide. In certain embodiments, R_{10} and R_{22} are selected from the group consisting of a nucleophile and a leaving group, wherein $R_{10} \neq R_{22}$.

Another general aspect of the present invention contemplates inhibiting the catalytic decatenation activity of topoisomerase IIα, comprising administering to a cell an effective amount of a bisanthrapyrazole.

Another general aspect of the present invention contemplates a method of treating a patient with cancer, comprising administering to the patient a therapeutically effective amount of a bisanthrapyrazole.

Yet another general aspect of the present invention contemplates a therapeutic kit comprising, in suitable container means, a pharmaceutically acceptable composition comprising a bisanthrapyrazole.

The present invention pertains also to molecular modeling methods that may show how a compound binds to DNA as well as provide estimates regarding the

binding strengths of various compounds. These methods may be used with any compound known in the art, including anthrapyrazoles, bisanthrapyrazoles and any other putative or known DNA intercalator.

In certain embodiments, then, the present invention also generally contemplates a method of estimating the binding strength of a compound to DNA using molecular modeling comprising modeling the compound docked into DNA and obtaining a GOLDScore. The method may, in certain embodiments, comprise the steps of:

(a) obtaining an x-ray structure of a known DNA-intercalator complex;

(b) optimizing the geometry of the compound;

(c) removing all water molecules and the intercalator from the DNA-intercalator complex;

(d) optionally adding hydrogens to the DNA;

(e) defining a binding site;

(f) docking the compound into the DNA; and

(g) obtaining a GOLDScore,

wherein the GOLDScore is an indication of the strength of the binding between the compound and the DNA: that is, the better the docking score, the stronger the DNA binding. Typically, and in certain embodiments, the compound is a DNA intercalator or a putative DNA intercalator. The compound may be, in certain embodiments, an anthrapyrazole or a bisanthrapyrazole. The compound may be a compound of formula (I) or (II), as discussed herein, for example.

As used herein, "alkyl" or "alk" refers to a straight, branched or cyclic carbon-carbon or hydrocarbon chain, optionally including alkene or alkyne bonding, containing 1-20 carbons. "Lower alkyl" refers to alkyl radicals comprising 1-5 carbons. In any embodiment wherein "alkyl" radicals may be employed, "lower alkyl" radicals may be employed, in certain embodiments. Non-limiting examples of lower alkyls include methyl, ethyl, propyl, cyclopropyl, butyl and isopropyl. "Substituted alkyl" refers to an alkyl radical substituted with at least one atom known to those of skill in the art. In certain embodiments, one or more substituents may be selected from the group consisting of hydrogen, halogen, oxo (*e.g.*, ether), hydroxy,

alkoxy, silyloxy, acyl, aryl, acetyl, carbonyl, cyano, heterocyclyl, amido, aminocarbonyl, amino, -NH-alkyl, -N(alkyl)$_2$, -NH-(substituted alkyl), -N-(substituted alkyl)$_2$, -NH-aryl, -N(aryl)$_2$, trialkylsilyloxy, acyloxy, acylamino, bis-acylamino, ester, NO, NO$_2$ and sulfo (*e.g.*, thioether, thioester, thiocarbonyl, sulfonamido, sulfonyl) and any combination thereof. In particular embodiments, a substituted alkyl is an aminoalkyl radical, such as –NH(CH$_2$)$_2$N(CH$_3$)$_2$ or –NH(CH$_2$)$_3$NH$_2$. In other particular embodiments, the subsituted alkyl is an hydroxyalkylamino radical, such as –NH(CH$_2$)$_3$NH(CH$_2$)$_2$OH. In other embodiments, a substituted alkyl is a haloalkyl. In other embodiments, a substituted alkyl is a hydroxyalkyl. In other embodiments, a substituted alkyl is a alkoxyalkyl.

As used herein the term "cycloalkyl" refers to carbocycle alkyl radicals of three or more atoms. A "substituted cycloalkyl" is a cycloalkyl group, the ring atoms of which comprise one or more functional group as substituents. A "heterocyclyl" refers to a cycloalkyl radical, the ring atoms of which are substituted with at least one heteroatom (*e.g.*, S, O, or N). A "substituted heterocycloalkyl" is a heterocycloalkyl group, the ring atoms of which comprise one or more functional group as substituents. Substituents may be selected, in some embodiments, from the group consisting of hydrogen, alkyl, halogen, oxo (*e.g.*, ether), hydroxy, alkoxy, silyloxy, acyl, aryl, acetyl, carbonyl, cyano, amido, aminocarbonyl, amino, -NH-alkyl, -N(alkyl)$_2$, -NH-(substituted alkyl), -N-(substituted alkyl)$_2$, -NH-aryl, -N(aryl)$_2$, trialkylsilyloxy, acyloxy, acylamino, bis-acylamino, ester, NO, NO$_2$ and sulfo (*e.g.*, thioether, thioester, thiocarbonyl, sulfonamido, sulfonyl) and any combination thereof.

As used herein, "aryl" refers to a carbocyclic aromatic group, including but not limited to those selected from the group consisting of phenyl, naphthyl, indenyl, indanyl, azulenyl, fluorenyl, and anthracenyl; or a heterocyclic aromatic group, including but not limited to those selected from the group consisting of furyl, furanyl, thienyl, pyridyl, pyrrolyl, oxazolyl, thiazolyl, imidazolyl, pyrazolyl, pyrazolinyl, pyrazolidinyl, isoxazolyl, isothiazolyl, oxadiazolyl, triazolyl, thiadiazolyl, pyridazinyl, pyrimidinyl, pyrazinyl, triazinyl, trithianyl, indolizinyl, indolyl, isoindolyl, indolinyl, thiophenyl, indazolyl, benzimidazolyl, benzthiazolyl, purinyl, quinolizinyl, quinolinyl, isoquinolinyl, innolinyl, phthalazinyl, quinazolinyl, quinoxalinyl, naphthyridinyl, pteridinyl carbazolyl, acridinyl, phenazinyl, phenothiazonyl, phenoxazinyl and any combination thereof.

"Aryl" groups, as defined in this application may independently contain one or more functional groups as substituents ("substituted aryl"). In certain embodiments, substituents may be selected from the group consisting of hydrogen, alkyl, halogen, oxo (*e.g.*, ether), hydroxy, alkoxy, silyloxy, acyl, aryl, acetyl, carbonyl, cyano, heterocyclyl, amido, aminocarbonyl, amino, -NH-alkyl, -N(alkyl)$_2$, -NH-(substituted alkyl), -N-(substituted alkyl)$_2$, -NH-aryl, -N(aryl)$_2$, trialkylsilyloxy, acyloxy, acylamino, bis-acylamino, ester, NO, NO_2 and sulfo (*e.g.*, thioether, thioester, thiocarbonyl, sulfonamido, sulfonyl) and any combination thereof.

As used herein, "halogen" refers to fluoro, chloro, bromo or iodo.

As used herein, the term "amino," alone or in combination, is used interchangeably with "amine" and refers to a primary amine, secondary amine or tertiary amine—that is, derivatives of ammonia (NH_3), in which one (primary), two (secondary) or three (tertiary) of the hydrogens have been replaced by carbon, wherein said carbon may be attached to any other atom(s).

As used herein, the term "cyclic group" refers to a cycloalkyl group, a substituted cycloalkyl group, a heterocyclyl group, a substituted heterocyclyl group, an aryl group, a substituted aryl group, or any combination thereof.

As used herein, the term "nucleophile" or "nucleophilic" generally refers to atoms bearing lone pairs of electrons. Such terms are well known in the art and include –NH_2, thiolate, carbanion and hydroxyl.

As used herein, the term "leaving group" generally refers to a group readily displaceable by a nucleophile, such as an amine, an alcohol, or a thiol nucleophile. Such leaving groups are well known and include carboxylates, N-hydroxysuccinimide, N-hydroxybenzotriazole, halogen (halides), triflates, tosylates, mesylates, alkoxy, thioalkoxy and the like.

The term "functional group" generally refers to how persons of skill in the art classify chemically reactive groups. Non-limiting examples of functional groups include hydroxyl, amine, sulfhydryl, amide, carboxyls, carbonyls, *etc.* While hydrogen and unsubstituted alkyl groups are not typically considered functional groups, as used herein hydrogen and/or unsubstituted alkyl groups may, in certain embodiments, be considered functional groups. In certain embodiments, hydrogen and/or unsubstituted alkyl groups are explicitly not considered functional groups.

As used herein, "protecting group" refers to a moiety attached to a functional group to prevent an otherwise unwanted reaction of that functional group. Protecting groups are well-known to those of skill in the art. Non-limiting exemplary protecting groups fall into categories such as hydroxy protecting groups, amino protecting groups, sulfhydryl protecting groups and carbonyl protecting groups. Such protecting groups may be found in Greene and Wuts, 1999 (incorporated herein by reference in its entirety).

As used herein, a "linker" is any diradical species that may covalently join one anthrapyzrazole to another anthrapyrazole such that the linker does not chemically react with either anthrapyrazole. Non-limiting examples include $(-CH_2-)_p$, wherein p is 1-10 (that is, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) or higher; $-(CH_2)_2N(CH_3)(CH_2)_2OC(O)(CH_2)_nC(O)O(CH_2)_2N(CH_3)(CH_2)_2-$, wherein n is 1-5 or higher (that is 1, 2, 3, 4, 5 or higher), $-(CH_2)_2N(CH_3)(CH_2)_2NHC(O)(CH_2)_nC(O)NH(CH_2)_2N(CH_3)(CH_2)_2-$, wherein n is 1-5 or higher, $-(CH_2)_2N(CH_3)(CH_2)_2OC(S)(CH_2)_nC(S)O(CH_2)_2N(CH_3)(CH_2)_2-$, wherein n is 1-5 or higher, and polyether diradicals (*e.g.*, $-(CH_2-O)_r-$, wherein r is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or higher. Other non-limiting examples of linkers are described herein and are known to those of skill in the art.

Persons of skill in the art will recognize that, when two atoms (*e.g.*, atom 1 and atom 2) are joined by either a single or a double bond as described herein (*e.g.*, L and Y_1 of the compound of formula (I)), depending what other atoms atom 1 and atom 2 are each bonded to, the choice of atom 1 and atom 2 becomes constricted due to bonding principles associated with atom 1 and atom 2. For instance, if L and Y_1 are double bonded to each other in formula (I), the choice of L is narrowed from carbon, -CH or nitrogen to carbon and nitrogen, and the choice of Y_1 is narrowed from of -CH, $-CH_2$, oxygen, nitrogen, -NH and sulfur to –CH or nitrogen. Those of skill in the art are familiar with bonding principles and preferences of atoms such as carbon, nitrogen, oxygen and sulfur.

Compounds as described herein may contain one or more asymmetric centers and thus can occur as racemates and racemic mixtures, single enantiomers, diastereomeric mixtures and individual diastereomers. All possible stereoisomers of the all the compounds described herein, unless otherwise noted, are contemplated as being within the scope of the present invention. The chiral centers of the compounds

of the present invention can have the S- or the R-configuration, as defined by the IUPAC 1974 Recommendations. The present invention is meant to comprehend all such isomeric forms of the compounds of the invention.

The claimed invention is also intended to encompass salts of any of the synthesized compounds of the present invention. The term "salt(s)" as used herein, is understood as being acidic and/or basic salts formed with inorganic and/or organic acids and bases. Zwitterions (internal or inner salts) are understood as being included within the term "salt(s)" as used herein, as are quaternary ammonium salts such as alkylammonium salts. Nontoxic, pharmaceutically acceptable salts are preferred as described below, although other salts may be useful, as for example in isolation or purification steps.

Non-limiting examples of acid addition salts include but are not limited to acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate and undecanoate.

Non-limiting examples of basic salts include but are not limited to ammonium salts; alkali metal salts such as sodium, lithium, and potassium salts; alkaline earth metal salts such as calcium and magnesium salts; salts comprising organic bases such as amines (*e.g.,* dicyclohexylamine, alkylamines such as *t*-butylamine and *t*-amylamine, substituted alkylamines, aryl-alkylamines such as benzylamine, dialkylamines, substituted dialkylamines such as N-methyl glucamine, trialkylamines, and substituted trialkylamines); and salts comprising amino acids such as arginine, lysine and so forth. The basic nitrogen-containing groups may be quaternized with agents such as lower alkyl halides (*e.g.,* methyl, ethyl, propyl, and butyl chlorides, bromides and iodides), dialkyl sulfates (*e.g.,* dimethyl, diethyl, dibutyl, and diamyl sulfates), long chain halides (*e.g.,* decyl, lauryl, myrtistyl and stearyl chlorides, bromides and iodides), arylalkyl halides (*e.g.,* benzyl and phenethyl bromides) and others known in the art.

As to any of the groups or compounds described herein that contain or optionally contain one or more substituents, it is understood, of course, that such groups do not contain any substitution or substitution patterns which are sterically impractical and/or synthetically non-feasible.

An "anti-cancer" agent is capable of negatively affecting cancer in a subject, for example, by killing one or more cancer cells, inducing apoptosis in one or more cancer cells, reducing the growth rate of one or more cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the blood supply to a tumor or one or more cancer cells, promoting an immune response against one or more cancer cells or a tumor, preventing or inhibiting the progression of a cancer, or increasing the lifespan of a subject with a cancer. Anti-cancer agents are well-known in the art and include, for example, chemotherapy agents (chemotherapy), such as DNA intercalators, radiotherapy agents (radiotherapy), a surgical procedure, immune therapy agents (immunotherapy), genetic therapy agents (gene therapy), reoviral therapy, hormonal therapy, other biological agents (biotherapy), and/or alternative therapies.

The term "effective," as that term is used in the specification and/or claims (*e.g.*, "an effective amount," means adequate to accomplish a desired, expected, or intended result.

"Treatment" and "treating" as used herein refer to administration or application of a therapeutic agent to a subject or performance of a procedure or modality on a subject for the purpose of obtaining a therapeutic benefit of a disease or health-related condition. For example, a subject (*e.g.*, a mammal, such as a human) having cancer may be subjected to a treatment comprising administration of one or more bisanthrapyrazoles of the present invention.

The term "therapeutic benefit" or "therapeutically effective" as used throughout this application refers to anything that promotes or enhances the well-being of the subject with respect to the medical treatment of a condition. This includes, but is not limited to, a reduction in the frequency or severity of the signs or symptoms of a disease. For example, a therapeutically effective amount of a bisanthrapyrazole of the present invention may be administered to a subject having a cancerous tumor, such that the tumor shrinks.

It is specifically contemplated that any limitation discussed with respect to one embodiment of the invention may apply to any other embodiment of the invention. Furthermore, any composition of the invention may be used in any method of the invention, and any method of the invention may be used to produce or to utilize any composition of the invention.

The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternative are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device and/or method being employed to determine the value.

As used herein the specification, "a" or "an" may mean one or more, unless clearly indicated otherwise. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

The following figures form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Bisanthrapyrazole **1D** shown bound in the minor groove of a 6 base-pair piece of DNA. The two doxorubicin molecules were removed from the Protein Data Bank 1da9.pb x-ray structure of the doxorubicin-DNA complex and **1D** was docked into the DNA with the genetic algorithm docking program GOLD.

FIG. 2. ΔT_m for DNA binding as a function of the number of CH_2 linker groups in bisanthrapyrazoles **1A-1E**.

FIGS. 3A, 3B. 3A: GOLD docking score as a function of the number of CH_2 linker groups in certain bisanthrapyrazoles. **3B:** GoldScore does not predict DNA ΔT_m.

FIG. 4: DNA ΔT_m, cytotoxicity and topoisomerase IIα inhibitor effects of bisanthrapyrazoles **1A-1E**.

FIG. 5: K562 cytotoxicity is not correlated with DNA ΔT_m.

FIG. 6: Structures of the anthrapyrazoles, mitoxantrone and doxorubicin. The core structure used for alignment of the anthrapyrazoles for the 3D-QSAR CoMFA and CoMSIA analyses is shown in bold.

FIG. 7: QSAR correlations of growth inhibition of K562 cells by the anthrapyrazoles and mitoxantrone. **FIG. 7A:** The IC_{50} for growth inhibition of K562 cells by the anthrapyrazoles and mitoxantrone was highly significantly correlated (p = 3 X 10^{-5}) with the strength of DNA binding as measured by their ability to increase the thermal melt temperature of DNA (ΔT_m). **FIG. 7B:** The IC_{50} for growth inhibition of K562 cells by the anthrapyrazoles and mitoxantrone was not significantly correlated (p = 0.2) with the inhibition of the catalytic decatenation activity of topoisomerase IIα. The anthrapyrazoles are identified by the numbers inside the symbols and losoxantrone, piroxantrone and mitoxantrone are identified as L, P, and M, respectively.

FIG. 8: Effect of anthrapyrazoles on the topoisomerase IIα-mediated cleavage of supercoiled pBR322 DNA. This fluorescent image of the ethidium bromide-stained gel shows that topoisomerase IIα relaxed supercoiled pBR322 plasmid DNA (SC) to relaxed DNA (RLX). As shown in lane 2 topoisomerase IIα completely relaxed pBR322 DNA (lane 1, no enzyme). Topoisomerase IIα was present in the reaction mixture for all other lanes. As shown in lane 13 etoposide treatment produced linear DNA (LIN) and inhibited the relaxation of supercoiled pBR322 DNA. A small amount of nicked circular (NC) is normally present in the pBR322 DNA. Bands identified as linear DNA are marked above and to their left with an "*". AP-1, AP-2, AP-6, AP-10, AP-11 and AP-12 are identified as having produced linear DNA above control levels (lane 2). The binding of some of the

fluorescent anthrapyrazoles (AP-3, AP-4, AP-8 and AP-9) to DNA obscured the band where the linear DNA would be expected to be found and also caused a mobility shift due to their binding. Topo IIα is topoisomerase IIα.

FIG. 9: Docking of the protonated anthrapyrazoles into DNA, their aligned docked structures and CoMSIA contour plots. **FIG. 9A:** The highest scoring structure of the most potent anthrapyrazole AP-10 (ball-and-stick structure) is shown docked into DNA (stick structure). The complex forms both base stacking and H-bonding interactions (green dotted lines). The side chains of AP-10 formed 3 hydrogen bonds (1 from the terminal OH and 2 others from the secondary and tertiary nitrogens) with DNA. The hydroxyl side chain of AP-10 was positioned in the minor groove, and the dimethyl amino side chain was in the major groove. The H-atoms are not shown for clarity. The DNA structure is 1DA9 from the Protein Data Bank and is a doxorubicin/DNA x-ray structure in which two doxorubicin molecules are bound to a 6-bp piece of DNA. Only the first 3 base pairs of the DNA are shown in the figure for clarity. One doxorubicin was removed and AP-10 was docked into its place with the genetic algorithm docking program GOLD. **FIG. 9B:** The structures of all the anthrapyrazoles and mitoxantrone that were docked into DNA and aligned to the core structure as shown in Figure 1 (bold bonds) are shown superimposed. **FIG. 9C:** CoMSIA stddev*coeff contour hydrogen bond donor plots for the K562 growth inhibition data are shown for AP-10, the most potent anthrapyrazole. The green contours indicate regions where hydrogen bond donors increase activity, and the red contours indicate regions where hydrogen bond donors decrease activity. The green contours were located near the nitrogens of the side chains indicating the importance of these groups. **FIG. 9D:** CoMSIA stddev*coeff contour electrostatic plots for the K562 growth inhibition data are shown for AP-10, the most potent anthrapyrazole. The green contours indicate regions where electrostatic interactions increase activity and the red contours indicate regions where electrostatic interactions decrease activity. The green contours were located near the protonated nitrogens of the side chains, indicating the importance of protonated nitrogens in the activity of the anthrapyrazoles.

FIG. 10: QSAR correlations of IC_{50} values for growth inhibition of K562 cells by the anthrapyrazoles and mitoxantrone with energy terms obtained by docking the anthrapyrazoles into a DNA x-ray structure. **FIG. 10A:** The IC_{50} for growth

inhibition of K562 cells by the anthrapyrazoles and mitoxantrone was highly correlated ($p = 2 \times 10^{-4}$) with the GOLDScore external van der Waals energy term in the GOLDScore fitness function indicating that increased van der Waals interactions with DNA increase the potency of these compounds. **FIG. 10B:** The IC_{50} for growth inhibition of K562 cells by the anthrapyrazoles and mitoxantrone was highly correlated ($p = 4 \times 10^{-4}$) with the GOLDScore internal energy term in the GOLDScore fitness function indicating that increased internal energy (sum of internal torsion and internal van der Waals energies) of the docked compound interactions with DNA decreases the potency of these compounds. **FIG. 10C:** The ΔT_m for the anthrapyrazoles and mitoxantrone were highly correlated ($p = 0.002$) with the GOLDScore external van der Waals energy term in the GOLDScore fitness function indicating that increased van der Waals interactions with DNA increase the DNA binding of these compounds. **FIG. 10D:** The ΔT_m for the anthrapyrazoles and mitoxantrone was highly correlated ($p = 0.001$) with the GOLDScore internal energy term in the GOLDScore fitness function indicating that increased internal energy (sum of internal torsion and internal van der Waals energies) of the docked compound interactions with DNA decreases the DNA binding of these compounds. The anthrapyrazoles are identified by the numbers inside the symbols and losoxantrone, piroxantrone and mitoxantrone are identified as L, P, and M, respectively. In GOLD a positive increase in an energy term corresponds to increased growth inhibitory activity or binding.

FIG. 11: CoMSIA predictions for the effect of the anthrapyrazoles and mitoxantrone on IC_{50} for growth inhibition of K562 cells (**FIG. 11A**) and the increase in the DNA melt temperature ΔT_m (**FIG. 11B**). The straight lines are the regression lines for the predictions. The anthrapyrazoles are identified by the numbers inside the symbols and losoxantrone, piroxantrone and mitoxantrone are identified as L, P, and M, respectively.

FIG. 12: Concentration dependence of ΔT_m for the bisanthrapyrazoles binding to DNA. (**FIG. 12A**) Concentration dependence of ΔT_m for the bisanthrapyrazoles **1A**, **1B**, **1C**, **1D**, and **1E** and parent monomer AP9 for comparison. The solid straight lines are linear least squares fits to the data. (**FIG. 12B**) Concentration dependence of the slopes ± SEM calculated from the data in (**FIG. 12A**). Except for **1A** (NS, not significant) the slopes of the plots in (**FIG. 12A**) for 2,

3, 4, and 5 are all significantly (***$p < 0.001$) different than the parent AP9. Compounds with slopes approximately twice that of the parent monomer AP9 indicate that they formed bisintercalation complexes with DNA.

FIG. 13: Effect of bisanthrapyrazoles **1A**, **1B**, **1C**, **1D**, and **1E** on the topoisomerase IIα-mediated cleavage of supercoiled pBR322 DNA. This fluorescent image of the ethidium bromide-stained gel shows that topoisomerase IIα (Topo IIα) relaxed supercoiled pBR322 plasmid DNA (SC) to relaxed (RLX) DNA (lane 2, the band running slightly ahead of the SC band). pBR322 DNA in the absence of topoisomerase IIα is shown in lane 1. Topoisomerase IIα was present in the reaction mixture for all other lanes. As shown in lane 8 etoposide treatment (100 μM) produced linear DNA (LIN). A small amount of nicked circular (NC) is normally present in the pBR322 DNA. BisAP identifies the bisanthrapyrazoles **1A**, **1B**, **1C**, **1D**, and **1E**. Based on densitometry, none of the bisanthrapyrazoles (50 μM) produced any significant amount of linear DNA above control levels (lane 2). Some of the fluorescent bisanthrapyrazoles present in the gel (lanes 3, 5, and 7) partially obscured the linear DNA band.

FIG. 14: Docking of the protonated bisanthrapyrazole **1B** into DNA. The highest scoring structure of the strongest DNA-binding bisanthrapyrazole **1B** (CPK structure) is shown docked into DNA (stick structure). The H-atoms of the DNA are not shown for clarity. The DNA structure is 1DA9 from the Protein Data Bank and is a DNA– $(doxorubicin)_2$ X-ray structure in which two doxorubicin molecules are bound to a 6-base pair piece of DNA. Both doxorubicin molecules were removed and **1B** was docked into its place with the genetic algorithm docking program GOLD.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention is based on the finding that certain bisanthrapyrazoles likely behave as DNA intercalating agents and topoisomerase IIα inhibitors, and show potent inhibition of cancer cells. In general, these bisanthrapyrazoles comprise two anthrapyrazoles operatively linked together. In certain embodiments, the linkage comprises one or more ester and/or amide groups. Some of these bisanthrapyrazoles bind to DNA more strongly than doxorubicin, a known DNA intercalator and anti-cancer drug. As such, the present invention provides for novel anti-cancer agents, their syntheses and methods of using them in chemotherapeutic treatments.

Molecular modeling methods are also set forth herein that provide insight into how compounds bind to DNA and what the strength of that bind is.

A. Topoisomerases

Topoisomerase is an isomerase enzyme that alters the supercoiling of DNA. The double-helical configuration of DNA strands makes them difficult to separate, and yet they must be separated by helicase proteins if other enzymes are to transcribe the sequences that encode proteins, or if chromosomes are to be replicated. In so-called circular DNA, in which double helical DNA is bent around and joined in a circle, the two strands are topologically linked, or knotted. Otherwise identical loops of DNA having different numbers of twists are topoisomers, and cannot be interconverted by any process that does not involve the breaking of DNA strands. Topoisomerases catalyze and guide the unknotting of DNA. This unlinking activity is termed "decatenation."

Topoisomerases are classified into two types separated by the number of strands cut in one round of action. Topoisomerase I cuts one strand, passes the other through it then reanneals the cut strand. Topoisomerase II cuts both strands, and passes an unbroken double strand through it then reanneals the cut strand. Mammalian topoisomerase II has been further classified into types IIα and IIβ. Some chemotherapy drugs work by interfering with topoisomerases in cancer cells. (Kornberg and Baker, DNA Replication, W. H. Freeman and Company, New York, 1991; Pommier *et al.*, 1998). For example, topoisomerase I is inhibited by irinotecan (Campto®) and topotecan (Hycamtin®). Topoisomerase II is inhibited by etoposide (Etopophos®, Vepesid®) and teniposide (Vumon®, VM-26®).

Drugs acting on topoisomerase II are divided into two main categories, topoisomerase II poisons and topoisomerase II catalytic inhibitors. The topoisomerase II poisons are associated with their ability to stabilize the enzyme-DNA cleavable complex and shift the equilibrium of the catalytic cycle towards cleavage, thereby increasing the concentration of the transient protein-associated breaks in the genome (Froelich-Ammon and Osheroff, 1995). That is, they trap the cleavable complexes, which converts the essential topoisomerase II enzyme into a lethal one (Chen and Liu, 1994). Topoisomerase poisons may bind to DNA, the topoisomerase, or either molecule at or near the region of the enzyme. Many topoisomerase poisons, such as the anthracyclines (discussed below) and actinomycin

D, are relatively planar hydrophobic compounds that intercalate DNA. Nonintercalating DNA binders can also poison topoisomerase (Chen, *et al.*, 1993). However, DNA binding is neither a necessary nor sufficient condition for topoisomerase poisoning (Bast, Kufe, Pollock, Weichselbaum, Holland, Frei and Gansler, Eds., Cancer Medicine, 5th Edition, BC Decker Inc., Hamilton, Ontario, 2000). Several antitumor agents in clinical use have potent activity as mammalian topoisomerase II poisons. These include doxorubicin (Adriamycin®), actinomycin D (Cosmegen®), daunomycin, etoposide (Etopophos®, Vepesid®) and teniposide (Vumon®, VM-26®).

The topoisomerase II catalytic inhibitors are an entirely different group of drugs. They act by interfering with the overall catalytic function, which can be accomplished in at least two ways. One is the inhibition of the initial binding of topoisomerase II to DNA, as in the case of chloroquine (Jensen *et al.*, 1994) and aclarubicin (Sehested and Jensen, 1996; Sørensen *et al.*, 1992). The other is by locking topoisomerase II in its closed-clamp step after religation, as appears to be the case for the ICRF-187 and its analogs (Tanabe *et al.*, 1991; Berger *et al.*, 1996; Roca *et al.*, 1994; Roca *et al.*, 1996). While the mechanisms of topoisomerase II poisons and inhibitors are different, one agent can act as both a topoisomerase II inhibitor and as a topoisomerase II poison.

Doxorubicin (Adriamycin®) and daunorubicin are anthracycline antibiotics that intercalate with DNA and are also topoisomerase IIα poisons. (Chaires, 1990a; Chaires, 1990b; Chaires, 1990c). Bisanthracyclines linked by a sugar moiety have also been studied. U.S. Patent No. 5,874,412. Although doxorubicin has an established place in the treatment of various solid and hematological neoplasms, one major drawback is its severe dose-related cardiotoxicity. While the cause of this cardiotoxicity is not exactly known, a tendency of the compound to form free radicals is suspected.

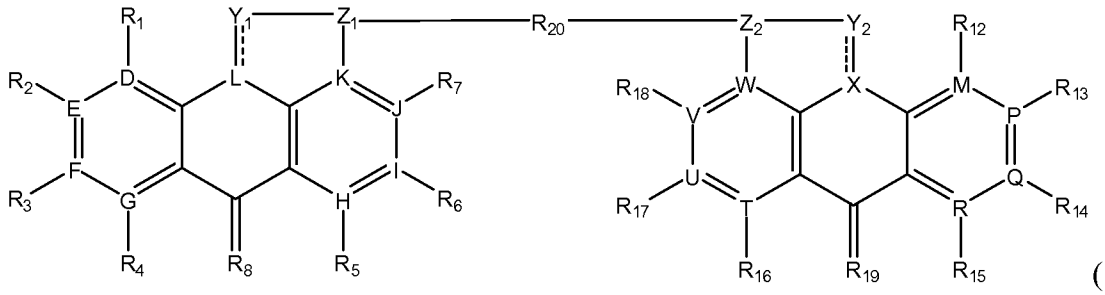
B. Bisintercalating Bisanthrapyrazoles

1. Design

As discussed herein, a series of new bisanthrapyrazole bisintercalating compounds have been designed and synthesized with a variety of linker chain lengths in order to obtain QSAR data for further drug development. Molecular modeling of a bisanthrapyrazole of the present invention docked into a DNA x-ray crystal structure

indicated that the bisanthrapyrazole was positioned in the DNA minor groove and docked at the same two positions as doxorubicin (see Example 2 and FIG. 1). This study implicates the bisanthrapyrazoles as DNA bisintercalators.

Bisanthrapyrazoles of the present invention generally comprise two anthrapyrazoles joined directly or *via* a linker. The bisanthrapyrazoles of the present invention may, in certain embodiments, be exemplified by the generic structure of formula (II):



(II)

wherein: R_1-R_7 and R_{12}-R_{18} are each independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl, phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group, or any one or more of Y_1-R_1, R_1-R_2, R_2-R_3, R_3-R_4, R_5-R_6, R_6-R_7, R_7-Z_1, Y_2-R_{12}, R_{12}-R_{13}, R_{13}-R_{14}, R_{14}-R_{15}, R_{16}-R_{17}, R_{17}-R_{18} or R_{18}-Z_2 together forms a cyclic group, or any combination of one or more of these groups; R_8 and R_{19} are each independently oxygen or sulfur; D, E, F, G, H, I, J, K, L, M, P, Q, R, T, U, V, W and X are each independently carbon, -CH or nitrogen; Y_1 and Y_2 are each independently selected from the group consisting of -CH, -CH_2, oxygen, nitrogen, -NH and sulfur; Z_1 and Z_2 are each independently selected from the group consisting of -CH and nitrogen; the L-Y_1 bond and the X-Y_2 bond are each independently a single or a double bond; and R_{20} is a linker.

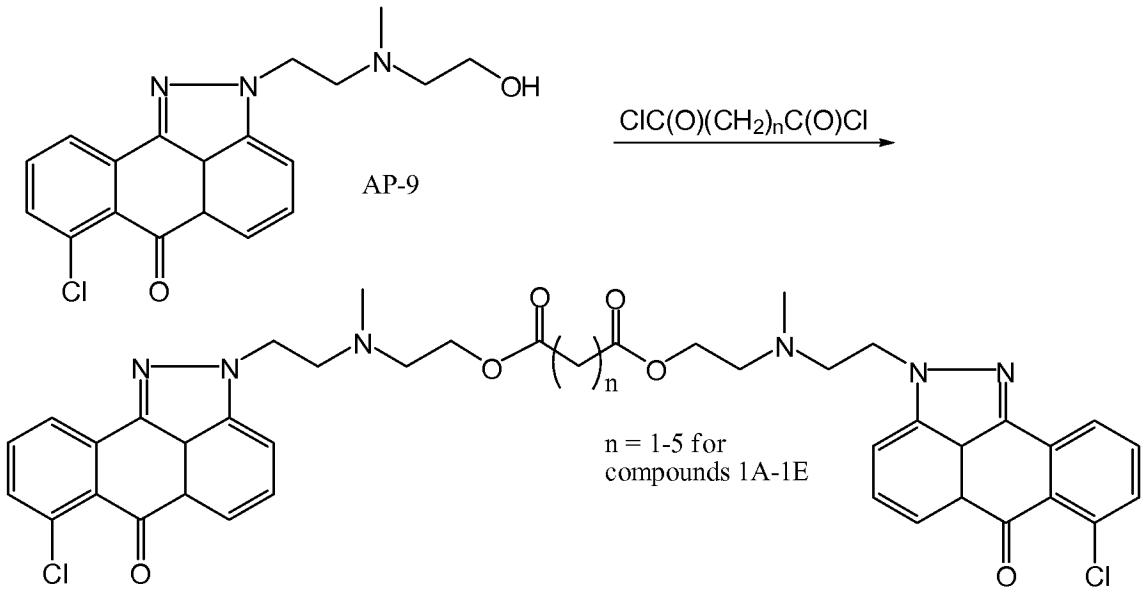
In certain embodiments, the present invention concerns methods for identifying further bisanthrapyrazoles, which are potential DNA intercalators, topoisomerase I or II inhibitors, and/or anti-cancer agents. It is contemplated that such identification will prove useful in the general identification of any compound that will serve the purpose of interacting with DNA in a manner similar to the exemplary bisanthrapyrazoles disclosed herein. The molecular modeling protocol explained in Example 2 provides one means of identifying additional intercalating

bisanthrapyrazoles. An alternative method for testing whether a bisanthrapyrazole is an intercalating agent is by using viscosity assays as described by Suh and Chaires (1995). Such assays are well known to those of skill in the art. In theory, for monointercalation, plots of the cubed root of the relative viscosity ($(\eta/\eta_0)^{1/3}$) versus the binding ratio (bound drug/DNA bp) ought to have a slope of 1.0. For bisintercalators, the slope is expected to be twice that observed for monointercalators, an expectation that has been verified for a variety of bisintercalating compounds (Wakelin, 1986). Thus in order to test the effectiveness of a candidate bisintercalator, the viscosity of a DNA solution is measured according to assays well known to those of skill in the art (Suh and Chaires, 1995) and compared to the viscosity of a known intercalator, such as daunorubicin. If the viscosity of the candidate substance is higher than that of the control monointercalator then the candidate substance is likely to be an effective bisintercalator.

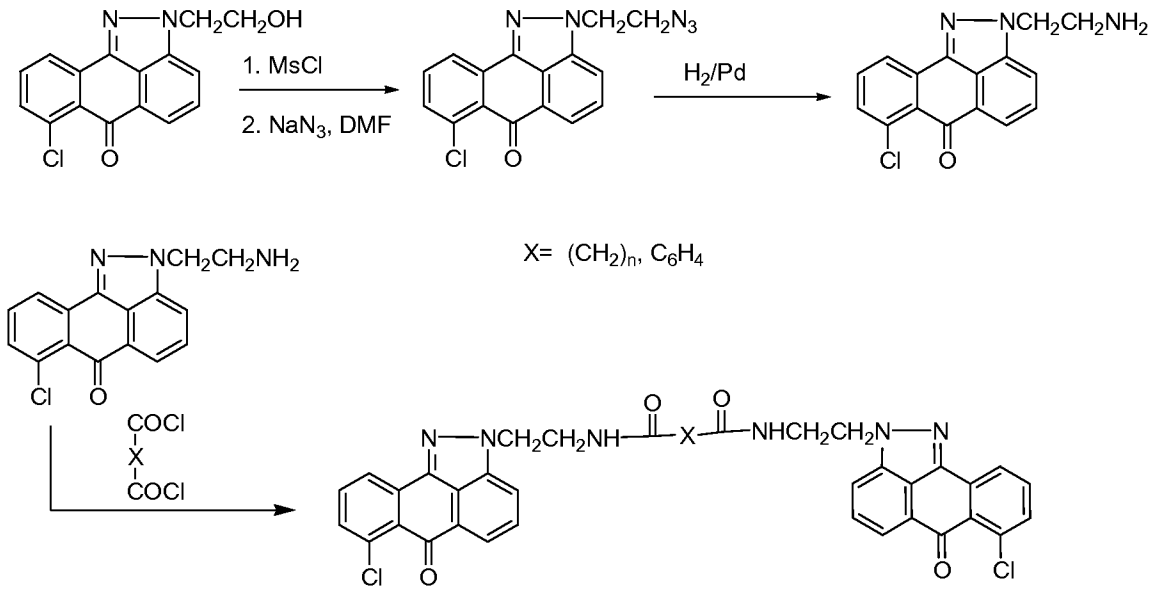
In certain embodiments, the most useful pharmacological compounds for identification through application of, *e.g.*, molecular modeling, will be compounds that are further metabolized before they are therapeutically active (*i.e.*, "prodrugs"). In certain embodiments, the active compounds may include fragments or parts of naturally-occurring compounds or may be only found as active combinations of known compounds which are otherwise inactive. In certain embodiments, the fragment may be an anthrapyrazole.

2. Synthesis

Preparations of various anthrapyrazoles are known to those of skill in the art. *See, e.g.,* Begleiter *et al.*, 2006; Liang *et al.*, 2006; Showalter *et al.*, 1987; and Showalter *et al.*, 1986 (each of these references are incorporated herein in their entirety). To obtain a bisanthrapyrazole, one may, for example, prepare an anthrapyrazole such as that of formula (I) above and incorporate a hydroxyl moiety at any of R_1-R_7 or R_{10}, such as at R_5. Reaction of such a compound with any of a variety of diacyl halides (*e.g.*, diacyl chloride, diacyl bromide) will yield a bisanthrapyrazole connected by a diester linkage. A non-limiting, representative bisanthrapyrazole synthetic scheme is shown below, wherein the preparation of the shown anthrapyrazole starting material (AP-9) has been previously reported (Liang *et al.*, 2006) (see also Example 1).



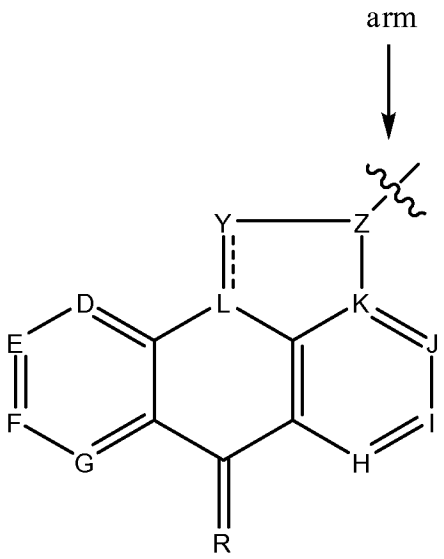
Bisanthrapyrazoles may be synthesized wherein instead of ester linkages bridging the two anthrapyrazoles, amide linkages are used. Such linkages could be generated by, for example, the scheme shown below:



MsCl is mesyl chloride
DMF is dimelthyl formamide

A non-limiting example of the preparation of an amide-linked bisanthrapyrazole of the present invention is presented in Example 17.

More generally, to generate a bisanthrapyrazole, one may, in certain embodiments: incorporate a nucleophile at a terminal end of one "arm" of a first anthrapyrazole; incorporate a leaving group at a terminal end of a second anthrapyrazole; and link the two together *via* conventional organic chemistry techniques. An "arm" is schemtically shown using the core skeleton structure of formula (A):



(A)

Conjugation of first and second bisanthrapyrazoles is not restricted to linkages between their "arms." Conjugation may take place at any position available for conjugation on either anthrapyrazole, such as a terminal end of the "arm" (which includes the terminal group of any branched group stemming from the backbone of the "arm"), any ring atom of rings that make up the anthrapyrazoles, or any substituent on any of the rings that make up the anthrapyrazoles. For example, a linkage may form between substitutions on the aromatic rings of the first and second anthrapyrazoles (*see* Begleiter *et al.*, 2006; Liang *et al.*, 2006).

In certain embodiments, the first and second anthrapyrazoles are the same. In certain embodiments, the first and second anthrapyrazoles are different. In the latter case, the different bisanthrapyrazoles that would form in the reaction would require separation. Separation techniques are well-known in the art and include, for example, column chromatography, HPLC, crystallization and dialysis.

In certain embodiments, the present invention provides for substantially pure bisanthrapyrazoles. As used herein, the term "substantially pure" indicates that the bisanthrapyrazoles of interest constitutes the predominant species in a mixture (that is, greater than 50%, or, in other words, at least 50% pure). A substantially pure bisanthrapyrazoles may be about or at least about 51%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 99.9% pure. In certain embodiments, a substantially pure bisanthrapyrazoles excludes any naturally occurring mixtures that contain the compound. A substantially pure bisanthrapyrazoles may also, or in the alternative, refer to the percent enantiomeric excess (% ee) of the compound. In certain embodiments, a substantially pure bisanthrapyrazoles may exhibit a % ee of about or at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65% or higher, or any range derivable therein, such as about 10% to about 62%.

In certain embodiments, bisanthrapyrazoles are prepared as salts, such as dihydrochloride salts. In certain embodiments, the bisanthrapyrazoles are fairly soluble, such as when they exist in their dihydrochloride salt form: in such a case, the dihydrochloride salt may be more soluble than the bisanthrapyrazole compound in its non-salt form.

The invention also contemplates formation of compounds that comprise three or more anthrapyrazoles. For example, upon formation of a bisanthrapyrazole, a third anthrapyrazole, which may be the same or different than the anthrapyrazoles which make up the bisanthrapyrazole, may be linked to the bisanthrapyrazole.

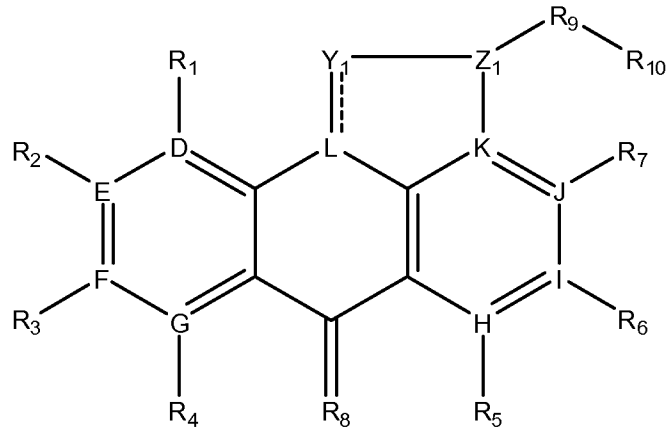
Modifications or derivatives of the compounds, agents, and active ingredients disclosed throughout this specification are contemplated as being useful with the methods and compositions of the present invention. Derivatives may be prepared and the properties of such derivatives may be assayed for their desired properties by any method known to those of skill in the art.

In certain aspects, "derivative" refers to a chemically modified compound that still retains the desired effects of the compound prior to the chemical modification. "bisanthrapyrazole derivatives," therefore, refers to a chemically modified compound that still retains the desired effects of the parent bisanthrapyrazole prior to its chemical modification. Such effects may be enhanced (*e.g.*, slightly more effective, twice as effective, etc.) or diminished (*e.g.*, slightly less effective, 2-fold less

effective, etc.) relative to the unmodified bisanthrapyrazole, but may still be considered a bisanthrapyrazole derivative. Such derivatives may have the addition, removal, or substitution of one or more chemical moieties on the parent molecule. Non-limiting examples of the types of modifications that can be made to the compounds and structures disclosed herein include the addition or removal of lower unsubstituted alkyls such as methyl, ethyl, propyl, or substituted lower alkyls such as hydroxymethyl or aminomethyl groups; carboxyl groups and carbonyl groups; hydroxyls; nitro, amino, amide, imide, and azo groups; sulfate, sulfonate, sulfono, sulfhydryl, sulfenyl, sulfonyl, sulfoxido, sulfonamide, phosphate, phosphono, phosphoryl groups, and halide substituents. Additional modifications can include an addition or a deletion of one or more atoms of the atomic framework, for example, substitution of an ethyl by a propyl; substitution of a phenyl by a larger or smaller aromatic group. Alternatively, in a cyclic or bicyclic structure, heteroatoms such as N, S, or O can be substituted into the structure instead of a carbon atom.

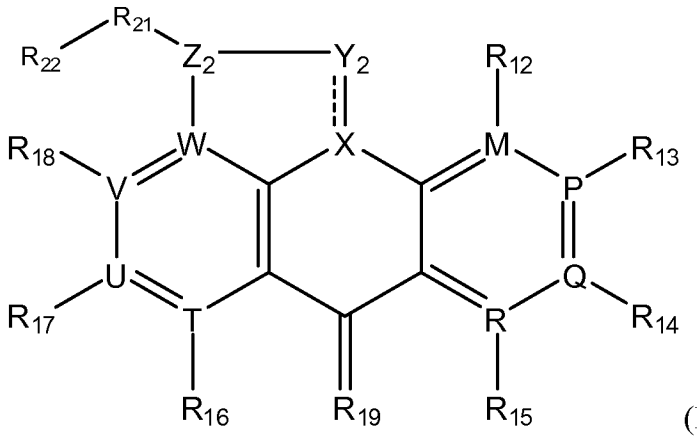
Another aspect of the present invention contemplates a method of preparing a bisanthrapyrazole comprising preparing a first anthrapyrazole, preparing a second anthrapyrazole, and conjugating the first anthrapyrazole to the second anthrapyrazole either directly or through a linker. The linker may join the two anthrapyrazoles at any two or more atoms of either anthrapyrazole. The linker may be any type of linker described herein, and known to those of skill in the art. The joined atoms of the first and second anthrapyrazoles may be any two atoms as described herein (*e.g.*, the Z atoms of the compounds of core skeleton formula (A), or the Z_1 atoms of the anthrapyrazoles of the compound of formula (I)). Typically, one of the two anthrapyrazoles comprises a nucleophile and the other comprises a leaving group, and the two anthrapyrazoles are linked together *via* reaction of the nucleophile and the atom to which the leaving group is attached.

Also contemplated by the present invention is a method of preparing a compound of formula (II) comprising: preparing a first anthrapyrazole of formula (I):



(I)

wherein: R_1-R_7 are each independently hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl, phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group, or any one or more of Y_1-R_1, R_1-R_2, R_2-R_3, R_3-R_4, R_5-R_6, R_6-R_7 or R_7-Z_1 together forms a cyclic group, or any combination of one or more of these groups; R_8 is either oxygen or sulfur; R_9 is either not present or is a linker; and R_{10} is either hydrogen, alkyl, a nucleophile or a leaving group; D, E, F, G, H, I, J, K and L are each independently carbon, -CH or nitrogen; Y_1 is selected from the group consisting of -CH, -CH_2, oxygen, nitrogen, -NH and sulfur; Z_1 is selected from the group consisting of –CH and nitrogen; and the bond between L and Y_1 is either a single or a double bond; preparing a second anthrapyrazole of formula (III):



(III)

wherein R_{12}-R_{18} are each independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl, phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group, or any one or more of Y_2-R_{12}, R_{12}-R_{13}, R_{13}-R_{14}, R_{14}-R_{15}, R_{16}-R_{17}, R_{17}-R_{18} or

R_{18}-Z_2 together forms a cyclic group, or any combination of one or more of these groups; R_{19} is either oxygen or sulfur; R_{21} is either not present or is a linker; and R_{22} is either H, alkyl, a nucleophile or a leaving group; M, P, Q, R, T, U V, W and X are each independently selected from the group consisting of carbon, -CH and nitrogen; Y_2 is selected from the group consisting of -CH, -CH_2, oxygen, nitrogen, -NH and sulfur; Z_2 is selected from the group consisting of –CH and nitrogen; and the bond between X and Y_2 is either a single or a double bond; and conjugating said first anthrapyrazole to said second anthrapyrazole either directly or through a linker. The conjugation may take place *via* any method known to those of skill in the art, and may take place between two or more atoms on either anthrapyrazole. In certain embodiments, the conjugation step comprises conjugating said first anthrapyrazole to said second anthrapyrazole through a linker that joins the Z_1 and Z_2 positions of each anthrapyrazole. In certain embodiments of this method, the linker comprises an alkyl group, a substituted alkyl group, a heterocyclyl group, an oxo group, a sulfo group, a thioether group, an aryl group, an amide group, a sulfonamide group, a carbonyl group, a thiocarbonyl group, a secondary amine group, a tertiary amine group, an ester group, a thioester group, a sulfonyl group, or any combination of one or more of these groups. In more particular embodiments, the linker may comprise an alkyl group, at least one ester, and/or at least one amide. In certain embodiments, R_{10} and R_{22} are selected from the group consisting of a nucleophile and a leaving group, wherein $R_{10} \neq R_{22}$.

3. Biological Activity

As discussed herein, the bisanthrapyrazoles of the present invention are likely bisintercalators. Antitumor activity and topoisomerase IIα decatenation activity of the bisanthrapyrazoles were each explored. These studies indicated that these compounds exert their cytotoxic effects by acting as topoisomerase II decatenation activity inhibitors (Begleiter *et al.*, 2006; Liang *et al.*, 2006). This is keeping with the inhibition activities of loxoxantrone and piroxantrone, but in contrast to the dual topoisomerase IIα inhibitor/poison behavior displayed by other monoanthrapyrazoles (Begleiter *et al.*, 2006; Liang *et al.*, 2006).

In particular embodiments, it will be necessary to determine the cytotoxic effect of a bisanthrapyrazole of the present invention in its role as an effective anticancer agent. In these embodiments, the present invention is directed to a method

for determining the ability of a bisanthrapyrazole to inhibit the growth of cancer cells. In certain embodiments, the method includes generally the steps of:

(a) obtaining a cancer cell responsive to DNA intercalators;

(b) admixing a bisanthrapyrazole with the cell; and

(c) determining the ability of the bisanthrapyrazole to inhibit the growth of the cell.

Cancer cells known to be responsive to DNA intercalators are well-known in the art.

Inhibition of growth of cancer cells may be measured by, for example, the cell culture and cytotoxicity assay as set forth in Example 3 below, comprising the MTS assay. *See also* Liang *et al.*, 2006. Inhibition of growth of cancer cells can also be measured by the MTT assay. Growth assays as measured by the MTT assay are well known in the art. Assays may be conducted as described by Mosmann *et al.*, 1983; Rubinstein *et al.*, 1990; and Green *et al.*, 1984 (each incorporated herein by reference). Therefore, if a candidate substance exhibited inhibition in this type of study, it would likely be a suitable compound for use in the present invention. A significant inhibition in growth is represented by decreases of about or at least about 30% (e.g., at least about or about 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90%, 95%, or more) as compared to uninhibited, with more significant decreases also being possible.

Quantitative *in vitro* testing of the bisanthrapyrazoles discussed herein is not a requirement of the invention as it is generally envisioned that the agents will often be selected on the basis of their known properties or by structural and/or functional comparison to those bisanthrapyrazoles or anthrapyrazoles already demonstrated to be effective. Therefore, the effective amounts will often be those amounts proposed to be safe for administration to animals in another context.

C. Molecular Modeling of DNA-Compound Interactions

The present invention also contemplates molecular modeling methods used to estimate the binding strength of a compound to DNA. This method also may be used, for example, to model how a compound may bind to DNA. Any compound may be studied and in particular embodiments, intercalators or putative intercalators may be modeled.

As described herein, a series of anthrapyrazole and bisanthrapyrazole compounds that are analogs of piroxantrone and losoxantrone were synthesized and their cell growth inhibitory effects, DNA binding, topoisomerase IIα-mediated (EC 5.99.1.3) cleavage of DNA and inhibition of DNA topoisomerase IIα decatenation catalytic activities were determined. Some of the analogs were designed with an alkylating group on the anthrapyrazole side chain in order to increase cytotoxicity by potentially covalently binding to DNA upon intercalation. Cell growth inhibitory activity was well correlated with DNA binding suggesting that these compounds may act by targeting DNA. However, cell growth inhibition was not well correlated with the inhibition of topoisomerase IIα catalytic activity, suggesting that these anthrapyrazoles did not act solely by inhibiting the catalytic activity of topoisomerase II. Most of the analogs were able to induce DNA cleavage and, thus, it was concluded that they acted, at least in part, as topoisomerase II poisons. Structure-based three-dimensional quantitative structure-activity analyses (3D-QSAR) were carried out on the aligned structures of the anthrapyrazoles and bisanthrapyrazoles docked into DNA using comparative molecular field analysis (CoMFA) and comparative molecular similarity index (CoMSIA) analyses in order to determine the structural features responsible for their activity. Both CoMFA and CoMSIA analyses yielded statistically significant models upon partial least squares analyses. The 3D-QSAR analyses showed that hydrogen bond donor interactions and electrostatic interactions with the protonated amino side chains of the anthrapyrazoles led to high cell growth inhibitory activity.

(a) Anthrapyrazoles

The present invention also discusses a study of a series of anthrapyrazole compounds showed that they potently inhibited the growth of K562 cells. The parent compounds of this group, losoxantrone and piroxantrone, which have both been tried in clinical trials (Showalter *et al.*, 1986; Judson, 1992; Gogas and Mansi, 1996; Diab *et al.*, 1999; Talbot *et al.*, 1991; Ingle *et al.*, 1994) likely exert their cell growth inhibitory effects by acting as DNA topoisomerase II poisons (Leteurtre *et al.*, 1994; Capranico *et al.*, 1994). The fact that cell growth inhibition was not correlated with inhibition of topoisomerase IIα-mediated catalytic decatenation activity suggests that the catalytic inhibition of topoisomerase II was not the primary mechanism by which these compounds act. This result does not rule out that the anthrapyrazoles were

acting as topoisomerase II poisons. Intercalating compounds typically inhibit the catalytic activity of topoisomerase II, presumably by interfering with the formation of the DNA-topoisomerase II complex (Fortune and Osheroff, 2000). Because the inventors did show that most of the anthrapyrazoles that could be evaluated were able to induce the formation of a linear DNA through stabilization of a covalent topoisomerase IIα-DNA cleavable complex in a DNA cleavage assay, this result suggested that most of the new anthrapyrazoles also acted as topoisomerase II poisons similar to losoxantrone and piroxantrone (Leteurtre *et al.*, 1994; Capranico *et al.*, 1994). This result was also supported by the general cross resistance (Table 2) that the anthrapyrazoles showed with the K/VP.5 cell line with a decreased level of topoisomerase IIα (Ritke and Yalowich, 1993; Ritke *et al.*, 1994a; Ritke *et al.*, 1994b; Fattman *et al.*, 1996) as cells with less topoisomerase II in the cell, cells produce fewer DNA strand breaks and topoisomerase II poisons are less lethal to these cells (Ritke *et al.*, 1994a; Fortune and Osheroff, 2000). The fact that a high level of cross resistance was not seen may be due to the fact that the anthrapyrazoles also target other DNA processing enzymes due to their ability to intercalate into DNA.

The good correlation of K562 cell IC_{50} values with the ΔT_m values indicates that their inhibitory activity was due, in part, to their ability to bind to DNA. Using an x-ray structure of a doxorubicin-DNA complex (1DA9) (Leonard *et al.*, 1993) the inventors showed that the structurally similar anthrapyrazoles could be docked into the doxorubicin binding pocket on DNA. The affinity of the anthrapyrazoles for DNA as measured by ΔT_m was positively correlated with the DNA-ligand van der Waals external energy term from the GOLDScore fitness function indicating that increased binding was associated with increased anthrapyrazole-DNA van der Waals interactions. Likewise the potency of the anthrapyrazoles was also positively correlated with the external energy term.

In order to further define the structural factors that result in high cell growth inhibitory potency for the anthrapyrazoles 3D-QSAR CoMFA and CoMSIA analyses were carried out in order to derive a model for the prediction of activity to aid in the synthesis of new more active analogs. Based on a common substructure alignment (FIG. 4B), both CoMFA and CoMSIA analyses gave high quality models based on their q^2 values. The largest contributions to the CoMSIA field were hydrogen bond donor and electrostatic contributions, in that order. Mapping of these field onto the

structure of AP-10, the most potent anthrapyrazole, showed that favorable contributions to the biological activity of these compounds was highly localized in the region around the protonated amino side chains. This conclusion is also supported by the docking results that showed that an amino side chain of AP-10 was able to act as hydrogen bond donor to DNA.

In summary, while the anthrapyrazoles were catalytic inhibitors of topoisomerase II, they likely exerted their cell growth inhibitory activity, at least in part, through their ability to act as topoisomerase II poisons. QSAR correlation and 3D-QSAR analyses showed the importance of anthrapyrazole-DNA van der Waals interactions, while the 3D-QSAR CoMFA and CoMSIA analysis showed that hydrogen bond donor interactions and electrostatic interactions with the protonated amino side chains of the anthrapyrazoles led to high cell growth inhibitory activity.

(b) Bisanthrapyrazoles

Certain bisanthrapyrazoles were designed using molecular modeling and docking into DNA in order to determine the optimal linker length for optimal binding to DNA and selection for their subsequent synthesis. An X-ray structure (1DA9) (Leonard *et al.*, 1993) of two molecules of doxorubicin separated by 4 base pairs bound to duplex DNA was used for the docking (FIG. 14). Five bisanthrapyrazoles based on AP9 with 1–5 methylene linkers (**1A**, **1B**, **1C**, **1D** and **1E**) were synthesized and chemically and biologically characterized. DNA melting temperatures ΔT_m (Table 5) were determined as a measure of strength of DNA binding and were significantly increased over that of the parent AP9, a result which suggests that the bisanthrapyrazoles **1B**, **1C**, **1D** and **1E** formed bisintercalation complexes with DNA. The concentration dependence of ΔT_m for compounds **1B**, **1C**, **1D** and **1E** (FIGS. 12A and 12B) indicated that these compounds formed mainly bisintercalation complexes with DNA. Our docking results predicted **1A** to have too short a linker to form a bisintercalation complex. This was confirmed by the results in FIG. 12B in which compound **1A** and monomeric AP9 had the same slope and thus both formed mono intercalation complexes. GOLDScores obtained from docking and the experimentally determined ΔT_m values both displayed broad maxima (*see, e.g.*, FIG. 2) when the bisanthrapyrazoles had 2–5 methylene linkers. The theoretical maximum value of ΔT_m, obtained from the quadratic fit to the experimentally determined ΔT_m values,

predicts that a bisanthrapyrazole with 3 methylene linkers was optimal for DNA binding. Compound **1C** with 3 methylene linkers had an experimentally measured ΔT_m value of 14.1 °C. This value is close to the experimentally measured value of 16.4 °C for **1B**, the bisanthrapyrazole with 2 methylene linkers that was experimentally observed to bind the strongest to DNA (Table 5). Thus, the calculated and experimental DNA-binding affinities were in good agreement when used to select a range of optimal methylene linker lengths for synthesis.

The bisanthrapyrazoles have a relatively large number of rotatable bonds (*e.g.*, up to 20 for **1E**) and thus are highly conformationally flexible molecules that can adopt large numbers of low energy configurations. This high degree of conformational flexibility makes it computationally difficult to obtain the best possible GOLDScore. This fact likely explains the lack of a smooth change in GOLDScore as the number of linker groups is varied (*see, e.g.*, FIG. 3). Again considering the size of the bisanthrapyrazoles and the large number of rotatable bonds, the docking produced chemically reasonable bisintercalation complexes as shown in FIG. 14. This result was likely due, in part, to the high affinity that the anthrapyrazole rings have for the doxorubicin-binding sites in which the bisanthrapyrazoles intercalate, thus anchoring the two ends of the bisanthrapyrazoles. Overall these results suggest that docking bisintercalators into X-ray structures of DNA is a useful tool for designing bisintercalating compounds with high affinity to DNA. DNA base specificity in bisanthrapyrazole binding to DNA may be another factor that may contribute to GOLDScores lacking the power to accurately predict DNA binding as measured by ΔT_m. The bisanthrapyrazoles were docked into a single X-ray structure (PDB ID: 1DA9) which does not have the set of base pairs which necessarily leads to optimum binding to DNA. The base pair specificity of the bisanthrapyrazoles for calf thymus DNA is unknown.

D. Pharmaceutical Preparations

Certain of the methods set forth herein pertain to methods involving the administration of a pharmaceutically effective amount of a bisanthrapyrazole for chemotherapeutic purposes.

In certain embodiments, the bisanthrapyrazoles of this invention may be administered to kill tumor cells by any method that allows contact of the active ingredient with the agent's site of action in the tumor. They can be administered by

any conventional methods available for use in conjunction with pharmaceuticals, either as individual therapeutically active ingredients or in a combination of therapeutically active ingredients. They may be administered alone, but are generally administered with a pharmaceutically acceptable carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The bisanthrapyrazoles may be extensively purified and/or dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle, where appropriate. Such methods are well-known in the art. The active compounds will then generally be formulated for administration by any known route, such as parenteral administration. Methods of administration are discussed in greater detail below.

Aqueous compositions of the present invention will typically have an effective amount of anthrapyrazole to kill or slow the growth of cancer cells. Further the potential recognition of genes can be accomplished by the synthesis of bisanthrapyrazoles with specific structures that allow for the recognition of specific parts of DNA. Such compositions will generally be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

Moreover, it will be generally understood that any bisanthrapyrazole can be provided in prodrug form, meaning that an environment to which a bisanthrapyrazole is exposed alters the prodrug into an active, or more active, form. It is contemplated that the term "precursor" covers compounds that are considered "prodrugs." In certain embodiments, a bisanthrapyrazole may act as a prodrug by *in situ* release of the individual anthrapyrazoles.

1. Pharmaceutical Formulations and Routes for Administration to Subjects

Pharmaceutical compositions of the present invention comprise an effective amount of one or more candidate substances (*e.g.*, a bisanthrapyrazole) or additional agents dissolved or dispersed in a pharmaceutically acceptable carrier. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of a pharmaceutical composition that contains at least one candidate

substance or additional active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference. Moreover, for animal (*e.g.*, human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (*e.g.*, antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, pp 1289-1329, 1990). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the therapeutic or pharmaceutical compositions is contemplated.

The candidate substance may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it need to be sterile for such routes of administration as injection. The present invention can be administered intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostaticaly, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, topically, intratumorally, intramuscularly, subcutaneously, subconjunctival, intravesicularlly, mucosally, intrapericardially, intraumbilically, intraocularally, orally, locally, via inhalation (*e.g.*, aerosol inhalation), via injection, via infusion, via continuous infusion, via localized perfusion bathing target cells directly, via a catheter, via a lavage, in cremes, in lipid compositions (*e.g.*, liposomes), or by other method or any combination of the foregoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 1990).

In particular embodiments, the composition is administered to a subject using a drug delivery device. Any drug delivery device is contemplated for use in delivering a pharmaceutically effective amount of a bisanthrapyrazole.

The actual dosage amount of a composition of the present invention administered to an animal patient can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

The dose can be repeated as needed as determined by those of ordinary skill in the art. Thus, in some embodiments of the methods set forth herein, a single dose is contemplated. In other embodiments, two or more doses are contemplated. Where more than one dose is administered to a subject, the time interval between doses can be any time interval as determined by those of ordinary skill in the art. For example, the time interval between doses may be about 1 hour to about 2 hours, about 2 hours to about 6 hours, about 6 hours to about 10 hours, about 10 hours to about 24 hours, about 1 day to about 2 days, about 1 week to about 2 weeks, or longer, or any time interval derivable within any of these recited ranges.

In certain embodiments, it may be desirable to provide a continuous supply of a pharmaceutical composition to the patient. This could be accomplished by catheterization, followed by continuous administration of the therapeutic agent. The administration could be intra-operative or post-operative.

In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of a bisanthrapyrazole. In other embodiments, the bisanthrapyrazole may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000

mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, *etc.*, can be administered, based on the numbers described above.

In any case, the composition may comprise various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (*e.g.*, methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal, or combinations thereof.

The candidate substance may be formulated into a composition in a free base, neutral, or salt form. Pharmaceutically acceptable salts, include the acid addition salts, *e.g.*, those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine, or procaine.

In embodiments where the composition is in a liquid form, a carrier can be a solvent or dispersion medium comprising but not limited to, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, liquid polyethylene glycol, *etc.*), lipids (*e.g.*, triglycerides, vegetable oils, liposomes) and combinations thereof. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin; by the maintenance of the required particle size by dispersion in carriers such as, for example liquid polyol or lipids; by the use of surfactants such as, for example hydroxypropylcellulose; or combinations thereof such methods. It may be preferable to include isotonic agents, such as, for example, sugars, sodium chloride, or combinations thereof.

In other embodiments, one may use eye drops, nasal solutions or sprays, aerosols or inhalants in the present invention. Such compositions are generally designed to be compatible with the target tissue type. In a non-limiting example,

nasal solutions are usually aqueous solutions designed to be administered to the nasal passages in drops or sprays. Nasal solutions are prepared so that they are similar in many respects to nasal secretions, so that normal ciliary action is maintained. Thus, in certain embodiments the aqueous nasal solutions usually are isotonic or slightly buffered to maintain a pH of about 5.5 to about 6.5. In addition, antimicrobial preservatives, similar to those used in ophthalmic preparations, drugs, or appropriate drug stabilizers, if required, may be included in the formulation. For example, various commercial nasal preparations are known and include drugs such as antibiotics or antihistamines.

In certain embodiments the candidate substance is prepared for administration by such routes as oral ingestion. In these embodiments, the solid composition may comprise, for example, solutions, suspensions, emulsions, tablets, pills, capsules (*e.g.*, hard or soft shelled gelatin capsules), sustained release formulations, buccal compositions, troches, elixirs, suspensions, syrups, wafers, or combinations thereof. Oral compositions may be incorporated directly with the food of the diet. In certain embodiments, carriers for oral administration comprise inert diluents, assimilable edible carriers or combinations thereof. In other aspects of the invention, the oral composition may be prepared as a syrup or elixir. A syrup or elixir, and may comprise, for example, at least one active agent, a sweetening agent, a preservative, a flavoring agent, a dye, a preservative, or combinations thereof.

In certain embodiments an oral composition may comprise one or more binders, excipients, disintegration agents, lubricants, flavoring agents, or combinations thereof. In certain embodiments, a composition may comprise one or more of the following: a binder, such as, for example, gum tragacanth, acacia, cornstarch, gelatin or combinations thereof; an excipient, such as, for example, dicalcium phosphate, mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate or combinations thereof; a disintegrating agent, such as, for example, corn starch, potato starch, alginic acid or combinations thereof; a lubricant, such as, for example, magnesium stearate; a sweetening agent, such as, for example, sucrose, lactose, saccharin or combinations thereof; a flavoring agent, such as, for example peppermint, oil of wintergreen, cherry flavoring, orange flavoring, *etc.*; or combinations thereof the foregoing. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, carriers such as a

liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar, or both.

Additional formulations which are suitable for other modes of administration include suppositories. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum, vagina, or urethra. After insertion, suppositories soften, melt or dissolve in the cavity fluids. In general, for suppositories, traditional carriers may include, for example, polyalkylene glycols, triglycerides, or combinations thereof. In certain embodiments, suppositories may be formed from mixtures containing, for example, the active ingredient in the range of about 0.5% to about 10%, and preferably about 1% to about 2%.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and/or the other ingredients. In the case of sterile powders for the preparation of sterile injectable solutions, suspensions or emulsion, certain methods of preparation may include vacuum-drying or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered liquid medium thereof. The liquid medium should be suitably buffered if necessary and the liquid diluent first rendered isotonic prior to injection with sufficient saline or glucose. The preparation of highly concentrated compositions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small area.

The composition must be stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria and fungi. It will be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less that 0.5 ng/mg protein.

In particular embodiments, prolonged absorption of an injectable composition can be brought about by the use in the compositions of agents delaying absorption, such as, for example, aluminum monostearate, gelatin, or combinations thereof.

2. Combination Therapy

In order to increase the effectiveness of a bisanthrapyrazole of the present invention, the bisanthrapyrazole may be combined with traditional drugs. It is contemplated that this type of combination therapy may be used *in vitro* or *in vivo*. In a non-limiting example, an anti-cancer agent may be used in combination with a bisanthrapyrazole.

For example, bisanthrapyrazoles of the present invention may be provided in a combined amount with an effective amount of an anti-cancer agent to reduce or block DNA replication in cancerous cells (*e.g.*, tissues, tumors). This process may involve administering the agents at the same time or within a period of time wherein separate administration of the substances produces a desired therapeutic benefit. This may be achieved by contacting the cell, tissue, or organism with a single composition or pharmacological formulation that includes two or more agents, or by contacting the cell with two or more distinct compositions or formulations, wherein one composition includes one agent and the other includes another.

The compounds of the present invention may precede, be co-current with and/or follow the other agents by intervals ranging from minutes to weeks. In embodiments where the agents are applied separately to a cell, tissue or organism, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agents would still be able to exert an advantageously combined effect on the cell, tissue or organism. For example, in such instances, it is contemplated that one may contact the cell, tissue or organism with two, three, four or more modalities substantially simultaneously (*i.e.*, within less than about a minute) as the candidate substance. In other aspects, one or more agents may be administered within of from substantially simultaneously, about 1 minute, about 5 minutes, about 10 minutes, about 20 minutes about 30 minutes, about 45 minutes, about 60 minutes, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours about 8 hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 13 hours, about 14 hours, about 15 hours, about 16 hours, about

17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 22 hours, about 23 hours, about 24 hours, about 25 hours, about 26 hours, about 27 hours, about 28 hours, about 29 hours, about 30 hours, about 31 hours, about 32 hours, about 33 hours, about 34 hours, about 35 hours, about 36 hours, about 37 hours, about 38 hours, about 39 hours, about 40 hours, about 41 hours, about 42 hours, about 43 hours, about 44 hours, about 45 hours, about 46 hours, about 47 hours, about 48 hours, about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days, about 10 days, about 11 days, about 12 days, about 13 days, about 14 days, about 15 days, about 16 days, about 17 days, about 18 days, about 19 days, about 20 days, about 21 days, about 1, about 2, about 3, about 4, about 5, about 6, about 7 or about 8 weeks or more, and any range derivable therein, prior to and/or after administering the candidate substance.

Various combination regimens of the agents may be employed. Non-limiting examples of such combinations are shown below, wherein a bisanthrapyrazole is "A" and a second agent, such as an anti-cancer agent, is "B":

A/B/A	B/A/B	B/B/A	A/A/B	A/B/B	B/A/A	A/B/B/B	B/A/B/B
B/B/B/A	B/B/A/B	A/A/B/B	A/B/A/B	A/B/B/A	B/B/A/A		
B/A/B/A	B/A/A/B	A/A/A/B	B/A/A/A	A/B/A/A	A/A/B/A		

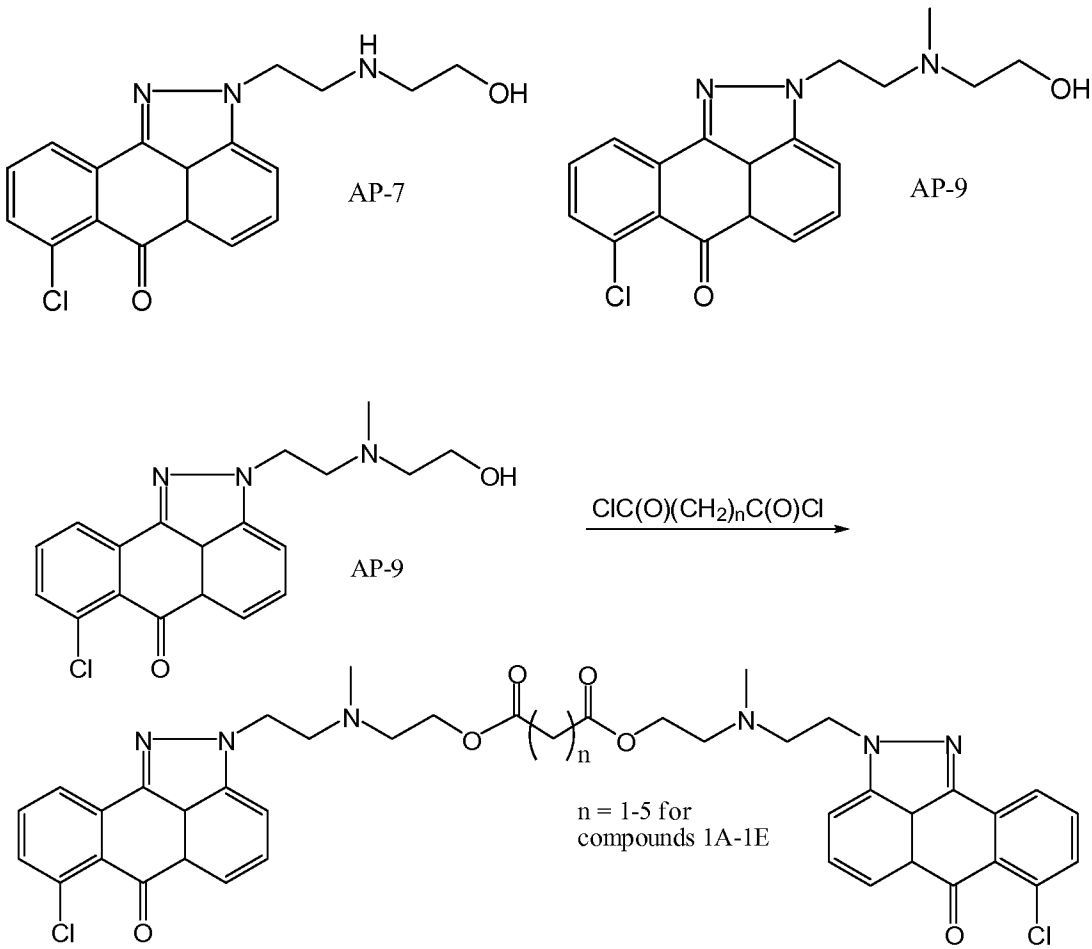
E. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Synthesis of Certain Bisanthrapyrazoles Of the Present Invention

General procedure as applied to anthrapyrazole AP-9, as an example:



AP-7 synthesis: 2-(2-Hydrazinoethyl)aminoethanol (2.91 g, 24.4 mmol) in acetonitrile (15 mL) was added to 1,5-dichloroanthraquinone (4.64 g, 16.6 mmol) in acetonitrile (19 mL) and DMF (2 mL). Triethylamine (2.0 mL) was added and the mixture heated to reflux for 48 h. The mixture was cooled to rt and the resulting crystals filtered. The filtrate was cooled overnight in a freezer and an additional crop of crystals was collected. The crystals were AP-7 hydrochloride which melted at 272-273°C (d). Typical yield was about 2.6 g, 46%. AP-7 HCl can be converted to the free base by taking up the crude mixture in $CHCl_3$ and extracting with excess NaOH solution.

AP-9 synthesis: The AP-7 free base (1.70 g, 5 mmol) was dissolved in formic acid (3.8 mL) and 37% aqueous formaldehyde (1.1 mL) was added. The mixture was heated to 110°C for 4 h – gas evolution begins at 95°C. After cooling to room temperature the mixture was poured onto cold water - $CHCl_3$ and the pH brought to ~13 with 2N NaOH. The chloroform phase was separated, and the aqueous phase

washed with 2 x 20 mL chloroform. The chloroform phases were dried and concentrated and the red residue recrystallized from methanol affording about 1.23 g orange needles, mp 119.5-121°C.

AP-9 (106 mg, 0.30 mmol) in $CHCl_3$ (5 mL) was treated with adipoyl chloride (0.75 mL of a 0.2 M solution in $CHCl_3$) and the mixture allowed to stir overnight. Within 1h a yellow precipitate began to form. At 18 h the mixture was diluted with ether (10 mL) and centrifuged. The resulting yellow crystals were dried in a nitrogen stream and then under vacuum overnight affording the bis-ester dihydrochloride (**1D**) (70 mg). The sample was recrystallized from methanol-ether.

EXAMPLE 2

Molecular Modeling of Compound 1D with DNA

The genetic algorithm-based molecular modeling program CCDC GOLD was used in docking bisanthrapyrazoles **1A-1E** and AP-9 into the Protein Data Bank 1d9a.pdb DNA(doxorubicin)$_2$ x-ray structure. Doxorubicin itself docked back into the DNA with a rms of 1.9 Å. As shown in FIG. 1, **1D** docked into the DNA minor groove and bound at the two doxorubicin binding sites. *See also* Example 14.

EXAMPLE 3

Cytotoxicity, DNA Binding and Topoisomerase IIα Inhibitory Activity of Bisanthrapyrazoles 1A-1E

***Experimental Conditions*:**

Topoisomerase IIα kDNA decatenation assay. Topoisomerase IIα decatenates kDNA in an ATP-dependent reaction to yield individual minicircles of DNA. kDNA was obtained from TopoGEN, Inc. (Port Orange, Florida). After reaction samples were centrifuged at 8000 g for 15 min, 20 µl of the supernatant were added to PicoGreen® dye in a 96-well plate. The fluorescence, which was proportional to the amount of kDNA, was measured in a fluorescence plate reader.

Topoisomerase IIα cleavage assay. pBR322 plasmid DNA (MBI Fermentas, Burlington, Ontario, Canada) was incubated with a compound and purified human topoisomerase IIα and the reaction was stopped with SDS to trap the cleavable complexes. The linear DNA produced was separated by gel electrophoresis.

Cell culture and cytotoxicity assay. K562 and K/VP.5 cell lines and topoisomerase IIα were a each gift from Jack Yalowich, University of Pittsburgh, Pittsburgh, Pennsylvania. K562 cells and etoposide-resistant K/VP.5 cells were grown in D-MEM/FCS. Cell growth was determined by MTS assay at 72 hr.

DNA ΔT_m determination. The strength of compound binding to sonicated calf thymus DNA was measured by determining the effect of 2 μM drug on the DNA melting temperature. ΔT_m was estimated from the maximum in the first derivative of the 280 nm absorbance in a temperature-programmed cell compartment.

***Results*:**

Compounds that act as DNA intercalators stabilize the DNA double helix, thereby increasing the temperature at which DNA denatures. This phenomenon is referred to as an increase in the "melting temperature," or ΔT_m, of the DNA. The bisanthrapyrazoles varied in their ability to increase the DNA melting temperature ΔT_m with **1B** binding the strongest to DNA (FIG. 4). **1B** bound to DNA more strongly than doxorubicin, suggesting a bisintercalation mode of binding. A clear maximum in ΔT_m at n = 2 was seen, indicating a $(CH_2)_2$ linker was optimal for DNA binding (FIG. 2). The *GoldScore* values were positively but poorly correlated (r^2 = 0.26 with ΔT_m for the bisanthrapyrazoles) (FIGS. 3A and 3B).

K562 cells are a cell line of human erythroleukemia cells. K/VP.5 cells are a K562 cell line with acquired resistance to etoposide because they contain one-fifth the amount of topoisomerase IIα. The bisanthrapyrazoles inhibited the growth of human leukemia K562 and low content topoisomerase IIα mutant K/VP.5 cells in the low micromolar concentration range (FIG. 4). The IC_{50} values only varied from 1.1 to 3.3 μM compared to 5.7 μM for AP-9. Thus, in spite of having $(CH_2)_n$ chain lengths from n = 1 – 5, the IC_{50} values only varied 3-fold. The K562 IC_{50} values were also poorly correlated with ΔT_m (FIG. 5). Together these results suggest that the ester-linked bisanthrapyrazoles may have been hydrolyzed by intercellular esterases to give AP-9.

All of the bisanthrapyrazoles inhibited topoisomerase IIα catalytic activity (FIG. 4). However, the K562 IC_{50} was only poorly correlated with the inhibition of topoisomerase IIα, which suggested that topoisomerase IIα was not the primary target of the bisanthrapyrazoles. Further, the K562 IC_{50} value was not significantly (p = 0.8)

negatively correlated with DNA melting temperature ΔT_m, which suggested that bisanthrapyrazoles **1A-1E** are either not cytotoxic through any ability to bind to DNA or that cellular esterases convert them to their monoanthrapyrazole form (AP-9).

While the K/VP.5 cells were slightly more cross resistant to the bisanthrapyrazoles, the degree of this cross resistance is not consistent with these compounds acting as topoisomerase II poisons like etoposide. None of the bisanthrapyrazoles were able to effect topoisomerase IIα-mediated cleavage of plasmid pBR322 DNA to produce linear DNA. These results indicate that the bisanthrapyrazoles were not topoisomerase II poisons like etoposide. It is to be kept in mind that a compound may act as both a topoisomerase IIα inhibitor and poison.

It is also contemplated that upon cleavage by esterases (or hydrolysis, or any other *in vivo* mechanism of operating on a bisanthrapyrazole), the resulting anthrapyrazole(s) may induce the cytotoxic effects discussed herein. For example, any of the anthrapyrazoles discussed in Liang *et al.*, 2006 that form upon cleavage of a bisanthrapyrazole would behave as a topoisomerase IIα poison. *In situ* production of a topoisomerase IIα poison would likely produce a more potent drug. Accordingly, in certain embodiments, the present invention contemplates bisanthrapyrazoles as prodrugs.

EXAMPLE 4

Treatment of Tumors with Bisanthrapyrazoles

Treatment with the anthrapyrazoles or the bisanthrapyrazoles of the present invention may be similar to the treatment regimes of other drugs, such as DNA intercalators (*e.g.*, the anthracyclines and their derivatives). For example, standard treatment with doxorubicin is described in *Remington's Pharmaceutical Sciences* as follows. Regarding Examples 4-7, bisanthrapyrazoles may be studied or anthrapyrazoles. Reference to bisanthrapyrazoles is made in Examples 4-7 for simplicity.

Doxorubicin is administered intravenously to adults at 60 to 75 mg/m^2 at 21-day intervals or 25 to 30 mg/m^2 on each of 2 or 3 successive days repeated at 3- or 4-week intervals or 20 mg/m^2 once a week. The lowest dose should be used in elderly patients, when there is prior chemotherapy or neoplastic marrow invasion, or when the drug is combined with other myelopoietic suppressant drugs. The dose should be

reduced by 50% if the serum bilirubin lies between 1.2 and 3 mg/dL and by 75% if above 3 mg/dL. The lifetime total dose should not exceed 550 mg/m^2 in patients with normal heart function and 400 mg/m^2 in patients with normal heart function and 400 mg/m^2 on each of 3 consecutive days, repeated every 4 weeks. Prescribing limits are as with adults. It has been reported that a 96-hr continuous infusion is as effective as and much less toxic than the same dose given by bolus injections.

Of course, modifications of the treatment regimes due to the unique nature of the bisanthrapyrazoles of the present invention are possible and well within the ability of one skilled in the art. Appropriate modifications may be ascertained by following the protocols in the following examples for *in vivo* testing and developments of human protocols.

EXAMPLE 5

In Vivo Prevention of Tumor Development Using Bisanthrapyrazoles

In an initial round of *in vivo* trials, a mouse model of human cancer with the histologic features and metastatic potential resembling tumors seen in humans (Katsumata *et al.*, 1995) is used. The animals are treated with bisanthrapyrazoles of the present invention to determine the suppression of tumor development. These studies are based on the discovery that bisanthrapyrazoles of the current invention have anti-cancer activity in cancer cells.

Bisanthrapyrazoles are tested *in vivo* for antitumor activity against murine leukemia L1210, P388 and P388 resistant to doxorubicin. In conjunction with these studies, the acute and sub-acute toxicity is studied in mice (LD10, LD50, LD90). In a more advanced phase of testing, the antitumor activity of bisanthrapyrazoles against human xenografts is assessed and cardiotoxicity studies performed is done in a rat or rabbit model.

Two groups of mice of a suitable cancer model are treated with doses of bisanthrapyrazoles. Several combinations and concentrations of bisanthrapyrazoles are tested. Control mice are treated with buffer only.

The effect of bisanthrapyrazoles on the development of tumors is compared with the control group by examination of tumor size, and histopathologic examination (tissue is cut and stained with hematoxylin and eosin) of the relevant tissue. With the

chemopreventive potential of bisanthrapyrazoles **1A-1E** and other bisanthrapyrazoles of the present invention, it is predicted that, unlike the control group of mice that develop tumors, the testing group of mice is resistant to tumor development.

EXAMPLE 6

Human Treatment with Bisanthrapyrazoles

This example describes a protocol to facilitate the treatment of cancer using bisanthrapyrazoles.

A cancer patient presenting cancer is treated using the following protocol. Patients may, but need not, have received previous chemo-, radio-, or gene therapeutic treatments. Optimally the patient exhibits adequate bone marrow function (defined as peripheral absolute granulocyte count of > 2,000/mm^3 and platelet count of 100, 000/mm^3, adequate liver function (bilirubin 1.5 mg/dl) and adequate renal function (creatinine 1.5 mg/dl).

A composition of the present invention is typically administered orally or parenterally in dosage unit formulations containing standard, well known non-toxic physiologically acceptable carriers, adjuvants, and/or vehicles as desired. The term "parenteral" as used herein includes subcutaneous injections, intravenous, intramuscular, intra-arterial injection, or infusion techniques. The bisanthrapyrazoles may be delivered to the patient before, after, or concurrently with any other anti-cancer agent(s), if desired.

A typical treatment course may comprise about six doses delivered over a 7 to 21 day period. Upon election by the clinician, the regimen may be continued six doses every three weeks or on a less frequent (monthly, bimonthly, quarterly, *etc.*) basis. Of course, these are only exemplary times for treatment, and the skilled practitioner will readily recognize that many other time-courses are possible.

To kill cancer cells using the methods and compositions described in the present invention, one will generally contact a target cell with a bisanthrapyrazole of the present invention. These compositions are provided in an amount effective to kill or inhibit the proliferation of the cell.

In certain embodiments, it is contemplated that one would contact the cell with agent(s) of the present invention about every 6 hours to about every one week. In

some situations however, it may be desirable to extend the time period for treatment significantly where several days (2, 3, 4, 5, 6, 7, or more) to several weeks (1, 2, 3, 4, 5, 6, 7, or more) lapse between respective administrations.

Regional delivery of a bisanthrapyrazole is an efficient method for delivering a therapeutically effective dose to counteract the clinical disease. Likewise, chemotherapy may be directed to a particular affected region. Alternatively systemic delivery of active agents may be appropriate. The therapeutic composition of the present invention may be administered to the patient directly at the site of the tumor. This is in essence a topical treatment of the surface of the cancer. The volume of the composition should usually be sufficient to ensure that the tumor is contacted by the bisanthrapyrazole.

In one embodiment, administration simply entails injection of the therapeutic composition into the tumor. In another embodiment, a catheter is inserted into the site of the tumor and the cavity may be continuously perfused for a desired period of time.

Clinical responses may be defined by acceptable measure. For example, a complete response may be defined by the disappearance of all measurable disease for at least a month. A partial response may be defined by a 50% or greater reduction of the sum of the products of perpendicular diameters of all evaluable tumor nodules or at least 1 month with no tumor sites showing enlargement. Similarly, a mixed response may be defined by a reduction of the product of perpendicular diameters of all measurable lesions by 50% or greater with progression in one or more sites.

Of course, the above-described treatment regimes may be altered in accordance with the knowledge gained from clinical trials, such as those described in Example 7. Those of skill in the art are able to take the information disclosed in this specification and optimize treatment regimes based on the results from the trials.

EXAMPLE 7

Clinical Trials Of the Use of Bisanthrapyrazoles in Treating Cancer

This example is concerned with the development of human treatment protocols using the bisanthrapyrazoles. These compounds are of use in the clinical treatment of various cancers in which transformed or cancerous cells play a role.

The various elements of conducting a clinical trial, including patient treatment and monitoring, are known to those of skill in the art in light of the present disclosure. The following information is being presented as a general guideline for studying bisanthrapyrazoles of the present invention in clinical trials.

Patients with cancer, such as human metastatic breast and/or epithelial ovarian carcinoma, colon cancer, leukemia, or sarcoma are chosen for clinical study. Measurable disease is not required; however the patient must have easily accessible pleural effusion and/or ascites. The patient may carry tumors that express a MDR (multi-drug resistant) phenotype. In an exemplary clinical protocol, patients may undergo placement of a Tenckhoff catheter, or other suitable device, in the pleural or peritoneal cavity and undergo serial sampling of pleural/peritoneal effusion. Typically, one will wish to determine the absence of known loculation of the pleural or peritoneal cavity, creatinine levels that are below 2 mg/dl, and bilirubin levels that are below 2 mg/dl. Baseline cellularity, cytology, LDH, and appropriate markers in the fluid (CEA, CA15-3, CA 125, p185) and in the cells (E1A, p185) may also be assessed and recorded. The patient should exhibit a normal coagulation profile.

In the same procedure, bisanthrapyrazoles may be administered. The administration may be in the pleural/peritoneal cavity, directly into the tumor, or in a systemic manner. The starting dose may be 0.5 mg/kg body weight. Three patients may be treated at each dose level in the absence of grade > 3 toxicity. Dose escalation may be done by 100% increments (0.5 mg, 1 mg, 2 mg, 4 mg) until drug related grade 2 toxicity is detected. Thereafter, dose escalation may proceed by 25% increments. The administered dose may be fractionated equally into two infusions, separated by six hours, if the combined endotoxin levels determined for the lot of bisanthrapyrazole exceeds 5 EU/kg for any given patient.

The bisanthrapyrazoles may be administered over a short infusion time or at a steady rate of infusion over a 7 to 21 day period. The bisanthrapyrazole infusion may be administered alone or in combination with, for example, another anti-cancer drug. The infusion given at any dose level is dependent upon the toxicity achieved after each. Hence, if Grade II toxicity was reached after any single infusion, or at a particular period of time for a steady rate infusion, further doses should be withheld or the steady rate infusion stopped unless toxicity improved. Increasing doses of bisanthrapyrazoles in combination with an anti-cancer drug is administered to groups

of patients until approximately 60% of patients show unacceptable Grade III or IV toxicity in any category. Doses that are 2/3 of this value could be defined as the safe dose.

Physical examination, tumor measurements and laboratory tests should, of course, be performed before treatment and at intervals of about 3-4 weeks later. Laboratory studies should include CBC, differential and platelet count, urinalysis, SMA-12-100 (liver and renal function tests), coagulation profile and any other appropriate chemistry studies to determine the extent of disease, or determine the cause of existing symptoms. Also, appropriate biological markers in serum should be monitored (*e.g.*, CEA, CA 15-3, p185 for breast cancer, and CA 125, p185 for ovarian cancer).

To monitor disease course and evaluate the anti-tumor responses, it is contemplated that the patients should be examined for appropriate tumor markers every 4 weeks, if initially abnormal, with twice weekly CBC, differential and platelet count for the 4 weeks; then, if no myelosuppression has been observed, then weekly. If any patient has prolonged myelosuppression, bone marrow examination is advised to rule out the possibility of tumor invasion of the marrow as the cause of pancytopenia. A coagulation profile shall be obtained every 4 weeks. An SMA-12-100 shall be performed weekly. Pleural/peritoneal effusion may be sampled 72 hours after the first dose, weekly thereafter for the first two courses, then every 4 weeks until progression or off study. Cellularity, cytology, LDH and appropriate markers in the fluid (CEA, CA15-3, CA 125, p185) and in the cells (p185) may be assessed. For an example of an evaluation profile, see Table 1. When measurable disease is present, tumor measurements are to be recorded every 4 weeks. Appropriate radiological studies should be repeated every 8 weeks to evaluate tumor response. Spirometry and DLCO may be repeated 4 and 8 weeks after initiation of therapy and at the time study participation ends. An urinalysis may be performed every 4 weeks.

Clinical responses may be defined by acceptable measure. For example, a complete response may be defined by the disappearance of all measurable disease for at least a month. Whereas a partial response may be defined by a 50% or greater reduction of the sum of the products of perpendicular diameters of all evaluable tumor nodules or at least 1 month with no tumor sites showing enlargement. Similarly, a mixed response may be defined by a reduction of the product of perpendicular

diameters of all measurable lesions by 50% or greater with progression in one or more sites.

TABLE 1

EVALUATIONS BEFORE AND DURING THERAPY

EVALUATIONS	PRE-STUDY	TWICE WEEKLY	WEEKLY	EVERY 4 WEEKS	EVERY 8 WEEKS
History	X			X	
Physical	X			X	
Tumor Measurements	X			X	
CBC	X	X[1]	X		
Differential	X	X[1]	X		
Platelet Count	X	X[1]	X		
SMA12-100 (SGPT, Alkaline Phosphatase, Bilirubin, Alb/Total Protein)	X		X		
Coagulation Profile	X			X	
Serum Tumor markers (CEA, CA15-3, CA-125, Her-2/neu)	X			X[3]	
Urinalysis	X			X	
X-rays:					
chest	X		X[4]		
others	X				X
Pleural/Peritoneal Fluids: (cellularity, cytology, LDH, tumor markers, E1A, HER-2/neu)	X		X[5]	X	
Spirometry and DLCO	X			X[6]	X[6]

[1] For the first 4 weeks, then weekly, if no myelosuppression is observed.
[2] As indicated by the patient's condition.
[3] Repeated every 4 weeks if initially abnormal.
[4] For patients with pleural effusion, chest X-rays may be performed at 72 hours after first dose, then prior to each treatment administration.
[5] Fluids may be assessed 72 hours after the first dose, weekly for the first two courses and then every 4 weeks thereafter.
[6] Four and eight weeks after initiation of therapy.

EXAMPLE 8

Materials and Methods for Examples 9-16

pBR322 plasmid DNA was obtained from MBI Fermentas (Burlington, Canada) and the kinetoplast plasmid DNA (kDNA) from TopoGEN (Columbus, OH). HindIII was from Invitrogen (Burlington, Canada). Unless indicated, other chemicals were from Sigma (Oakville, Canada). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) CellTiter 96® AQueous One Solution Cell Proliferation Assay kit was obtained from Promega (San Luis Obispo, CA). Losoxantrone and piroxantrone were obtained from the National Cancer Institute (Bethesda, MD). The linear least squares analysis was done with SigmaStat, (Systat, Point Richmond, CA).

EXAMPLE 9

Synthesis Of Certain Anthrapyrazoles Of the Present Invention

The synthesis of AP-3, AP-7, AP-10 and AP-12 have been described (Showalter *et al.*, 1987; Showalter *et al.*, 1986a). The chloroanthrapyrazole derivatives AP-3 and AP-7 were prepared from the commercially available 1,4- and 1,5-dichloroanthroquinone by reaction with 2-[(hydrazinoethyl)amino]ethanol in refluxing acetonitrile (Showalter *et al.*, 1986b). AP-12 was prepared by reaction of 1,4-dichloroanthroquinone with 2-hydrazinoethanol at 110°C. The N-methylated derivatives AP-1 and AP-9 were prepared by reaction of the corresponding unmethylated compounds (AP-3 and AP-7) with formaldehyde in formic acid at 110°C. The N,N'- disubstituted anthrapyrazoles AP-10, AP-11, AP-13 and AP-14 were prepared from the corresponding chloroanthrapyrazoles by reaction with excess N,N-dimethylethylenediamine at reflux. Chlorinated derivatives AP-2, AP-4, AP-6 and AP-8 were prepared from the corresponding hydroxy compounds by reaction with excess thionyl chloride at room temperature. Anthrapyrazole hydrochlorides and dihydrochlorides were prepared by addition of excess methanolic hydrogen chloride followed by crystallization of the resulting solids from methanol/ether. All structures were confirmed by ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy.

EXAMPLE 10

Cell Culture and Growth Inhibition Assays

Human leukemia K562 cells, obtained from the American Type Culture Collection and K/VP.5 cells (a 26-fold etoposide-resistant K562-derived sub-line with decreased levels of topoisomerase IIα mRNA and protein) (Ritke and Yalowich, 1993; Ritke *et al.*, 1994a; Ritke *et al.*, 1994b; Fattman *et al.*, 1996) were maintained as suspension cultures in DMEM (Dulbecco's Modified Eagle Medium, Invitrogen, Burlington, Canada) containing 10% fetal calf serum (FCS) and 2 mM L-glutamine. The spectrophotometric 96-well plate cell growth inhibition MTS assay, which measures the ability of the cells to enzymatically reduce MTS after drug treatment, has been described (Hasinoff *et al.*, 1997; Hasinoff *et al.*, 2001) (each incorporated by reference in its entirety). The drugs were dissolved in DMSO. The final concentration of DMSO did not exceed 0.5% (v/v) and was an amount that had no detectable effect on cell growth. The cells were incubated with the drugs for the times indicated and then assayed with MTS. IC_{50} values for growth inhibition in both assays were measured by fitting the absorbance-drug concentration data to a four-parameter logistic equation as described (Hasinoff *et al.*, 1997).

(a) Anthrapyrazoles

As shown in Table 2 all of the anthrapyrazoles inhibited the growth of K562 and K/VP.5 cells. AP-10 was the most potent of the compounds and AP-12, which has no amino side chains, was the least potent indicating the importance of the amino side chains. It can be seen from the data in Table 2 that when a chlorine was substituted for an hydroxyl group on the pyrazole side chains in the following pairs: AP-1/AP-2, AP-3/AP-4, AP-9/AP-6, AP-7/AP-8, the growth inhibitory activity was either decreased or unaffected. Thus, it can be concluded that the design goal of increasing cytotoxicity by having an alkylating group on the anthrapyrazole side chain was not achieved. The effect of converting the secondary amine group on the pyrazole side chain into a tertiary methyamine was mixed. For the AP-13/AP-14 pair, in which a hydrogen was substituted by a methyl group, the growth inhibitory effect was increased, but for the AP-3/AP-1, AP-4/AP-2, AP-8/AP-6, AP-7/AP-9 and AP-10/AP-11 pairs for this same substitution, growth inhibitory effects were either unchanged or slightly reduced. It can also be seen from the AP-10/AP-13, AP-11/AP-

14 pairs that moving the amino side chain from the 5- to the 7-ring position decreased or did not affect growth inhibitory effects, respectively.

One method by which cancer cells increase their resistance to topoisomerase II poisons is by lowering their level or activity of topoisomerase II (Ritke *et al.*, 1994a; Fortune and Osheroff, 2000). With less topoisomerase II in the cell, cells produce fewer DNA strand breaks and topoisomerase II poisons are less lethal to cells. These cell lines provide a convenient way to test whether a drug that inhibits topoisomerase II acts as a topoisomerase II poison (Hasinoff *et al.*, 2005). Conversely, a lack of change in sensitivity of a putative topoisomerase II poison to a cell line with a lowered topoisomerase II level can be taken to indicate that poisoning of topoisomerase II was not an important mechanism for this particular agent. The inventors previously showed that the K/VP.5 cell line with acquired resistance to etoposide contained one-fifth the topoisomerase IIα content of the parental K562 cells (Ritke and Yalowich, 1993; Ritke *et al.*, 1994a; Ritke *et al.*, 1994b; Fattman *et al.*, 1996). The IC_{50} for growth inhibition of K562 cells and K/VP.5 cells, as measured with the MTS assay, after a 72 h continuous treatment with a range of anthrapyrazole concentrations are compared in Table 2. While almost all of the anthrapyrazoles except AP-2 were somewhat cross resistant, none were as cross resistant as mitoxantrone or doxorubicin (4- to 5-fold). Interestingly the anthrapyrazoles AP-10 and AP-11 that were the most potent at inhibiting cell growth were also the most cross resistant. Thus, from these results and the results of the cleavage assay (FIG. 7) it can be concluded that most of the anthrapyrazoles inhibit cell growth, at least in part, by acting as topoisomerase II poisons.

(b) Bisanthrapyrazoles

The IC_{50} for growth inhibition of K562 cells and K/VP.5 cells, measured as previously described (Liang *et al.*, 2006) with the MTS assay, after a 72 h continuous treatment with a range of bisanthrapyrazole concentrations are compared in Table 5. Most of the bisanthrapyrazoles were slightly to moderately cross resistant (up to 2.6-fold for **1C**), but none were as cross resistant as mitoxantrone or doxorubicin (4- to 5-fold). Thus, these results and the results of the cleavage assay (FIG. 13) offer only moderate support for the conclusion that the bisanthrapyrazoles inhibited cell growth by acting as topoisomerase II poisons.

Table 5. DNA ΔT_m cell growth inhibition and topoisomerase IIα inhibitory effects of the bisanthrapyrazoles.

		MTS cell growth inhibition			topo II inhibition
Agent	ΔT_m (°C)	K562 IC_{50} (μM)	K/VP.5 IC_{50} (μM)	Resistance factor[a]	IC_{50} (μM)
1A	7.4	2.5	4.2	1.7	25
1B	16.4	2.7	4.2	1.6	50
1C	14.1	1.1	2.9	2.6	32
1D	12.7	3.3	3.9	1.2	8.4
1E	9.7	1.8	2.7	15	33
AP9[b]	6.1	5.7	6.4	1.1	7.2

[a] The resistance factor was calculated from the ratio of the IC_{50} value for the K/VP.5 cell line divided by that for the K562 cell line.

[b] Data from reference (Liang *et al.*, 2006).

EXAMPLE 11

Topoisomerase IIα kDNA Decatenation Inhibition Assays

A spectrofluorometric decatenation assay was used to determine the inhibition of topoisomerase IIα by the anthrapyrazoles and bisanthrapyrazoles (Hasinoff *et al.*, 2004; Barnabé and Hasinoff, 2001; Hasinoff *et al.*, 2005). kDNA consists of highly catenated networks of circular DNA. Topoisomerase IIα decatenates kDNA in an ATP-dependent reaction to yield individual minicircles of DNA. The 20 μl reaction mixture contained 0.5 mM ATP, 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 30 μg/ml bovine serum albumin, 50 ng kDNA, test compound (0.5 μl in dimethyl sulfoxide) and 20 ng of topoisomerase IIα protein (the amount that gave approximately 80% decatenation). Using a high copy yeast expression vector, full-length human topoisomerase IIα was expressed, extracted and purified as described previously (Hasinoff *et al.*, 2005). The final dimethyl sulfoxide concentration of 2.5% (v/v) was shown in controls not to affect the activity of topoisomerase IIα. The assay incubation was carried out at 37°C for 20 min and was terminated by the addition of

12 µl of 250 mM Na_2EDTA. Samples were centrifuged at 8000 *g* at 25°C for 15 min and 20 µl of the supernatant was added to 180 µl of 600-fold diluted PicoGreen dye (Molecular Probes, Eugene, OR) in a 96-well plate. The fluorescence, which was proportional to the amount of kDNA, was measured in a Fluostar Galaxy (BMG, Durham, NC) fluorescence plate reader using an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

(a) Anthrapyrazoles

As shown in Table 2 all of the anthrapyrazoles inhibited the decatenation activity of human topoisomerase IIα. This assay is a measure of the ability of these compounds to inhibit the catalytic activity only and was not a measure of whether these compounds acted as topoisomerase II poisons as do losoxantrone and piroxantrone (Leteurtre *et al.*, 1994; Capranico *et al.*, 1994) and some widely used anticancer drugs (Fortune and Osheroff, 2000; Li and Liu, 2001). It can be seen from the data in Table 2 that AP-10, which was the most potent in terms of growth inhibition, was the weakest inhibitor of topoisomerase IIα.

(b) Bisanthrapyrazoles

As shown in Table 5 all of the bisanthrapyrazoles inhibited the decatenation activity of human topoisomerase IIα in the low micromolar concentration range, but only **1D** achieved a potency nearly equal to that of the parent AP9. This assay is a measure of the ability of these compounds to inhibit the catalytic activity only, and was not a measure of whether these compounds acted as topoisomerase II poisons as do losoxantrone and piroxantrone (Leteurtre *et al.*, 1994; Capranico *et al.*, 1994) and some widely used anticancer drugs (Fortune and Osheroff, 2000; Li and Liu, 2001).

EXAMPLE 12

pBR322 DNA Relaxation and Cleavage Assays

Topoisomerase II-cleaved DNA complexes produced by anticancer drugs may be trapped by rapidly denaturing the complexed enzyme with sodium dodecyl sulfate (SDS) (Burden *et al.*, 2001). The anthrapyrazole-induced cleavage of double-stranded closed circular pBR322 DNA to form linear DNA was followed by separating the SDS-treated reaction products using ethidium bromide gel electrophoresis as described (Burden *et al.*, 2001). The 20 µl cleavage assay reaction mixture contained 100 µM of the drug, 150 ng of topoisomerase IIα protein, 80 ng pBR322 plasmid

DNA (MBI Fermentas, Burlington, Canada), 0.5 mM ATP in assay buffer (10 mM Tris-HCl, 50 mM KCl, 50 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2.5% (v/v) glycerol, pH 8.0, and drug (0.5 µl in dimethyl sulfoxide). The order of addition was assay buffer, DNA, drug, and then topoisomerase IIα. The reaction mixture was incubated at 37°C for 10 min and quenched with 1% (v/v) SDS/25 mM Na_2EDTA. The reaction mixture was treated with 0.25 mg/ml proteinase K (Sigma) at 55°C for 30 min to digest the protein. The linear pBR322 DNA cleaved by topoisomerase IIα was separated by electrophoresis (2 h at 8 V/cm) on a TAE (Tris base (4 mM)/glacial acetic acid (0.11% (v/v))/Na_2EDTA (2 mM) buffer)/ethidium bromide (0.5 µg/ml)/agarose gel (1.2%, wt/v)). Ethidium bromide was used in the gel and running buffer in order that the inhibition of relaxation activity could be measured along with formation of cleaved linear DNA. The DNA in the gel was imaged by its fluorescence on a Alpha Innotech (San Leandro, CA) Fluorochem 8900 imaging system equipped with a 365 nm UV illuminator and a CCD camera.

(a) Anthrapyrazoles

Several widely used anticancer agents, including doxorubicin and the other anthracyclines, mitoxantrone and etoposide (Fortune and Osheroff, 2000; Li and Liu, 2001), as well as losoxantrone and piroxantrone (Leteurtre *et al.*, 1994; Capranico *et al.*, 1994), are thought to be cytotoxic by virtue of their ability to stabilize a covalent topoisomerase II-DNA intermediate (the cleavable complex) and act as what are called topoisomerase II poisons. Topoisomerase II alters DNA topology by catalyzing the passing of an intact DNA double helix through a transient double-stranded break made in a second helix and is critical for relieving torsional stress that occurs during replication and transcription and for daughter strand separation during mitosis (Fortune and Osheroff, 2000; Li and Liu, 2001). Thus, DNA cleavage assay experiments (Burden *et al.*, 2001) as the inventors previously described (Hasinoff *et al.*, 2006) were carried out using 100 µM etoposide as a control to see whether 100 µM of the test compounds stabilized the cleavable complex. As shown in FIG. 8 the addition of etoposide (lane 13) to the reaction mixture containing topoisomerase IIα and supercoiled pBR322 DNA induced formation of linear pBR322 DNA and inhibited relaxation of supercoiled pBR322 DNA. Linear DNA was identified by comparison with linear pBR322 DNA produced by action of the restriction enzyme HindIII acting on a single site on pBR322 DNA. The anthrapyrazoles were mixed in their ability to induce linear DNA formation. The interpretation of the results was

problematic for some of the anthrapyrazoles, both because binding of the anthrapyrazoles to the DNA retarded the migration of the linear DNA band, and also because of interference from the fluorescence of bound anthrapyrazole. Identification of the linear DNA bands in the former cases were aided by the identification of the shift of the nicked circular DNA. The latter effect could be largely, but not completely, ameliorated by destaining the gel overnight and restaining with ethidium bromide. For the anthrapyrazoles that could be evaluated these experiments showed that AP-1, AP-2, AP-6, AP-10, AP-11, and AP-12 were able to induce formation of linear DNA (marked on the gel image of FIG. 8 with an "*"), indicating that these anthrapyrazoles, at least, were able to stabilize the covalent topoisomerase II-DNA intermediate. It is interesting that AP-12 which does not detectably intercalate into DNA (Table 2) produced the largest amount of linear DNA. It is likely that the anthrapyrazoles that strongly bind DNA inhibit the formation of linear DNA due to intercalation at higher concentrations as has been shown for doxorubicin (Tewey *et al.*, 1984).

(b) Bisanthrapyrazoles

DNA cleavage assay experiments (Burden *et al.*, 2001) as previously described (Liang *et al.*, 2006; Hasinoff *et al.*, 2006) were carried out using 100 μM etoposide as a positive control to see whether 50 μM of the test bisanthrapyrazoles stabilized the cleavable complex. As shown in FIG. 13 the addition of etoposide (lane 8) to the reaction mixture containing topoisomerase IIα and supercoiled pBR322 DNA induced formation of linear pBR322 DNA. Linear DNA was identified by comparison with linear pBR322 DNA produced by action of the restriction enzyme HindIII acting on a single site on pBR322 DNA (not shown). Although it may have appeared that the bisanthrapyrazoles were capable of inducing some linear DNA formation in the presence of topoisomerase IIα, the fluorescence of the bisanthrapyrazoles related to their binding to DNA made accurate determination of the amount of linear DNA problematic (FIG. 13). Similar problems were encountered with the anthrapyrazoles of our previous study. (Liang *et al.*, 2006) As shown in lanes 5 and 7 both compounds 3 and 5 displayed significant fluorescence that interfered with quantitation of the linear DNA band. Based on integrated band intensities of linear DNA in FIG. 13 none of the bisanthrapyrazoles appeared to induce formation of linear DNA comparable to that induced by etoposide (lane 8). However, given the problems in measuring the amount of linear DNA in these experiments, it cannot firmly be concluded that the bisanthrapyrazoles do not poison topoisomerase II as found with some of the anthrapyrazoles of our previous study. (Liang *et al.*, 2006) The relaxed DNA band observed in lane 2 for the topoisomerase IIα control ran ahead of the supercoiled DNA in lane 1 (pBR322 control). Etoposide and all 5 bisanthrapyrazoles (**1A**, **1B**, **1C**, **1D** and **1E**) inhibited the relaxation of supercoiled pBR322 DNA of topoisomerase IIα, thus preventing the DNA 200 band from migrating in a similar manner as in lane 2. Together these results indicated that etoposide inhibited topoisomerase IIα catalytic activity and induced DNA cleavage while the bisanthrapyrazoles acted primarily by inhibition of topoisomerase IIα strand passage reactions.

EXAMPLE 13

Thermal Denaturation of DNA Assay

Compounds that intercalate into DNA stabilize the DNA double helix and increase the temperature at which the DNA is denatured (Sissi *et al.*, 2004; Priebe *et al.*, 2001; McGhee, 1976). The effect of 2 μM of the compounds on the DNA thermal melt temperature (ΔT_m) of sonicated calf thymus DNA (5 μg/ml) was measured in 10 mM Tris-HCl buffer (pH 7.5) in a Cary 1 (Varian, Mississauga, Canada) double beam spectrophotometer by measuring the absorbance increase at 260 nm upon the application of a temperature ramp of 1°C/min. The maximum of the first derivative of the absorbance-temperature curve was used to obtain the ΔT_m. Doxorubicin (2 μM), which is a strong DNA intercalator, was used as a positive control (Priebe *et al.*, 2001). Under limiting conditions the value of ΔT_m is directly proportional to the logarithm of the equilibrium constant for ligand binding to DNA (McGhee, 1976) and thus the value of ΔT_m was used directly in the free energy correlation analyses.

Doxorubicin (2 μM), which is a well-known DNA intercalating drug, was used as a control and was observed to increase the ΔT_m of sonicated DNA by 13.2 °C from 71.0 °C. Under limiting conditions the value of ΔT_m is 90 directly proportional to the logarithm of the equilibrium constant for ligand binding to DNA (McGhee, 1976) and thus the value of ΔT_m was used directly in the free energy correlation analyses.

(a) Anthrapyrazoles

Of the anthrapyrazoles studied (Table 2) AP-10 had the largest effect on ΔT_m, and thus bound the strongest to DNA. By contrast AP-12, which has no amino side chains (FIG. 6), did not increase ΔT_m, which indicates that the amino side chains were important for DNA binding. It can be seen from the data in FIG. 6 and Table 2 that when a chlorine was substituted for an hydroxyl group on the pyrazole side chains in the following pairs: AP-1/AP-2, AP-3/AP-4, AP-9/AP-6 and AP-7/AP-8, the ΔT_m was either decreased or unaffected, and thus it can be concluded that the hydroxyl group did not make a large contribution to the binding to DNA. For the AP-13/AP-14 pair, in which a hydrogen was substituted by a methyl group, the ΔT_m was increased, but for the AP-3/AP-1, AP-4/AP-2, AP-8/AP-6, AP-7/AP-9 and AP-10/AP-11 pairs for this substitution, the ΔT_m values were essentially unchanged. It can also be seen from the AP-10/AP-13, AP-11/AP-14 pairs that moving the amino side chain from the 5- to

the 7-ring position decreased ΔT_m which suggests that having the two amino side chains on the same side of the anthrapyrazole as in losoxantrone, piroxantrone, and mitoxantrone favours binding to DNA.

Table 2. DNA ΔT_m, cytotoxicity and topoisomerase II inhibitory effects of the anthrapyrazoles.

		MTS cell growth inhibition			topo II inhibition
Agent	ΔT_m (°C)	K562 IC_{50} (μM)	K/VP.5 IC_{50} (μM)	Resistance factor[a]	IC_{50} (μM)
AP-1	7.0	4.9	6.2	1.3	2.9
AP-2	6.0	4.4	6.0	1.4	0.25
AP-3	9.1	1.8	2.5	1.4	13
AP-4	5.2	11	12	1.1	12
AP-6	6.3	6.2	6.2	1.0	0.87
AP-7	7.0	3.3	4.3	1.3	18
AP-8	8.0	6.3	8.6	1.4	14
AP-9	6.1	5.7	6.4	1.1	7.2
AP-10	25.0	0.11	0.26	2.4	23
AP-11	24.0	0.26	0.86	3.3	16
AP-12	0	11.9	21	1.7	9.2
AP-13	4.1	1.7	3.1	1.8	11
AP-14	17.0	0.30	0.60	2.0	19
Losoxantrone	18.1	0.12	0.22	1.8	7.3
Piroxantrone	19.0	4.0	6.7	1.7	4.6
Mitoxantrone	18.4	0.42	1.68	4.0	5.3
Doxorubicin	13.2	0.08	0.41	5.1	ND

[a] The resistance factor was calculated from the ratio of the IC_{50} value for the K/VP.5 cell line divided by that for the K562 cell

(b) Bisanthrapyrazoles

In order to determine whether the bisanthrapyrazoles formed bisintercalation complexes with DNA, the concentration dependence of ΔT_m was measured from 0.1 to 2 μM anthrapyrazole or bisanthrapyrazole as described above in 10 mM Tris–HCl buffer (pH 8.0) at a DNA concentration of 20 μM (base pair basis). The slopes of the plots for monomer AP9 were compared with that of the bisanthrapyrazoles 1A–1E using a t-test comparison of the slopes. (Jones, D., Pharmaceutical Statistics; Pharmaceutical Press: London, 2002, p. 585).

Of the bisanthrapyrazoles studied **1B** had the largest effect on ΔT_m, and thus bound the strongest to DNA (Table 5). Compounds **1B** and **1C** all bound to DNA in a stronger manner than the strongly binding DNA intercalator doxorubicin. Of the five bisanthrapyrazoles synthesized **1A** bound to DNA was the weakest and increased ΔT_m about the same as the parent AP9. The other four bisanthrapyrazoles synthesized all bound more strongly than the parent AP9. The fact that 2, 3, and 4 all had ΔT_m values much greater than AP9 suggests that these bisanthrapyrazoles may form bisintercalation complexes with DNA.

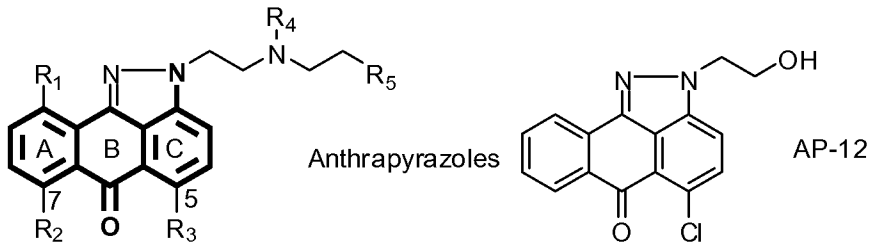
In a study (Bjorndal and Fygenson, 2002) on the bisintercalator dye YOYO and its monomeric form YO-PRO it was shown that the slope of a plot of ΔT_m versus drug concentration is approximately twice that for the bisintercalator compared to the mono intercalator due to the bisintercalator occupying twice the number of intercalation sites on the DNA. The concentration dependence of ΔT_m for monomeric AP9 and the bisanthrapyrazoles **1A**, **1B**, **1C**, **1D**, and **1E** is shown in FIG. 12A. The slopes ± SEM are plotted in FIG. 12B. A comparison of the slopes by t-test showed that all bisanthrapyrazoles but compound 1 ($p > 0.5$, not significant) had slopes that were significantly different ($p < 0.001$) than monomer AP9. From the data in FIG. 12A and a propagation propagation of errors analysis compound 1 gave a ratio of the slopes of compound **1A** to AP9 of 1.0 ± 0.1 indicating mono intercalation. A similar calculation for compound **1B** gave a ratio of slopes of 2.5 ± 0.2 which is in reasonable agreement with the theoretical value of **1B**. Similarly compounds **1C**, **1D**, and **1E** give ratio of slopes of 2.0 ± 0.2, 1.5 ± 0.1, and 1.6 ± 0.2, respectively. These results suggest that all but **1A** formed bisintercalation complexes with DNA. A ratio of

slopes less than **1B** for **1D** and **1E** may be due to these bisanthrapyrazoles forming mixed bis and mono intercalation complexes.

EXAMPLE 14

Molecular Modeling

The following protocol can be used to model DNA with both anthrapyrazoles and bisanthrapyrazoles to evalute the binding ability and positioning of these compounds. (Liang *et al.*, 2006, incorporated herein in its entirety). The following methods can also be expanded to model any putative intercalator. While the protocols recited below pertain to anthrapyrazoles, they may be suitably modified to examine bisanthrapyrazoles and any other intercalator. Structures of tested anthrapyrazoles are shown:



Compound	R_1	R_2	R_3	R_4	R_5
AP-1	H	H	Cl	CH_3	OH
AP-2	H	H	Cl	CH_3	Cl
AP-3	H	H	Cl	H	OH
AP-4	H	H	Cl	H	Cl
AP-6	H	Cl	H	CH_3	Cl
AP-7	H	Cl	H	H	OH
AP-8	H	Cl	H	H	Cl
AP-9	H	Cl	H	CH_3	OH
AP-10	H	H	$NH(CH_2)_2N(CH_3)_2$	H	OH
AP-11	H	H	$NH(CH_2)_2N(CH_3)_2$	CH_3	OH
AP-13	H	$NH(CH_2)_2N(CH_3)_2$	H	H	OH
AP-14	H	$NH(CH_2)_2N(CH_3)_2$	H	CH_3	OH
Losoxantrone	H	OH	$NH(CH_2)_3NH(CH_2)_2OH$	H	OH
Piroxantrone	OH	OH	$NH(CH_2)_3NH_2$	H	OH

All molecular modeling was done using SYBYL 7.0 (Tripos, St. Louis, MO) on a Hewlett-Packard XW4100 PC workstation with a Redhat Enterprise 3 Linux

operating system. All molecules except the DNA were built using SYBYL. AP9 and the bisanthrapyrazoles, were docked into the doxorubicin binding site of a 6 bp x-ray crystal structure of 2 molecules of doxorubicin bound to double stranded DNA, d(TGGCCA)/doxorubicin, (world wide web at .rcsb.org/pdb/ ; PDB ID: 1DA9) (Leonard *et al.*, 1993) using the genetic algorithm docking program GOLD version 2.2 or 3.1 (CCDC Software, Cambridge, UK) using the default GOLD parameters and atom types and with 100 starting runs (Verdonk *et al.*, 2003). GOLDScore was used as the fitness function with flipping options of amide bonds, planar and pyramidal nitrogens and internal hydrogen bonds being allowed. No early termination was allowed.

The 1DA9 x-ray structure shows that the first and second base pairs buckle out to accommodate bound doxorubicin (Leonard *et al.*, 1993; Berman *et al.*, 2000). Thus the 1DA9 x-ray structure of the doxorubicin-DNA complex was used for the docking experiments, rather than constructing DNA in SYBYL because it was reasoned that this DNA structure would be a more realistic model for binding of anthrapyrazoles and bisanthrapyrazoles because of their structural similarity to doxorubicin.

In the 1DA9 X-ray structure the two doxorubicin molecules are separated by four intervening base pairs. This structure was chosen because it was reasoned that DNA molecules with a smaller number of intervening base pairs would have to distort the DNA double helix (with an accompanying energy penalty) in order to bind.

The protonated anthrapyrazoles were first geometry optimized with the Tripos force field using a conjugate gradient with a convergence criterion of 0.01 kcal/mol and Gasteiger-Huckel charges and a distance-dependent dielectric constant. The SYBYL CONFORT module was then used to find the lowest energy conformation. The DNA structure was prepared by removing one of the bound doxorubicin molecules and removing all water molecules to avoid potential interference with the docking. Hydrogens were added to the DNA and the SYBYL Biopolymer module and sometimes used to add Kollman-All charges to the DNA. Using bound doxorubicin as the reference ligand, the binding site was defined as being within 5 Å of the reference ligand or defined using an atom in the center of the DNA molecule and was large enough that it encompassed both of the doxorubicin-binding sites.

The anthrapyrazoles, losoxantrone, piroxantrone and mitoxantrone were all docked into the DNA structure to obtain the top 10 scoring GOLDScore structures for each molecule. Each of these structures were then rescored using a local optimization (simplexing) to obtain the final GOLDScore and their individual component energy terms. Rescoring did not affect the initial relative scoring order.

Initial docking runs showed that not all bisanthrapyrazoles docked with the linker located in the DNA minor groove. Thus, docking runs were also carried out with the major groove blocked with a small molecule in order to compare all bisanthrapyrazoles docked into the minor groove. The bisanthrapyrazoles (n = 0–9) were docked into the DNA structure to obtain the top 10 scoring GOLDScore structures for each molecule and their individual component energy terms. The top structures were then rescored using a local optimization (simplexing) to obtain the final GOLDScore. Rescoring did not affect the initial relative scoring order. As a test of the docking procedure doxorubicin was docked back into the DNA structure with a heavy atom root-meansquared distance of 1.3 Å compared to the X-ray structure. (Leonard e*t al.*, 1993) Values of 2.0 Å or less in the extensive GOLD test set are considered to be good. (Verdonk *et al.*, 2003). The graph was prepared with DS Visualizer 1.7 (Accelrys, San Diego, CA).

The GOLD fitness function (GOLDScore) is the sum of its four components: DNA-ligand hydrogen bond energy (external H-bond); DNA-ligand van der Waals (vdw) energy (external vdw); ligand internal vdw energy (internal vdw); and ligand torsional strain energy (internal torsion) and were output for QSAR correlation analysis. The ligand intramolecular hydrogen bond energy (internal H-bond) term was zero for each docked ligand as there were no internal H-bonds found in the docked structures. For the QSAR correlation analysis the sum of the internal torsion and internal vdw terms was combined into the single internal energy term *S*(int) as these two terms were highly cross-correlated ($p = 2 \times 10^{-6}$). As a test of the docking procedure doxorubicin was docked back into the DNA structure with a heavy atom root-mean-squared distance of 1.3 Å compared to the x-ray structure (Leonard *et al.*, 1993). Values of 2.0 Å or less in the extensive GOLD test set are considered to be good (Verdonk *et al.*, 2003).

The structure of AP-10, the most potent anthrapyrazole, docked into DNA is shown in FIG. 9A. AP-10 shows both stacking interactions and H-bond interactions

through both amino side chains with the DNA base pairs between which it intercalates. Additionally, the side chain hydroxyl group forms an H-bond with the oxygen of a DNA sugar residue. The conformation of all the anthrapyrazoles and mitoxantrone docked into DNA have been superimposed in FIG. 9B. Most of the anthrapyrazoles docked into the DNA with a similar configuration, both with respect to the pyrazole rings and the amino side chains. However, some of the docked anthrapyrazoles were slightly rotated about the central DNA axis.

Linear regression (FIGS. 10A and 10B) was carried out on the logarithm of the K562 IC_{50} values vs. the GOLD fitness function and each of its four component terms: (DNA-ligand hydrogen bond energy (external H-bond); DNA-ligand van der Waals (vdw) energy (external vdw); ligand internal vdw energy (internal vdw); and ligand torsional strain energy (internal torsion). Regression analysis of the ΔT_m data was also carried out (FIGS. 7C and 7D). Because ΔT_m and the logarithm of the K562 IC_{50} values were highly correlated ($p = 4 \times 10^{-5}$), the conclusions reached from the K562 IC_{50} analysis likewise apply to the ΔT_m data shown in FIG. 10C. While the overall GOLD fitness function was itself not well correlated ($p = 0.2$), two of its component terms (external van der Waals and S(int) (the sum of the two internal energy terms) were (FIG. 10A). As shown in FIG. 10A an increase in the contribution to the external van der Waals energy term to the GOLD score was well correlated ($p = 2 \times 10^{-4}$) with increasing growth inhibitory potency. Likewise an increase in the external van der Waals energy term also corresponded to an increased binding affinity to DNA as shown in FIG. 10C. For the ΔT_m data this is a reasonable result as increased van der Waals interactions with DNA, in particular, would be expected to lead to increased binding affinity and increased growth inhibitory effects. FIG. 10B, however, shows that an increase in the internal energy of the anthrapyrazoles decreases cell growth inhibitory properties (and also in weaker binding to DNA as shown in FIG. 10D). The internal energy term reflects the internal torsional and van der Waals increases in energy due to conformational changes that the anthrapyrazoles must make to be able to dock into DNA. Thus, it is reasonable that an increase in the internal energy is negatively correlated with both ΔT_m and growth inhibitory effects.

EXAMPLE 15

Structure-based 3D-QSAR Analyses Of Certain Anthrapyrazoles

The following protocol can be used to model DNA with both anthrapyrazoles and bisanthrapyrazoles to evalute the binding ability and positioning of these compounds. (Liang *et al.*, 2006, incorporated herein in its entirety). The following methods can also be expanded to model any putative intercalator. While the protocols recited below pertain to anthrapyrazoles, they may be suitably modified to examine bisanthrapyrazoles and any other intercalator.

CoMFA and CoMSIA analysis requires that the 3D structures of the molecules be aligned to a core conformational template that is their presumed active form. The molecular conformations which were used for alignment were the conformers that had the best GOLDScore value after docking by GOLD. All the molecules were then aligned to template molecule AP-10, the most potent compound, (shown in FIG. 6 with the template atoms joined by heavy lines) by using the Align Database option in SYBYL. CoMFA and CoMSIA analyses were also carried out on aligned structures in the lowest energy conformations obtained with the SYBYL CONFORT module. For the CoMFA calculation, steric and electrostatic field energies were calculated using an sp^3 carbon with a van der Waals radius of 1.52 Å as the steric probe and a +1 charge as an electrostatic probe. Steric and electrostatic interactions were calculated using the Tripos force field with a distance-dependent dielectric constant at all lattice points of a regular spaced (2 Å) grid. The energy cutoff was 30 kcal/mol. The alignment and lattice box used for the CoMFA calculation were also used to calculate similarity index fields for the CoMSIA analysis. Steric, electrostatic, hydrophobic, hydrogen bond donor and acceptor fields were evaluated in CoMSIA analysis. Similarity indices were computed using a probe atom with +1 charge, radius 1 Å, hydrophobicity +1, hydrogen bond donating +1, hydrogen bond acceptor +1, attenuation factor α 0.3 for the Gaussian-type distance.

A partial least-squares (PLS) statistical approach, which is an extension of multiple regression analysis in which the original variables are replaced by a set of their linear combinations, was used to obtain the 3D-QSAR results. All models were investigated using the leave-one-out (LOO) method, which is a cross-validated partial least-squares method. The CoMFA and CoMSIA descriptors were used as

independent variables and pIC_{50} or ΔT_m were used as dependent variables to derive 3D-QSAR models. The q^2 (cross-validated correlation coefficient r^2) and the optimum number of components (N) were obtained by the LOO method. The final model (non-cross-validated conventional analysis) was developed and yielded the non-cross-validated correlation coefficient r^2 with the optimum number of components. Because it has been shown that losoxantrone was most closely related to the anthracenedione mitoxantrone and other topoisomerase II poisons in the NCI COMPARE analysis (Leteurtre *et al.*, 1994), and because mitoxantrone has a core structure and side chains the same or similar to the other anthrapyrazoles, it was included in the analysis of the other 15 anthrapyrazoles studied.

The results of the CoMFA and CoMSIA analyses on the K562 growth inhibitory IC_{50} and ΔT_m data of Table 2 are summarized in Table 3. No compounds were excluded as outliers. Due to the relatively small numbers of compounds in the data set (16) all structures were used in the analyses rather than dividing them into training and validation sets. For these variables the optimum number of components was one. The predicted and experimental values for the K562 IC_{50} and ΔT_m data are plotted in FIG. 9A and 9B, respectively. The CoMFA and CoMSIA analyses of the K562 IC_{50} data gave fairly high q^2 values (0.757 and 0.705, respectively) which indicates that the model and the alignment used was a good predictor of the activity. The CoMFA and CoMSIA q^2 values of 0.593 and 0.570, respectively, for the ΔT_m data were, however, smaller than for the biological data. CoMFA and CoMSIA analyses were also carried out on the topoisomerase IIα IC_{50} data and much lower q^2 values of 0.305 and 0.147, respectively were found (Table 3). This result was consistent with the lack of correlation between the K562 IC_{50} - topoisomerase IIα IC_{50} data shown in FIG. 7B. CoMFA and CoMSIA analyses of the K562 IC_{50} data carried out on aligned structures in the lowest energy conformations obtained with the SYBYL CONFORT module yielded lower q^2 values (0.530 and 0.401, respectively) than the q^2 values from the alignments obtained from the docking of the anthrapyrazoles into DNA. This result can be interpreted to mean that the conformations obtained from the docking into DNA are closer to the biologically active conformations.

Table 3. PLS statistics and field contributions of CoMFA and CoMSIA models for the prediction of pIC_{50} for K562 cell growth inhibition and for ΔT_m for binding of anthrapyrazoles to DNA

PLS statistics						
Parameter	K562 pIC_{50}		ΔT_m (°C)		topo II pIC_{50}	
	CoMFA	CoMSIA	CoMFA	CoMSIA	CoMFA	CoMSIA
q^2	0.757	0.705	0.593	0.570	0.305	0.147
N	1	1	1	1	3	1
S.E.P.	0.355	0.391	0.384	0.394	0.491	0.503
r^2	0.870	0.842	0.785	0.776	0.845	0.527
S.E.E.	0.260	0.286	0.279	0.285	0.232	0.357
F	93	75	51	48	21.7	15.6
Field contributions						
steric	0.428	0.111	0.386	0.103	0.681	0.128
electrostatic	0.572	0.255	0.620	0.225	0.319	0.294
hydrophobic		0.176		0.188		0.165
donor		0.327		0.340		0.288
acceptor		0.131		0.143		0.125

pIC_{50}, the negative logarithm of the molar IC_{50} concentration for cell growth inhibition; q^2, the cross-validated correlation coefficient; *N*: optimal number of components; *S.E.P.*: standard error of prediction; r^2,the non-cross-validated correlation coefficient; *S.E.E.,* standard error of estimate; *F*: *F*-test value.

EXAMPLE 16

Structure-based 3D-QSAR Analyses Of Certain Anthrapyrazoles

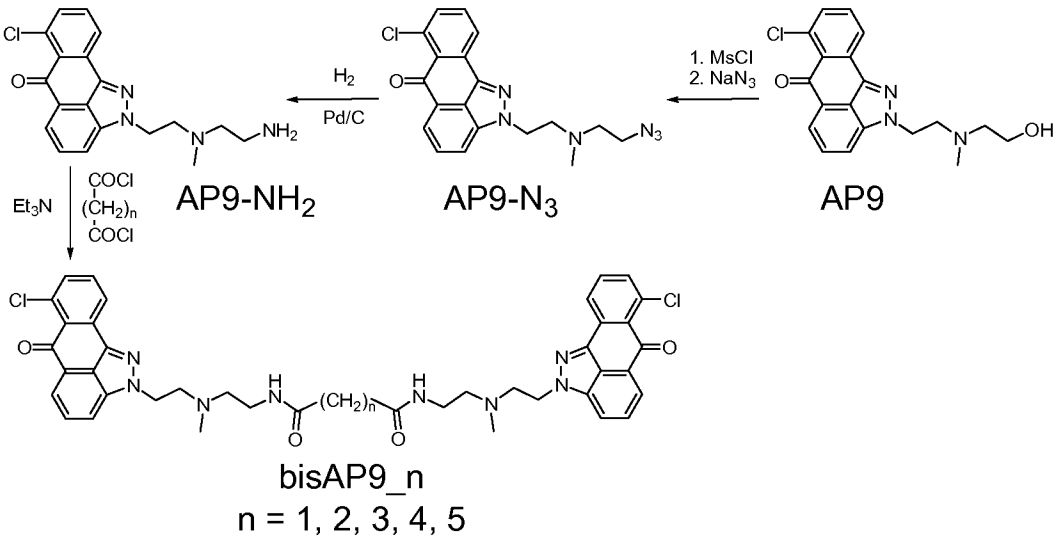
As shown in FIG. 7A the logarithm of the K562 50% cell growth inhibitory concentration (IC_{50}) data was well correlated with ΔT_m ($r^2 = 0.72$, $p = 3 \text{ X } 10\text{-}^5$) which indicates that high binding affinity for DNA results in high potency. This result

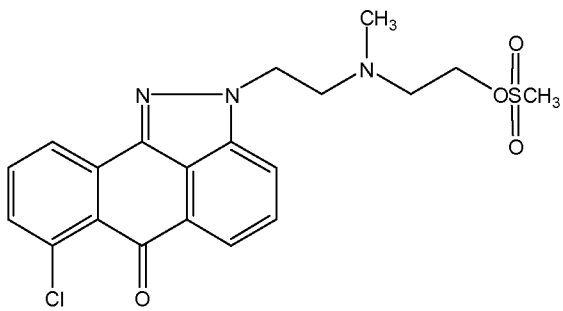
suggests that these compounds target DNA or interferes with some enzyme that processes DNA. The logarithm of the K562 IC_{50} data was, however, poorly correlated with the IC_{50} for the catalytic inhibition of topoisomerase IIα (r^2 = 0.12, p = 0.2) (FIG. 7B). The lack of correlation with topoisomerase IIα IC_{50} does not mean that these compounds did not act on topoisomerase II as topoisomerase II poisons, but only that they did not act solely through their inhibition of the catalytic activity of topoisomerase IIα.

EXAMPLE 17

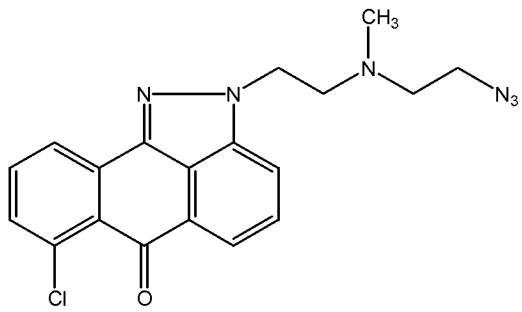
Synthesis of Certain Amide-Coupled Bisanthrapyrazoles Of the Present Invention

General scheme for synthesis of certain amide-linked bisanthrapyrazoles of the present invention

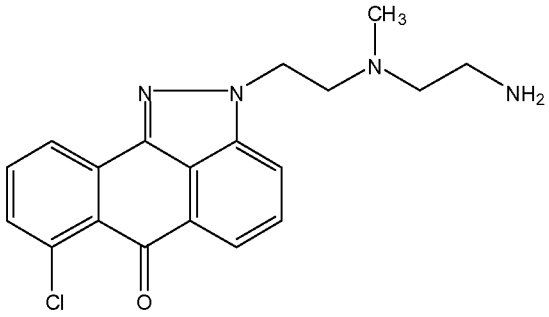




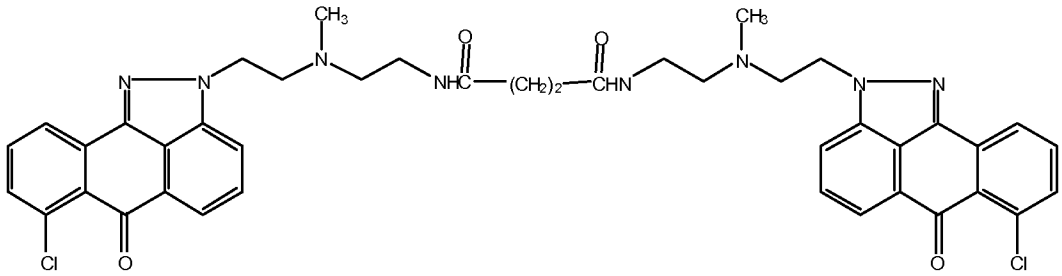
Preparation of AP9-OMs – Mesylchloride (0.789 mL, 10.196 mmol) and triethylamine (1.42 mL, 10.196 mmol) were added to a solution of AP-9 (0.907 g, 2.549 mmol) in chloroform (15 mL) while on ice. The mixture was allowed to stir for 1 week under nitrogen. The product was then extracted into chloroform with sodium hydroxide, dried over sodium sulfate, condensed, and dried on a vacuum pump, affording 0.940 g (2.166 mmol) in 85% yield. 1H NMR ($CDCl_3$) δ 8.20 (dd, 1H); 7.99 (d, 1H); 7.73-7.53 (m, 4H); 4.61 (t, 2H); 3.43 (t, 2H); 3.09 (t, 2H); 2.79 (t, 2H); 2.39 (s, 3H). 13C NMR ($CDCl_3$) δ ~183; 139.57; 137.43; 134.89; 133.18; 132.81; 129.34; 128.90; 127.15; 122.01; 121.26; 115.14; 77.67; 77.24; 76.82; 59.41; 57.30; 48.64; 42.67; 41.48. IR (KBr) υ 3416.22; 1660.91; 1646.02; 1589.42; 1281.44; 1263.85; 1020.72; 792.32; 693.30 cm^{-1}.



Preparation of AP9-N3 – AP-9-OMs (0.550 g, 1.268 mmol) was added to sodium azide (0.824 g, 12.675 mmol) in dimethylformamide (5 mL) and heated to 60°C under nitrogen for 24 hours. The solution was then cooled to room temperature, added to a beaker of water (50 mL), and allowed to stir for 30 minutes. A solid precipitated and was filtered, affording 0.439 g (1.153 mmol) in 91% yield. 1H NMR ($CDCl_3$) δ 8.20 (dd, 1H); 8.00 (dd, 1H); 7.74-7.52 (m, 4H); 4.62 (t, 2H); 3.23 (t, 2H); 3.08 (t, 2H); 2.66 (t, 2H); 2.39 (s, 3H). IR (KBr) υ 3413; 2953; 2808; 2104; 1660; 1646; 1589; 1447; 1282; 1020; 795; 766; 694 cm^{-1}.



Catalytic Hydrogenation of AP9-N3 – AP-9-N3 (0.438 g, 1.15 mmol) was dissolved in methanol (100 mL) and hydrochloric acid (2.530 mmol). Palladium on charcoal (0.082 g, 0.767 mmol) was added and the mixture was placed under a positive hydrogen atmosphere for 2 hours. The mixture was then filtered through a Celite pad. The methanol filtrate was condensed, extracted into chloroform with base, dried over sodium sulfate, condensed and dried on a vacuum pump, affording 0.485 g (1.367 mmol) of a yellow solid in 119% yield. 1H NMR ($CDCl_3$) δ 8.202 (dd, 1H); 7.99 (d, 1H); 7.70 (quintet, mH); 7.54 (q, mH); 4.61 (t, 2H); 3.01 (t, 2H); 2.65 (t, 2H); 2.48 (t, 2H); 2.34 (s, 3H). 13C NMR ($CDCl_3$) δ 182.97; 139.55; 138.58; 138.43; 137.45; 134.93; 133.22; 132.78; 129.32; 128.86; 127.19; 122.88; 122.02; 121.24; 115.02; 60.68; 57.68; 48.73; 42.61. IR (KBr) υ 3422.2; 1663.7; 1590.2; 1497.4; 1450.1; 1262.4; 1021.7; 790.9; 694.2 cm^{-1}.



Preparation of Bis-Amide bisAP9_2 – Succinyl chloride (31.04 μL, 0.282 mmol) was added dropwise to a clear, dark orange solution of the starting amine (0.200 g, 0.564 mmol) dissolved in dichloromethane (2 mL). Immediately, a light, white orange precipitate formed. After stirring overnight, the solution was diluted with ether (2 mL) and suspended. The stir bar was removed and the solution was centrifuged. The supernatant was removed and the solid was washed with two additional portions of ether. The solid was dried under a stream of nitrogen and then place on a vacuum pump. The solid was then extracted into chloroform with base and dried on sodium sulfate, condensed, and dried on a vacuum pump. The solid was then

separated on a short chromatography column, affording 0.025 g (0.0316 mmol, 11% yield) of the yellow bis-amide product. 1H NMR ($CDCl_3$) δ 8.16 (dd, 2H); 7.95 (d, 2H); 7.60 (m, 8H); 5.86 (t, 2H); 4.58 (t, 4H); 3.16 (q, 4H); 3.00 (t, 4H); 2.51 (t, 4H); 2.33 (s, 6H); 2.00 (s, 4H).

EXAMPLE 18

Biological Evaluation Of An Amide-Coupled Biasanthrapyrazole Of the Present Invention

In preliminary results, bisAP9_2 has also been partially evaluated for K562 growth inhibition and DNA binding (Table 4). The results of Table 4 show that bisAP9_2 is about 7-fold more cytotoxic than monomeric AP9. The ΔT_m values for the anthrapyrazoles, which is a measure of strength of DNA binding, correlates well with cytotoxicity. The ΔT_m results also indicate that the design goal of making a bisintercalator was achieved.

Table 4. DNA ΔT_m and cell growth inhibitory effects of the amide-linked bisanthrapyrazole bisAP9_2 compared to monomer AP9 and doxorubicin.

		MTS K562 cell growth inhibition
Compound	ΔT_m[a] (°C)	IC_{50} (μM)
bisAP9_2	20.5	0.86
AP9	12.2	5.7
Doxorubicin	15.5	0.08

[a] The change in DNA melting temperature ΔT_m was measured at a drug concentration of 5 μM. The fact that the ΔT_m for bisAP9_2 is approximately that of the monomer AP9 indicates that bisAP9_2 is a bisintercalator.

EXAMPLE 19

Syntheses of Certain Biasanthrapyrazoles Of the Present Invention

Bis(2-((2-(7-chloro-6-oxo-6H-dibenzo[*cd,g*]indazol-2-yl)-ethyl)-methyl-amino)-ethyl) malonate (1A). AP9 (53.2 mg, 0.149 mol) reacted with malonyl chloride (0.37 mL, 0.075 mmol) affording **1A** as a yellow solid. Yield 25.5 mg (21.5%); mp: 138.0–141.0 °C; ^{1}H NMR δ 8.25–7.55 (m, 12H, ArH), 5.13 (m, 4H, 2x $NNCH_2$), 4.50 (m, 4H, 4x OCH_2), 3.90–3.40 (m, 8H), 3.45 (s, 2H, $COCH_2CO$), 2.92 (s, 6H, 2x NCH_3); ^{13}C NMR δ 182.6, 182.3, 169.5, 168.9, 167.7, 167.1 (6x CO), 140.2, 140.1, 139.1, 138.9, 137.0, 137.0, 135.2, 135.1, 135.0, 134.9, 133.9, 133.8, 130.4, 130.2, 129.5, 129.4, 127.1, 126.9, 123.2, 123.1, 123.1, 123.0, 1221.1, 122.0, 117.8, 117.7 (ArC), 60.3, 60.1 (2x OCH_2), 56.1, 55.2, 55.0, 54.8, 45.3, 43.1, 42.6, 42.2, 41.4 ($COCH_2CO$); MS (ESI m/z): calcd for $[C_{41}H_{36}Cl_2N_6O_6+H]^+$ 779.2, found 779.3.

Bis(2-((2-(7-chloro-6-oxo-6H-dibenzo[*cd,g*]indazol-2-yl)-ethyl)-methyl-amino)-ethyl) succinate (1B). AP9 (48.1 mg, 0.135 mmol) reacted with succinyl chloride (0.34 mL, 0.068 mmol) affording **1B** as a yellow solid. Yield 41.7 mg (38.8%); mp: 180 °C (decomposed); ^{1}H NMR δ 8.24–7.57 (m, 12H, ArH), 5.11 (br s, 4H, 2x $NNCH_2$), 4.44 (br s, 4H, 2x OCH_2), 3.86–3.45 (m, 8H), 2.92 (s, 6H, 2x NCH_3), 2.64 (br s, 4H, $COCH_2CH_2CO$); ^{13}C NMR δ 181.8, 181.6, 171.9 (CO), 139.3, 138.2, 136.2, 134.4, 134.3, 134.1, 133.0, 129.4, 128.6, 126.2, 122.3, 121.1, 116.9 (ArC), 57.7 (OCH_2), 55.5, 54.8, 54.2, 44.4 (NCH_3), 29.0 ($COCH_2CH_2CO$). MS (ESI m/z): calcd for $[C_{42}H_{38}Cl_2N_6O_6+H]^+$ 793.2, found 793.3.

Bis(2-((2-(7-chloro-6-oxo-6H-dibenzo[*cd,g*]indazol-2-yl)-ethyl)-methyl-amino)-ethyl) glutarate (1C). AP9 (81.9 mg, 0.230 mol) reacted with glutaryl chloride (0.58 mL, 0.115 mmol) affording **1C** as a yellow solid. Yield 104.5 mg (57.2%); mp: 111.5–113.0 °C; ^{1}H NMR δ 8.03–7.12 (m, 12H, ArH), 4.92 (s, 4H, 2x NNCH2), 4.25 (4H, 2x OCH_2), 3.63, 3.55, 3.42, 3.31 (4s, 8H), 2.73 (s, 6H, 2x NCH_3), 2.23, 2.01 (2s, 4H, 2x $COCH_2$), 1.60 (m, 2H, $COCH_2CH_2$); ^{13}C NMR d 181.6, 181.4, 1774.3, 172.5 (CO), 139.4, 139.1, 138.2, 138.1, 136.1, 136.0, 134.3, 134.3, 134.2, 134.1, 133.0, 129.5, 129.4, 128.6, 128.4, 126.2, 126.0, 122.3, 122.3, 122.2, 122.1, 121.2, 121.1,

116.9, 116.7 (ArC), 58.5 (OCH_2), 54.2 (NCH_2), 44.4 (NCH_3), 32.9, 20.8 ($COCH_2CH_2CH_2CO$); MS (ESI m/z): calcd for $[C_{43}H_{40}Cl_2N_6O_6+H]^+$ 807.2, found 807.4.

Bis(2-((2-(7-chloro-6-oxo-6H-dibenzo[*cd,g*]indazol-2-yl)-ethyl)-methyl-amino)-ethyl) adipate dihydrochloride (1D). AP9 (106 mg, 0.30 mmol) in $CHCl_3$ (2.5 mL) was treated at 0 °C with adipoyl chloride (0.75 mL of a 0.2 M solution in $CHCl_3$) and the mixture allowed to come to room temperature while stirring overnight. Within 1 h a yellow precipitate began to form. At 18 h the mixture was diluted with ether (10 mL) and centrifuged. The resulting yellow crystals were dried in a nitrogen stream and then under vacuum overnight affording the dihydrochloride of **1D**. The sample was recrystallized from methanol-ether affording yellow crystals. Yield: 70 mg (53%), ^{1}H NMR δ 8.24–7.60 (m, 12H, ArH), 5.05 (s, 4H, 2x $NNCH_2$), 4.35 (br s, 4H, 2x OCH_2), 3.80 (br s, 4H), 2.90 (s, 6H, 2x NCH_3), 2.20 (br s, 4H, 2x $COCH_2$), 1.25 (br s, 2H, $COCH_2CH_2$), MS (ESI m/z): calcd for $[C_{44}H_{42}Cl_2N_6O_6+H]^+$ 821.3, found 821.4.

Bis(2-((2-(7-chloro-6-oxo-6H-dibenzo[*cd,g*]indazol-2-yl)-ethyl)-methyl-amino)-ethyl) pimelate (1E). AP9 (51.5 mg, 0.145 mol) reacted with pimeloyl chloride (0.36 mL, 0.115 mmol) affording **1E** as a yellow solid. Yield 64.5 mg (56.1%); mp: 102.0–104.0 °C; ^{1}H NMR δ 8.26–7.57 (m, 12H, ArH), 5.18 (s, 4H, 2x $NNCH_2$), 4.48 (s, 4H, 2x OCH_2), 3.89, 3.81, 3.67, 3.56 (4s, 8H), 2.99 (s, 6H, 2x NCH_3), 2.34 (t, J = 7.3 Hz, 4H, 2x $COCH_2$), 1.50 (m, 4H, 2x $COCH_2CH_2$), 1.24 (m, 2H, $COCH_2CH_2CH_2$); ^{13}C NMR δ 181.4, 174.7, 172.7 (CO), 139.2, 138.1, 136.1, 134.2, 134.1, 133.0, 129.4, 128.5, 126.0, 122.2, 122.1, 121.1, 116.9 (ArC), 58.5 (OCH_2), 54.1 (NCH_2), 44.4 (NCH_3), 33.5, 28.1, 24.1 ($COCH_2CH_2CH_2$); MS (ESI m/z): calcd for $[C_{45}H_{44}Cl_2N_6O_6+H]^+$ 835.3, found 835.3.

EXAMPLE 20

Materials and Methods for Synthesis Examples 1, 9, 17 and 19

Melting points were taken on a Gallenkamp (Loughborough, England) melting point apparatus and are uncorrected. Electrospray ionization mass spectra (ESI-MS) were acquired on an Applied Biosystems API 2000 Triple Quadrupole mass spectrometer (Thornhill, Toronto, Canada) equipped with a syringe pump. Samples (~1 mM in acetonitrile) were injected into the ion source at a flow rate of 5 μL/min. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 300 K in 5 mm NMR tubes on a Bruker Avance 500 spectrometer operating at 500.14 MHz for ^{1}H NMR and 125.8 MHz for ^{13}C NMR, respectively, on solutions in dimethyl sulfoxide-d_6 (DMSO-d_6), unless otherwise indicated. Chemical shifts are given in parts per million (ppm) (±0.01 ppm) relative to that of tetramethylsilane (TMS) (0.00 ppm) in the case of the ^{1}H NMR spectra, and to the central line of DMSO-d_6 (δ 39.5) for the ^{13}C NMR spectra. The ^{1}H NMR spectrum of **1D** was run on a Varian Mercury 300 MHz NMR. TLC was performed on aluminum-backed plates bearing 200 μm silica gel 60 F254 (Silicycle, Quebec City, Canada). Compounds were visualized by quenching of fluorescence by UV light (254 nm) where applicable. The reaction conditions were not optimized for reaction yields. Dichloromethane and triethylamine were refluxed with calcium hydride and distilled. AP9 (7-chloro-2-[2-[(2-hydroxyethyl)methylamino]ethyl]anthra[1,9-*cd*]pyrazol-6(2H)-one) was prepared from 1,5-dichloroanthraquinone as previously described (Liang *et al.*, 2006) (incorporated herein by reference in its entirety).

* * * * *

All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and

modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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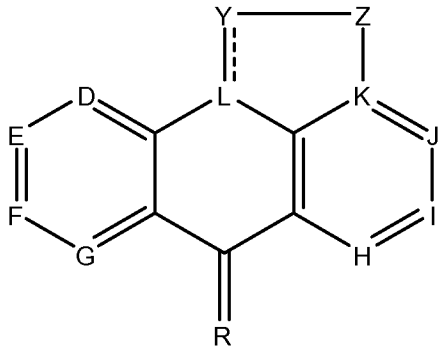
CLAIMS

1. A compound comprising a first anthrapyrazole operatively linked to a second anthrapyrazole.

2. The compound of claim 1, wherein the first anthrapyrazole is operatively linked to a second anthrapyrazole via a linker.

3. The compound of claim 2, wherein the core skeleton of each of the first and second anthrapyrazoles is represented by structure (A), and wherein a linker links each Z atom of the first and second anthrapyrazoles:



(A),

wherein:

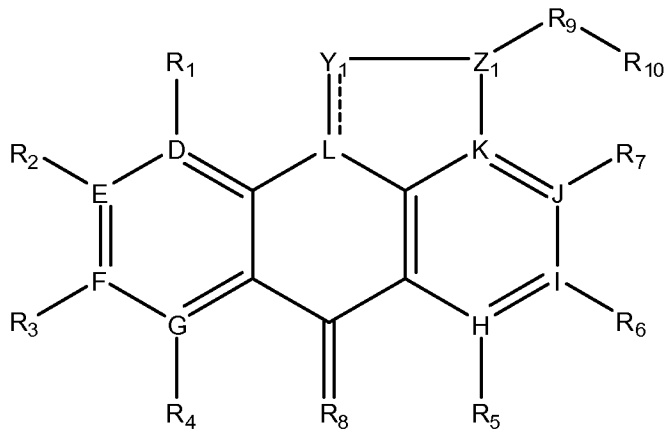
ring atoms D, E, F, G, H, I, J, K, L, Y and Z are each independently carbon or nitrogen in each of the first and second anthrapyrazoles;

the bond between L and Y may be a double or single bond; and

R is oxygen or sulfur;

and wherein the first and second anthrapyrazoles have the same or different core skeleton.

4. The compound of claim 1, wherein the first and second anthrapyrazoles are each independently selected from the compound of formula (I):



(I)

wherein:

R_1-R_7 are each independently hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl, phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group,

or any one or more of Y_1-R_1, R_1-R_2, R_2-R_3, R_3-R_4, R_5-R_6, R_6-R_7 or R_7-Z_1 together forms a cyclic group,

or any combination of one or more of these groups;

R_8 is either oxygen or sulfur;

R_9 is either not present or is a linker; and

R_{10} is either hydrogen, alkyl, a nucleophile or a leaving group;

D, E, F, G, H, I, J, K and L are each independently carbon, -CH or nitrogen;

Y_1 is selected from the group consisting of -CH, -CH_2, oxygen, nitrogen, -NH and sulfur;

Z_1 is selected from the group consisting of –CH and nitrogen; and

the bond between L and Y_1 is either a single or a double bond.

5. The compound of claim 4, wherein R_1 is hydrogen or OH.

6. The compound of claim 4, wherein R_4 is hydrogen, halogen, hydroxyl, or substituted alkyl.

7. The compound of claim 6, wherein the substituted alkyl is an aminoalkyl.

8. The compound of claim 7, wherein the aminoalkyl is further defined as –$NH(CH_2)_2N(CH_3)_2$.

9. The compound of claim 4, wherein R_5 is hydrogen, halogen, or substituted alkyl.

10. The compound of claim 9, wherein the substituted alkyl is an aminoalkyl.

11. The compound of claim 7, wherein the aminoalkyl is further defined as —$NH(CH_2)_2N(CH_3)_2$ or –$NH(CH_2)_3NH_2$.

12. The compound of claim 10, wherein the substituted alkyl is hydroxyalkylamino.

13. The compound of claim 12, wherein the hydroxyalkylamino is further defined as –$NH(CH_2)_3NH(CH_2)_2OH$.

14. The compound of claim 4, wherein R_2, R_3, R_6 and R_7 are each hydrogen.

15. The compound of claim 4, wherein R_8 is oxygen.

16. The compound of claim 4, wherein R_9 is not present and R_{10} is hydroxyl.

17. The compound of claim 4, wherein R_9 is –$N(R_{11})(CH_2)_2$-, wherein R_{11} is selected from the group consisting of hydrogen, alkyl and substituted alkyl.

18. The compound of claim 17, wherein R_1, R_2, R_3, R_5, R_6 and R_7 is hydrogen, R_4 is Cl, R_8 is oxygen, R_{10} is hydroxyl and R_{11} is CH_3.

19. The compound of claim 4, wherein R_{10} is hydroxyl, azido or amino.

20. The compound of claim 3, wherein the number of linear atoms linking the first and second anthrapyrazoles is 7 or higher.

21. The compound of claim 20, wherein the linear atoms are selected from the group consisting of carbon, nitrogen, oxygen, sulfur and phosphorus.

22. The compound of claim 4, wherein R_9 comprises an alkyl group, a substituted alkyl group, a heterocyclyl group, an oxo group, a sulfo group, a thioether group, an aryl group, an amide group, a sulfonamide group, a carbonyl group, a thiocarbonyl group, a secondary amine group, a tertiary amine group, an ester group, a thioester group, a sulfonyl group, or any combination of one or more of these groups.

23. The compound of claim 2, wherein the linker comprises an alkyl group.

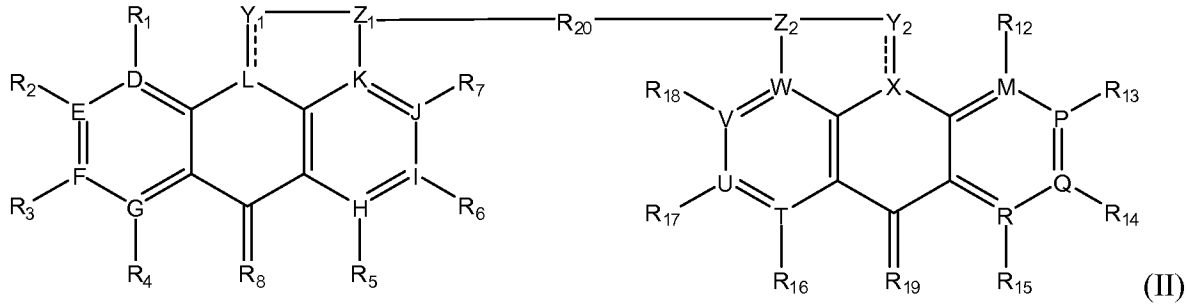
24. The compound of claim 2, wherein the linker comprises at least one ester.

25. The compound of claim 2, wherein the linker comprises at least one amide.

26. The compound of claim 2, wherein the linker is further defined as $-(CH_2)_2N(CH_3)(CH_2)_2OC(O)(CH_2)_nC(O)O(CH_2)_2N(CH_3)(CH_2)_2-$, wherein n = 1-5.

27. The compound of claim 2, wherein the linker is further defined as $-(CH_2)_2N(CH_3)(CH_2)_2NHC(O)(CH_2)_nC(O)NH(CH_2)_2N(CH_3)(CH_2)_2-$, wherein n = 1-5.

28. A compound of formula (II):



(II)

wherein:

R_1-R_7 and R_{12}-R_{18} are each independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl, phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group,

or any one or more of Y_1-R_1, R_1-R_2, R_2-R_3, R_3-R_4, R_5-R_6, R_6-R_7, R_7-Z_1, Y_2-R_{12}, R_{12}-R_{13}, R_{13}-R_{14}, R_{14}-R_{15}, R_{16}-R_{17}, R_{17}-R_{18} or R_{18}-Z_2 together forms a cyclic group,

or any combination of one or more of these groups;

R_8 and R_{19} are each independently oxygen or sulfur;

D, E, F, G, H, I, J, K, L, M, P, Q, R, T, U, V, W and X are each independently selected from the group consisting of carbon, -CH and nitrogen;

Y_1 and Y_2 are each independently selected from the group consisting of -CH, -CH_2, oxygen, nitrogen, -NH or sulfur;

Z_1 and Z_2 are each independently selected from the group consisting of -CH and nitrogen;

the L-Y_1 bond and the X-Y_2 bond are each independently a single or a double bond; and

R_{20} is a linker.

29. The compound of claim 28, wherein R_1-R_7 and R_{12}-R_{18} are each independently hydrogen or halogen.

30. The compound of claim 29, wherein the halogen is chlorine.

31. The compound of claim 28, wherein R_8 and R_{19} are each oxygen.

32. The compound of claim 28, wherein D, E, F, G, H, I, J, K, L, M, P, Q, R, T, U, V, W and X are each carbon or -CH.

33. The compound of claim 28, wherein Y_1, Y_2, Z_1 and Z_2 are each carbon or -CH.

34. The compound of claim 28, wherein the L-Y_1 bond and the X-Y_2 bond are each double bonds.

35. The compound of claim 28, wherein the linker is selected from the group consisting of an alkyl group, a substituted alkyl group, a heterocyclyl group, an oxo group, a sulfo group, a thioether group, an aryl group, an amide group, a sulfonamide group, a carbonyl group, a thiocarbonyl group, a secondary amine group, a tertiary amine group, an ester group, a thioester group, a sulfonyl group, or any combination of one or more of these groups.

36. The compound of claim 28, wherein the linker comprises an alkyl.

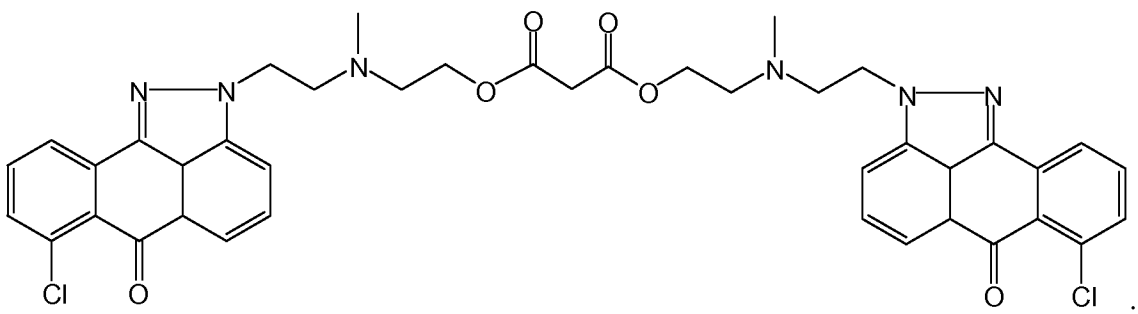
37. The compound of claim 28, wherein the linker comprises at least one ester.

38. The compound of claim 28, wherein the linker comprises at least one amide.

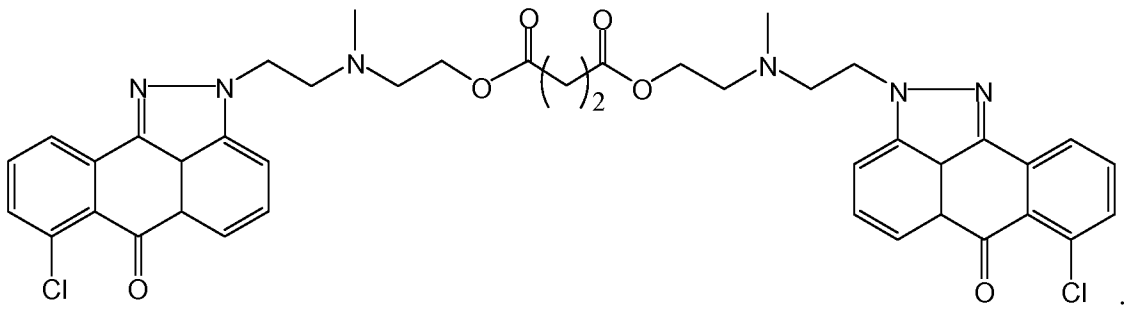
39. The compound of claim 28, wherein R_{20} is further defined as $-(CH_2)_2N(CH_3)(CH_2)_2OC(O)(CH_2)_nC(O)O(CH_2)_2N(CH_3)(CH_2)_2-$, wherein n = 1-5.

40. The compound of claim 28, wherein R_{20} is further defined as $-(CH_2)_2N(CH_3)(CH_2)_2NHC(O)(CH_2)_nC(O)NH(CH_2)_2N(CH_3)(CH_2)_2-$, wherein n = 1-5.

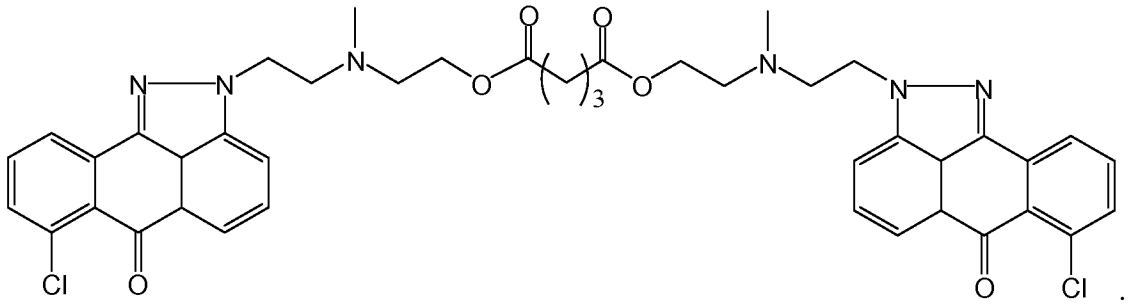
41. The compound of claim 28, further defined as:



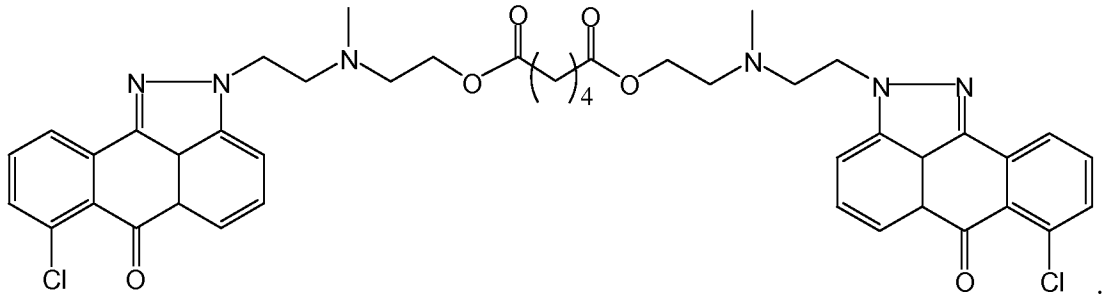
42. The compound of claim 28, further defined as:



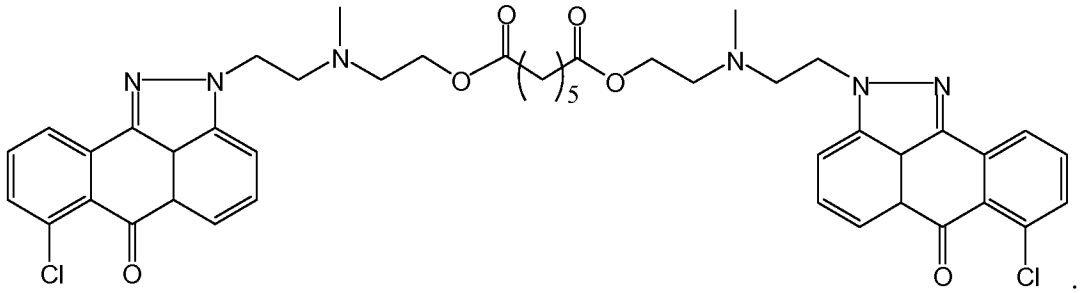
43. The compound of claim 28, further defined as:



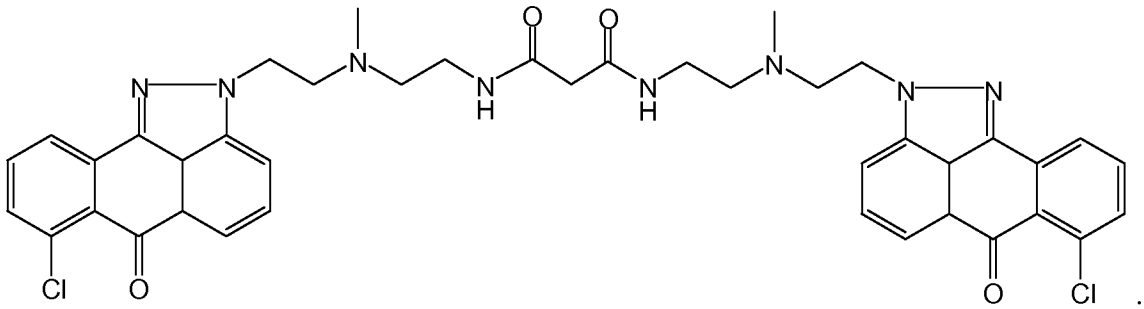
44. The compound of claim 28, further defined as:



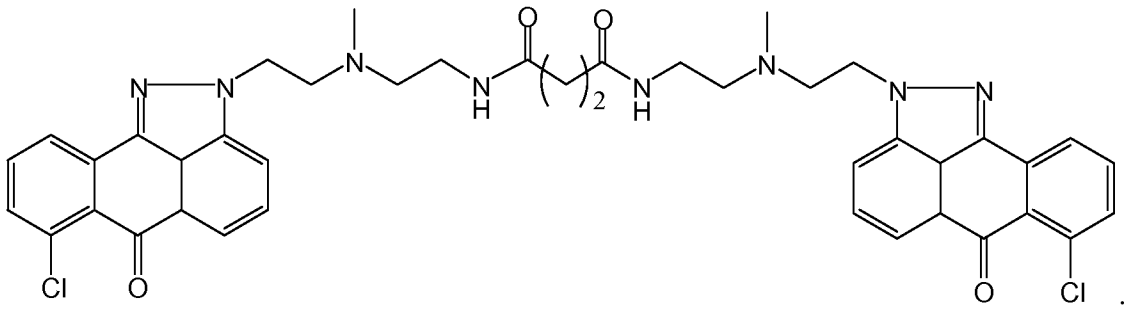
45. The compound of claim 28, further defined as:



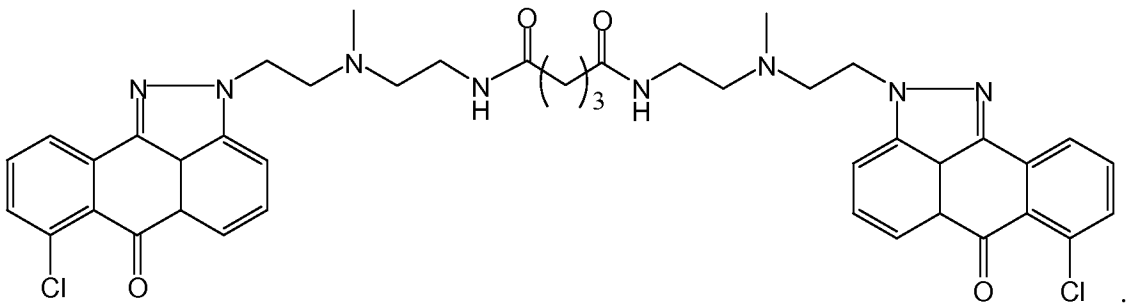
46. The compound of claim 28, further defined as:



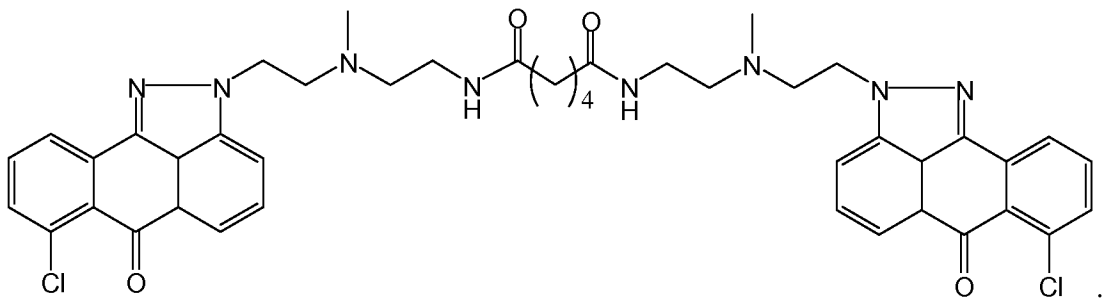
47. The compound of claim 28, further defined as:



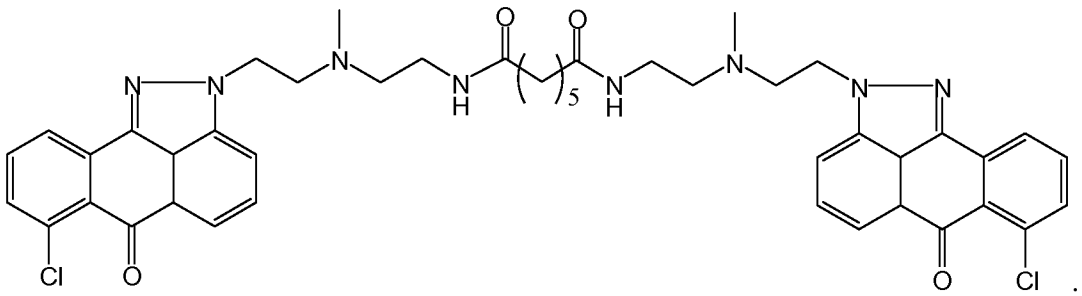
48. The compound of claim 28, further defined as:



49. The compound of claim 28, further defined as:



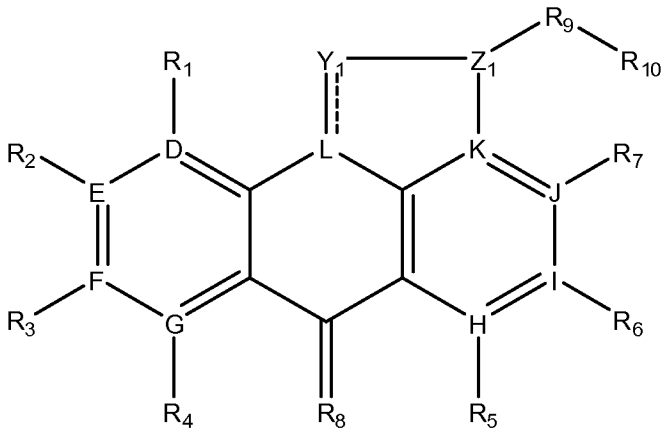
50. The compound of claim 28, further defined as:



51. The compound of claim 28, further defined as a prodrug.

52. A method of preparing a bisanthrapyrazole comprising preparing a first anthrapyrazole, preparing a second anthrapyrazole, and conjugating the first anthrapyrazole to the second anthrapyrazole either directly or through a linker.

53. A method of preparing a compound of claim 28, comprising:
preparing a first anthrapyrazole of formula (I):



(I),

wherein:

R_1-R_7 are each independently hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl, phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group,

or any one or more of Y_1-R_1, R_1-R_2, R_2-R_3, R_3-R_4, R_5-R_6, R_6-R_7 or R_7-Z_1 together forms a cyclic group,

or any combination of one or more of these groups;

R_8 is either oxygen or sulfur;

R_9 is either not present or is a linker; and

R_{10} is either hydrogen, alkyl, a nucleophile or a leaving group;

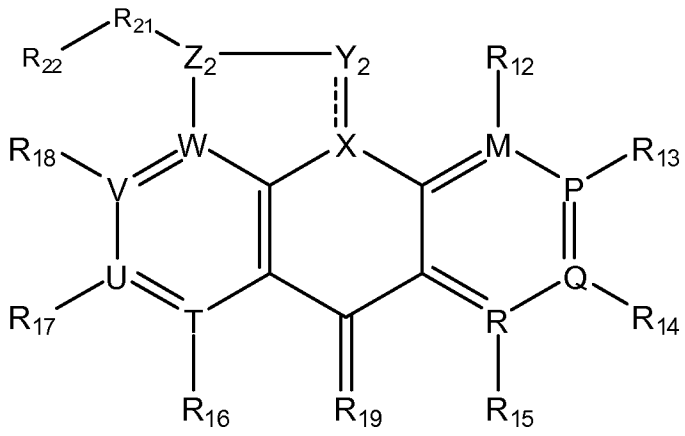
D, E, F, G, H, I, J, K and L are each independently selected from the group consisting of carbon, -CH and nitrogen;

Y_1 is selected from the group consisting of -CH, -CH_2, oxygen, nitrogen, -NH and sulfur;

Z_1 is selected from the group consisting of –CH and nitrogen; and

the bond between L and Y_1 is either a single or a double bond;

preparing a second anthrapyrazole of formula (III):



(III),

wherein:

R_{12}-R_{18} are each independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl, phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group,

or any one or more of Y_2-R_{12}, R_{12}-R_{13}, R_{13}-R_{14}, R_{14}-R_{15}, R_{16}-R_{17}, R_{17}-R_{18} or R_{18}-Z_2 together forms a cyclic group,

or any combination of one or more of these groups;

R_{19} is either oxygen or sulfur;

R_{21} is either not present or is a linker; and

R_{22} is either H, alkyl, a nucleophile or a leaving group;

M, P, Q, R, T, U V, W and X are each independently selected from the group consisting of carbon, -CH and nitrogen;

Y_2 is selected from the group consisting of -CH, -CH_2, oxygen, nitrogen, -NH and sulfur;

Z_2 is selected from the group consisting of –CH and nitrogen; and

the bond between X and Y_2 is either a single or a double bond; and

conjugating said first anthrapyrazole to said second anthrapyrazole either directly or through a linker.

54. The method of claim 53, wherein the conjugation step comprises conjugating said first anthrapyrazole to said second anthrapyrazole through a linker that joins the Z_1 and Z_2 positions of each anthrapyrazole.

55. The method of claim 53, wherein the linker comprises an alkyl group, a substituted alkyl group, a heterocyclyl group, an oxo group, a sulfo group, a thioether group, an aryl group, an amide group, a sulfonamide group, a carbonyl group, a thiocarbonyl group, a secondary amine group, a tertiary amine group, an ester group, a thioester group, a sulfonyl group, or any combination of one or more of these groups.

56. The method of claim 53, wherein the linker comprises an alkyl group.

57. The method of claim 56, wherein the linker further comprises at least one ester.

58. The method of claim 56, wherein the linker further comprises at least one amide.

59. The method of claim 56, wherein R_{10} and R_{22} are selected from the group consisting of a nucleophile and a leaving group, wherein $R_{10} \neq R_{22}$.

60. A method of inhibiting the catalytic decatenation activity of topoisomerase IIα, comprising administering to a cell an effective amount of a bisanthrapyrazole.

61. A method of treating a patient with cancer, comprising administering to the patient a therapeutically effective amount of a bisanthrapyrazole.

62. A therapeutic kit comprising, in suitable container means, a pharmaceutically acceptable composition comprising a bisanthrapyrazole.

63. A method of estimating the binding strength of a compound to DNA using molecular modeling comprising modeling the compound docked into DNA and obtaining a GOLDScore.

64. The method of claim 63, further comprising the steps of:

(a) obtaining an x-ray structure of a known DNA-intercalator complex;

(b) optimizing the geometry of the compound;

(c) removing all water molecules and the intercalator from the DNA-intercalator complex;

(d) optionally adding hydrogens to the DNA;

(e) defining a binding site;

(f) docking the compound into the DNA; and

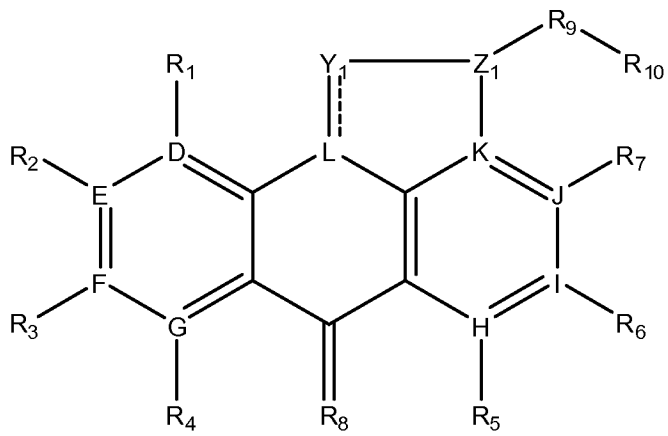
(g) obtaining a GOLDScore,

wherein the GOLDScore is an indication of the strength of the binding between the compound and the DNA.

65. The method of claim 63, wherein the compound is a DNA intercalator or a putative DNA intercalator.

66. The method of claim 63, wherein the compound is an anthrapyrazole or a bisanthrapyrazole.

67. The method of claim 66, wherein the compound is a compound of formula (I):



(I)

wherein:

R_1-R_7 are each independently hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl, phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group,

or any one or more of Y_1-R_1, R_1-R_2, R_2-R_3, R_3-R_4, R_5-R_6, R_6-R_7 or R_7-Z_1 together forms a cyclic group,

or any combination of one or more of these groups;

R_8 is either oxygen or sulfur;

R_9 is either not present or is a linker; and

R_{10} is either hydrogen, alkyl, a nucleophile or a leaving group;

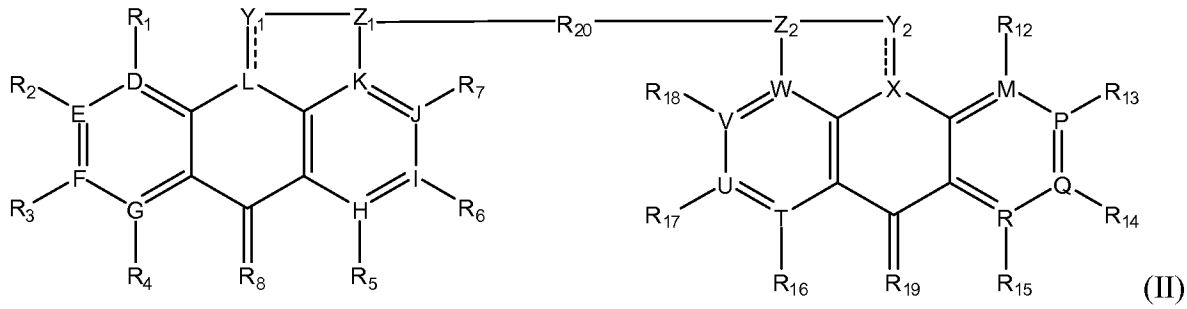
D, E, F, G, H, I, J, K and L are each independently selected from the group consisting of carbon, -CH and nitrogen;

Y_1 is selected from the group consisting of -CH, -CH_2, oxygen, nitrogen, -NH and sulfur;

Z_1 is selected from the group consisting of –CH and nitrogen; and

the bond between L and Y_1 is either a single or a double bond;

or formula (II):



(II)

wherein:

R_1-R_7 and R_{12}-R_{18} are each independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, halogen, oxo, hydroxy, silyl, phosphoro, acyl, aryl, acetyl, carbonyl, cyano, amido, amino, ester, NO, NO_2, azido, sulfo, or a protecting group,

or any one or more of Y_1-R_1, R_1-R_2, R_2-R_3, R_3-R_4, R_5-R_6, R_6-R_7, R_7-Z_1, Y_2-R_{12}, R_{12}-R_{13}, R_{13}-R_{14}, R_{14}-R_{15}, R_{16}-R_{17}, R_{17}-R_{18} or R_{18}-Z_2 together forms a cyclic group,

or any combination of one or more of these groups;

R_8 and R_{19} are each independently oxygen or sulfur;

D, E, F, G, H, I, J, K, L, M, P, Q, R, T, U, V, W and X are each independently carbon, -CH or nitrogen;

Y_1 and Y_2 are each independently selected from the group consisting of -CH, -CH_2, oxygen, nitrogen, -NH and sulfur;

Z_1 and Z_2 are each independently selected from the group consisting of -CH and nitrogen;

the L-Y_1 bond and the X-Y_2 bond are each independently a single or a double bond; and

R_{20} is a linker.

FIG. 1

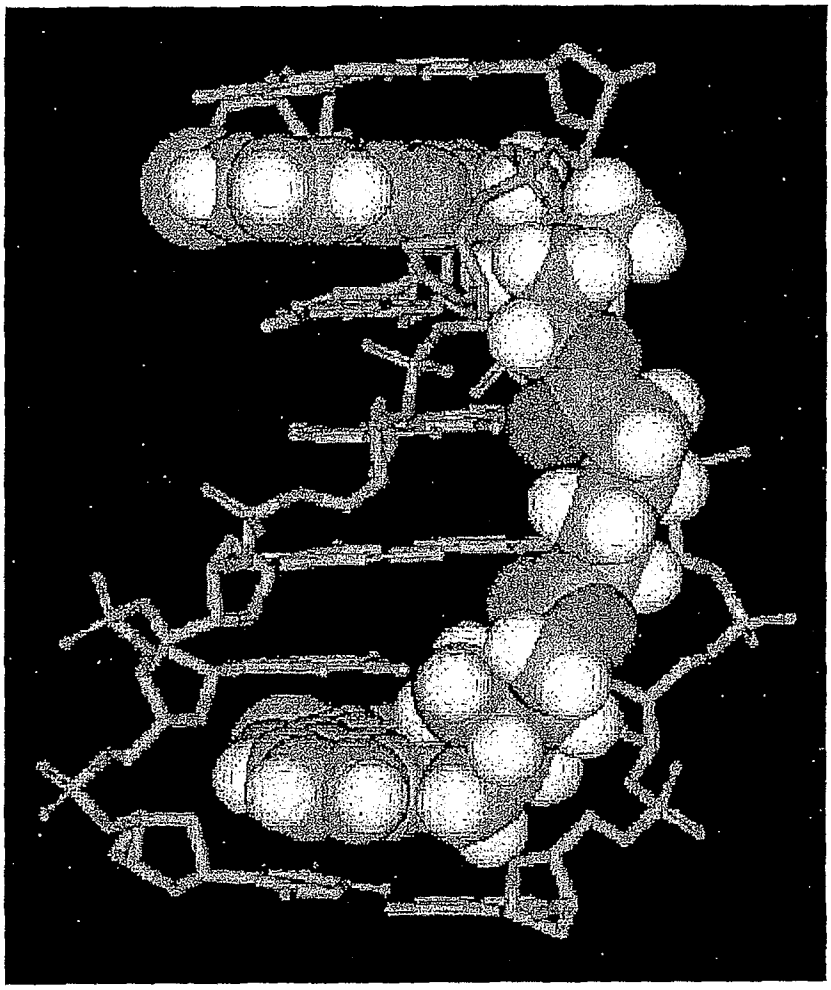


FIG. 2

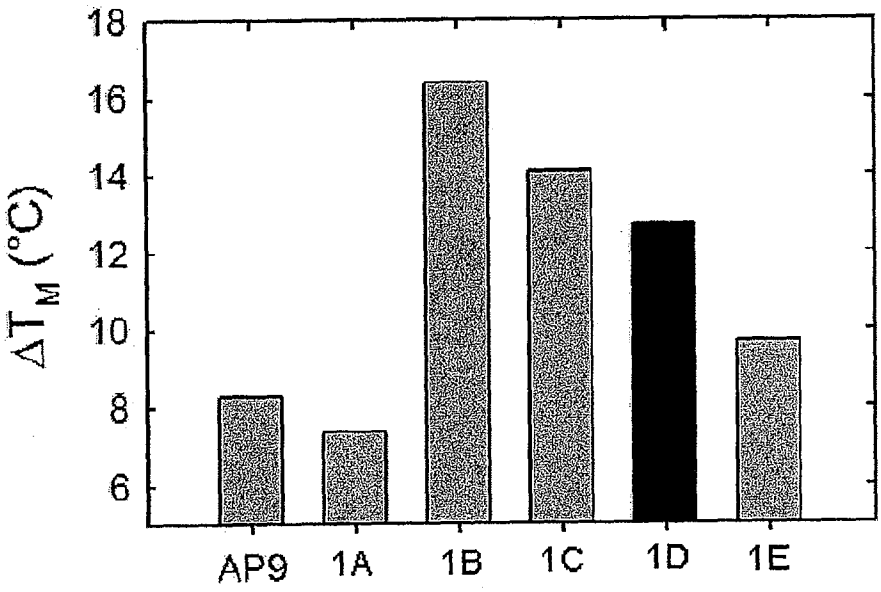


FIG. 3A

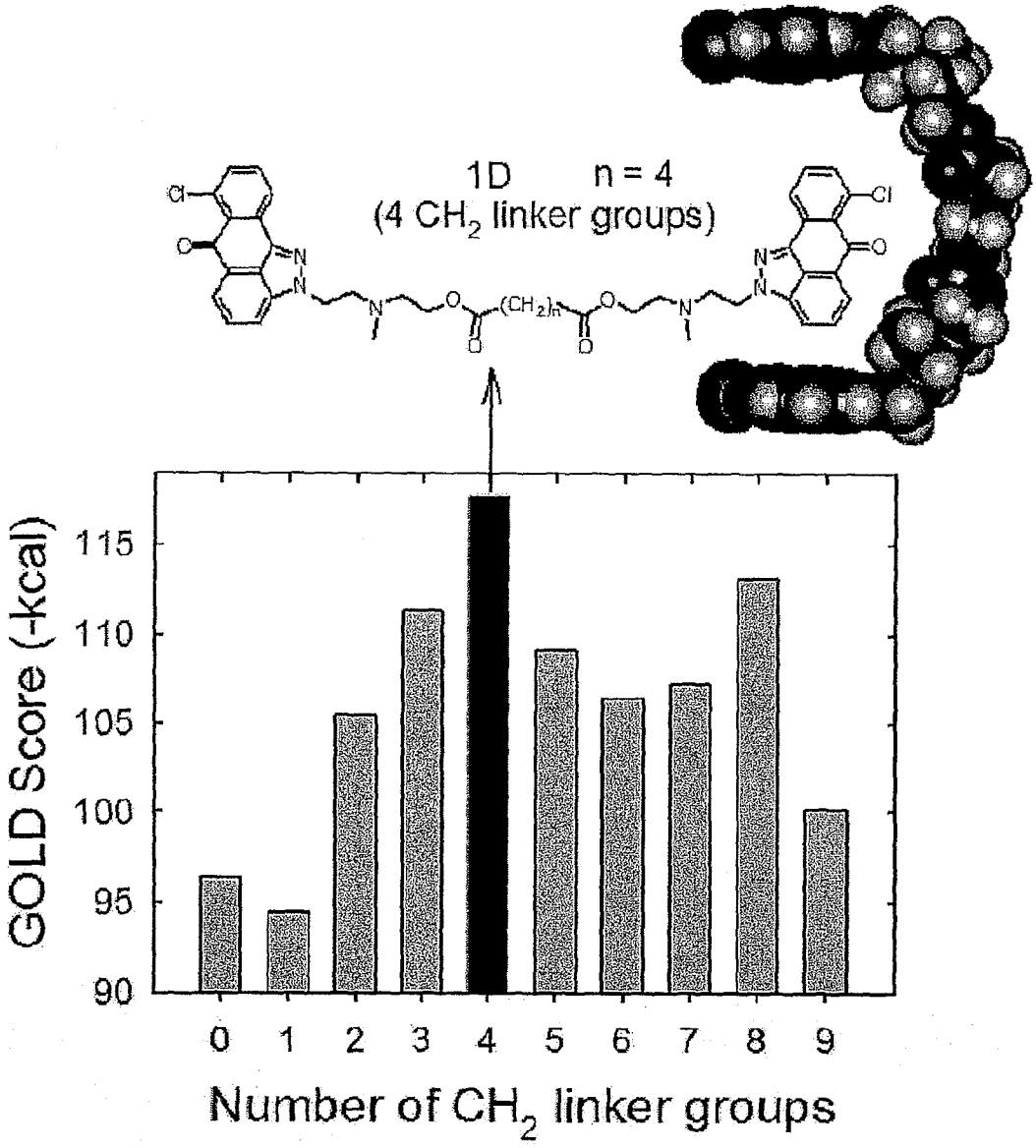

1D n = 4
(4 CH2 linker groups)
Cl
O
N
O
(CH2)n
GOLD Score (-kcal)
115
110
105
100
95
90
0 1 2 3 4 5 6 7 8 9
Number of CH2 linker groups

FIG. 3B

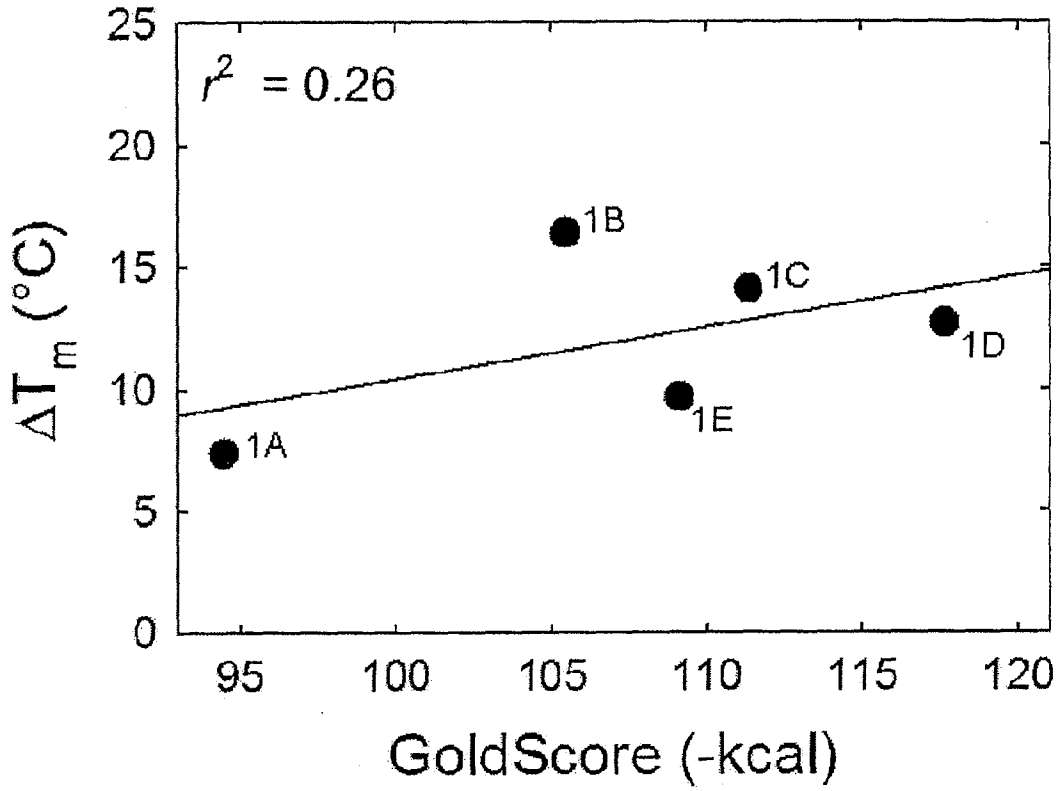

r^2 = 0.26
ΔT_m (°C)
GoldScore (-kcal)
25
20
15
10
5
0
95
100
105
110
115
120
1A
1B
1C
1D
1E

FIG. 4

Agent	ΔT_m (°C)	MTS cell growth inhibition: K562 IC_{50} (μM)	MTS cell growth inhibition: K/VP.5 IC_{50} (μM)	topo II inhibition: IC_{50} (μM)
AP9	8.3	5.7	6.4	7.2
1A	7.4	2.5	4.2	25
1B	16.4	2.7	4.2	50
1C	14.1	1.1	2.9	32
1D	12.7	3.3	3.9	8.4
1E	9.7	1.8	2.7	33
Losoxantrone	18.1	0.12	0.22	7.3
Piroxantrone	19.0	4.0	6.7	4.6
Mitoxantrone	18.4	0.42	1.68	5.3
Doxorubicin	14.2	0.08	0.41	ND

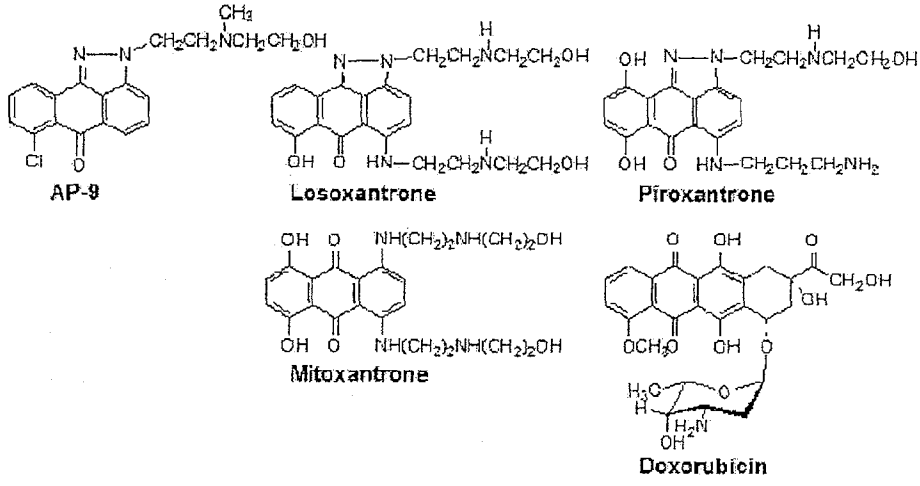


FIG. 5

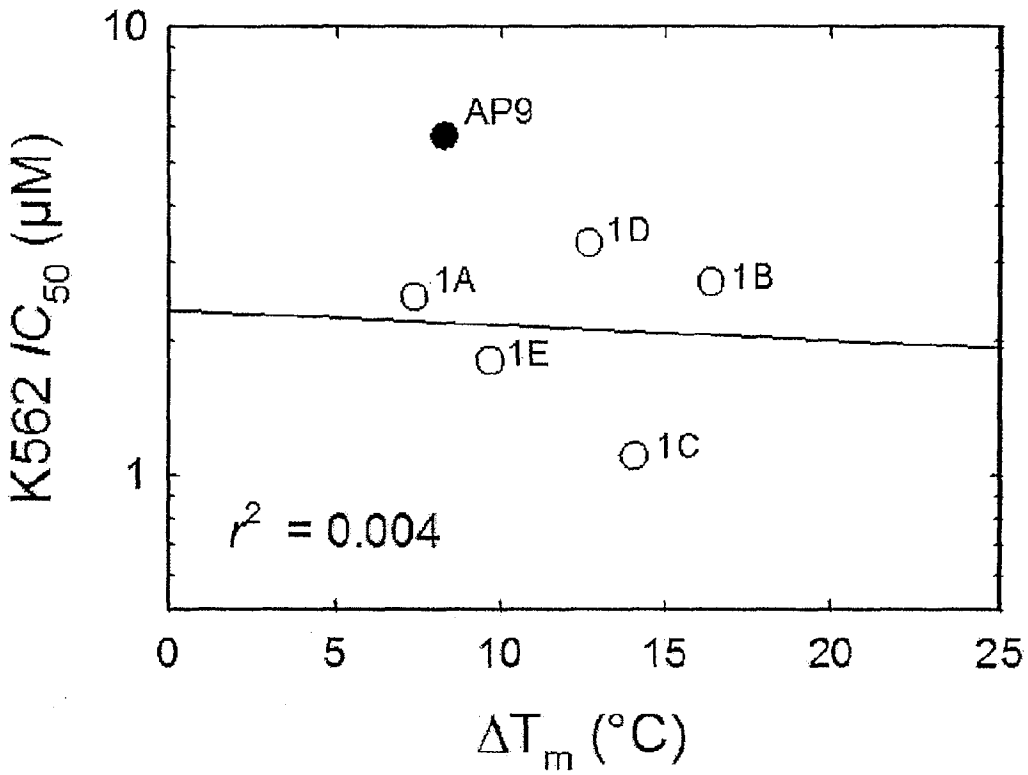
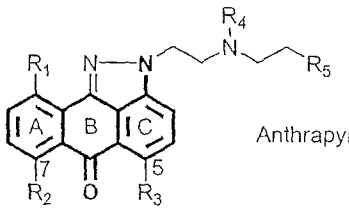
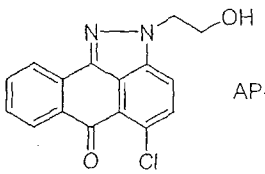

10
1
K562 IC_{50} (μM)
AP9
1D
1A
1B
1E
1C
r^2 = 0.004
0
5
10
15
20
25
ΔT_m (°C)

FIG. 6

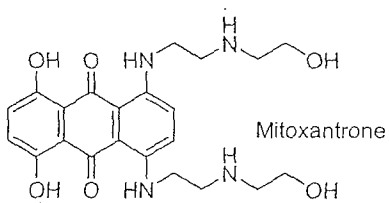
Compound	R_1	R_2	R_3	R_4	R_5
AP-1	H	H	Cl	CH_3	OH
AP-2	H	H	Cl	CH_3	Cl
AP-3	H	H	Cl	H	OH
AP-4	H	H	Cl	H	Cl
AP-6	H	Cl	H	CH_3	Cl
AP-7	H	Cl	H	H	OH
AP-8	H	Cl	H	H	Cl
AP-9	H	Cl	H	CH_3	OH
AP-10	H	H	$NH(CH_2)_2N(CH_3)_2$	H	OH
AP-11	H	H	$NH(CH_2)_2N(CH_3)_2$	CH_3	OH
AP-13	H	$NH(CH_2)_2N(CH_3)_2$	H	H	OH
AP-14	H	$NH(CH_2)_2N(CH_3)_2$	H	CH_3	OH
Losoxantrone	H	OH	$NH(CH_2)_3NH(CH_2)_2OH$	H	OH
Piroxantrone	OH	OH	$NH(CH_2)_3NH_2$	H	OH



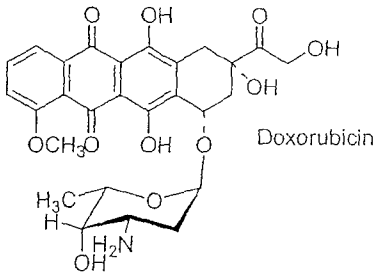
Anthrapyrazoles



AP-12

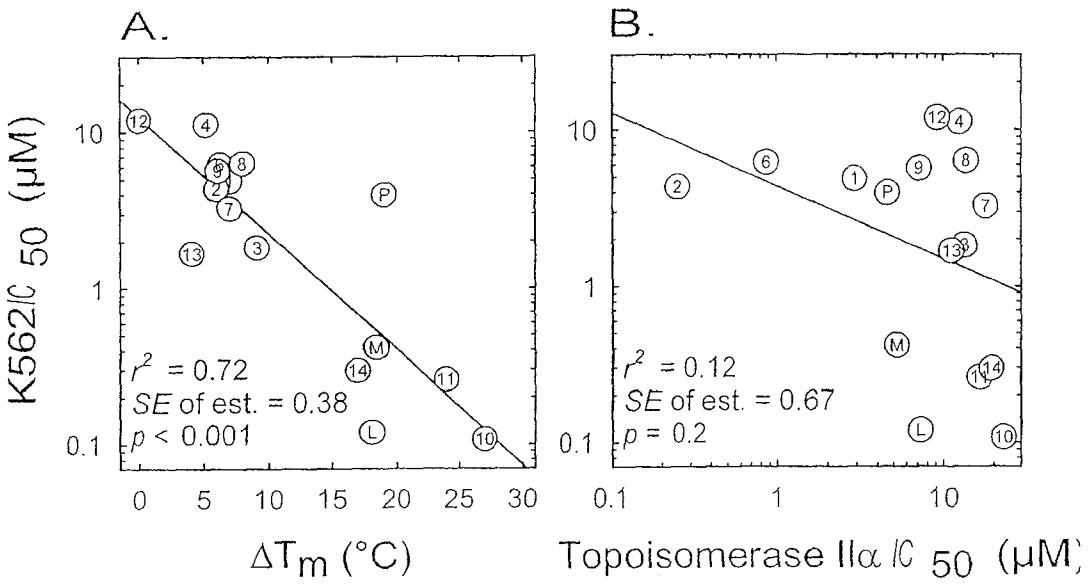


Mitoxantrone



Doxorubicin

FIG. 7


A.
B.
K562 IC 50 (μM)
10
1
0.1
0
5
10
15
20
25
30
ΔTm (°C)
r² = 0.72
SE of est. = 0.38
p < 0.001
r² = 0.12
SE of est. = 0.67
p = 0.2
0.1
1
10
Topoisomerase IIα IC 50 (μM)

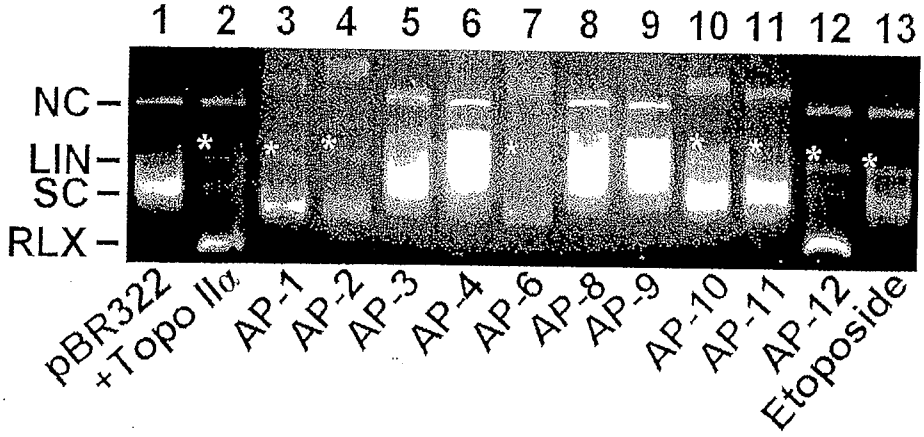

1 2 3 4 5 6 7 8 9 10 11 12 13
NC
LIN
SC
RLX
pBR322
+Topo IIα
AP-1
AP-2
AP-3
AP-4
AP-6
AP-8
AP-9
AP-10
AP-11
AP-12
Etoposide

FIG. 8

FIG. 9

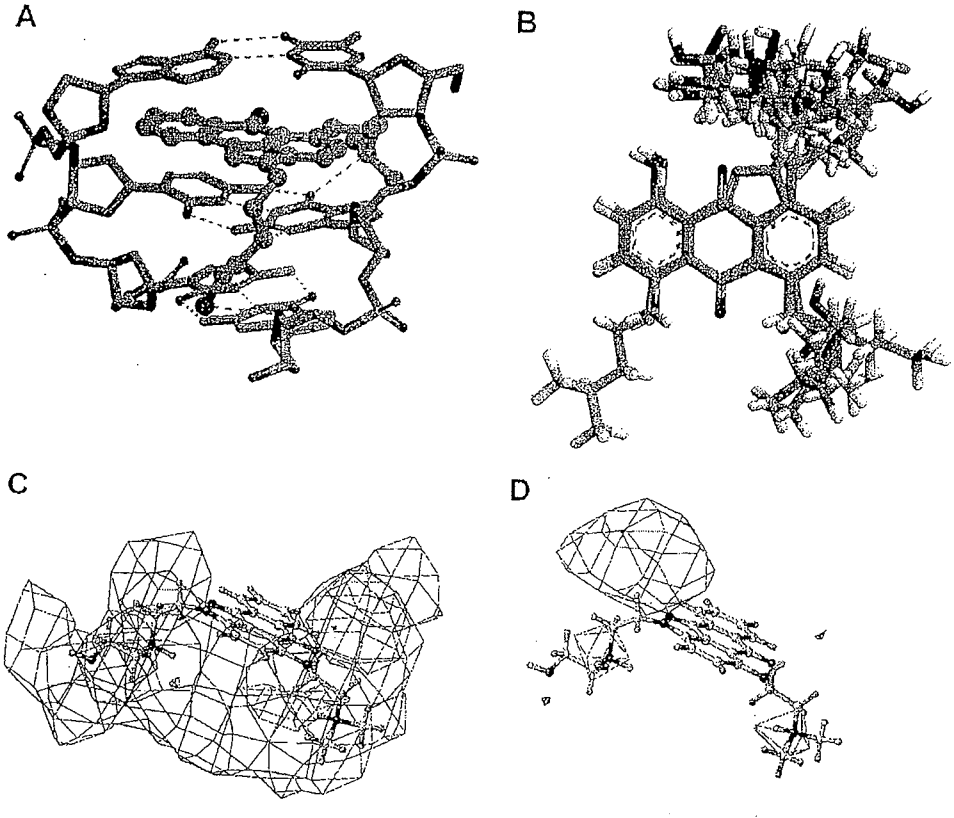


FIG. 10

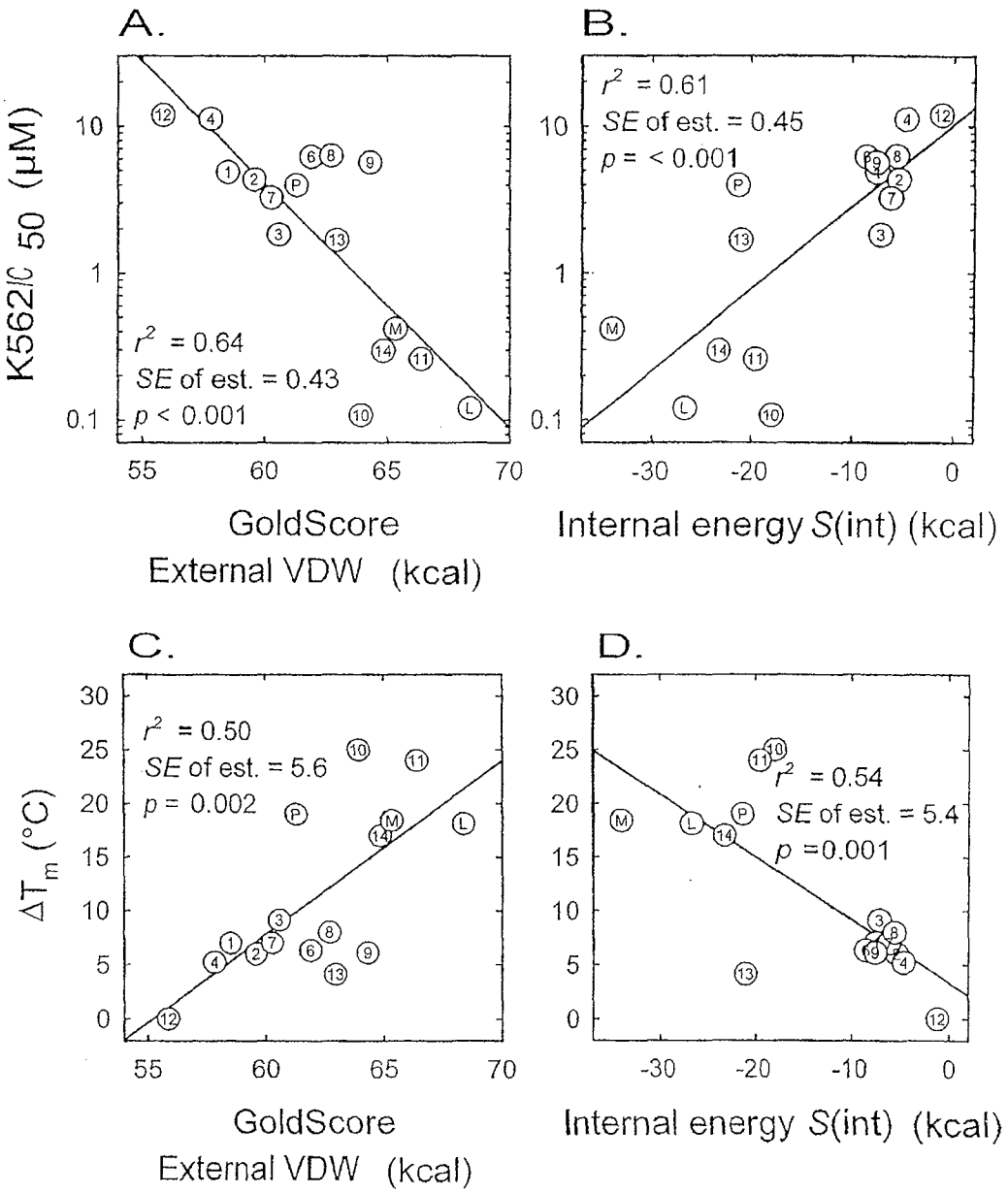
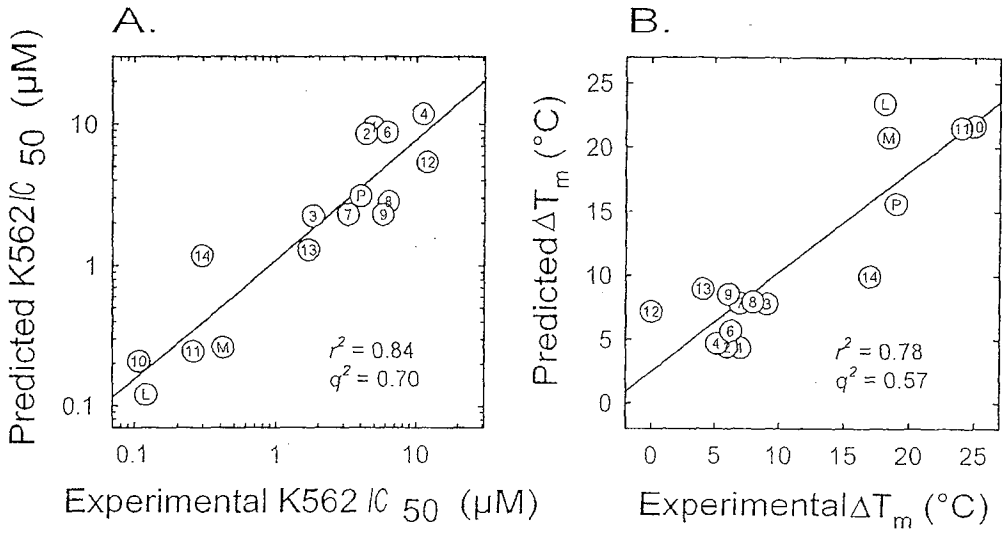

A.
B.
C.
D.
K562/C 50 (μM)
10
1
0.1
r^2 = 0.64
SE of est. = 0.43
p < 0.001
55
60
65
70
GoldScore
External VDW (kcal)
r^2 = 0.61
SE of est. = 0.45
p = < 0.001
-30
-20
-10
0
Internal energy S(int) (kcal)
ΔT$_m$ (°C)
30
25
20
15
10
5
0
r^2 = 0.50
SE of est. = 5.6
p = 0.002
r^2 = 0.54
SE of est. = 5.4
p =0.001

FIG. 11


A.
B.
Predicted K562 IC 50 (μM)
Experimental K562 IC 50 (μM)
r^2 = 0.84
q^2 = 0.70
Predicted ΔT$_m$ (°C)
Experimental ΔT$_m$ (°C)
r^2 = 0.78
q^2 = 0.57

FIGS. 12A-B

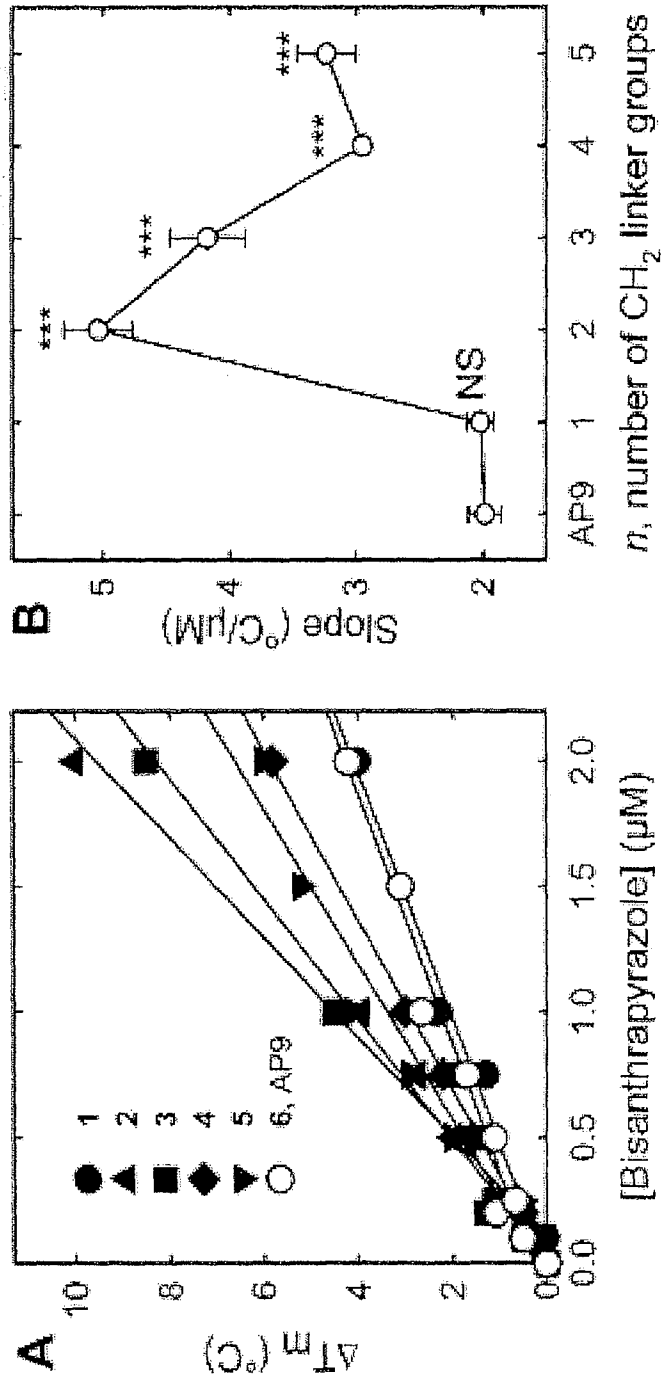

A
ΔTm (°C)
10
8
6
4
2
0
0.0
0.5
1.0
1.5
2.0
[Bisanthrapyrazole] (μM)
1
2
3
4
5
6, AP9
B
Slope (°C/μM)
5
4
3
2
***
***
***
NS
AP9
1
2
3
4
5
n, number of CH2 linker groups

FIG. 13

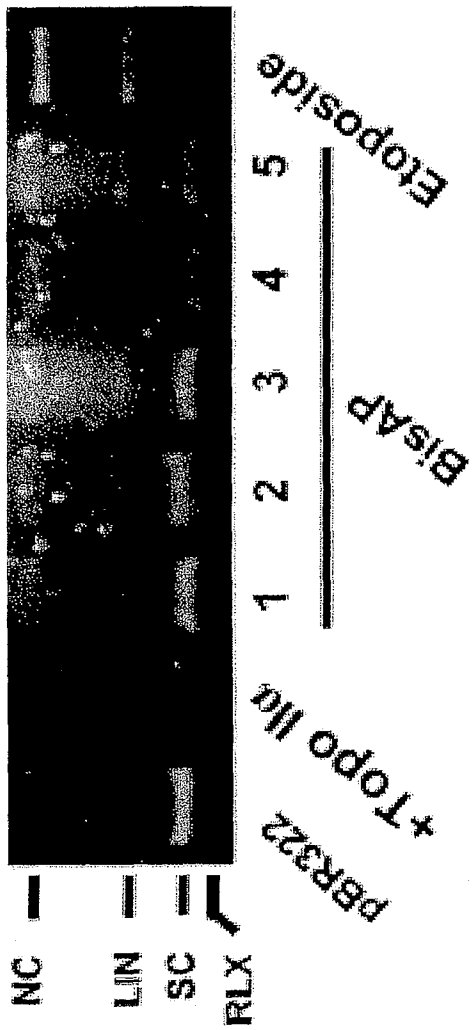

NC
LIN
SC
RLX
pBR322
+Topo III
1 2 3 4 5 6 7 8
BisAP
1 2 3 4 5
Etoposide

FIG. 14