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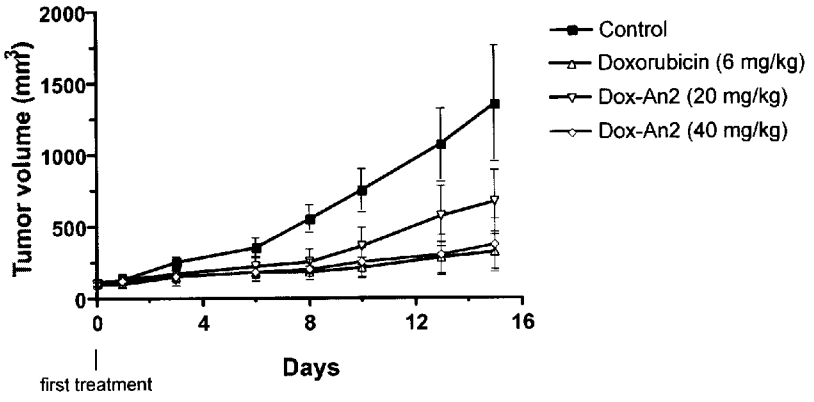
(54) Title: ETOPOSIDE AND DOXORUBICIN CONJUGATES FOR DRUG DELIVERY

Fig. 1



Mice (n=5 per group) were treated once a week for 3 weeks

(57) Abstract: The invention relates to improvements in the field of drug delivery. More particularly, the invention relates to polypeptides having a hydrolyzable covalent bond to a therapeutic agent that includes, etoposide, etoposide 4'- dimethylglycine or doxorubicin. These polypeptide conjugates can be used as vectors to transport the podophyllotoxin derivative across the blood brain barrier (BBB) or into particular cell types such as ovary, liver, lung, or kidney. The invention also relates to pharmaceutical compositions that include the compounds of the invention and to uses thereof in methods of treatment.

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ETOPOSIDE AND DOXORUBICIN CONJUGATES FOR DRUG DELIVERY

Cross-reference to Related Applications

This application claims benefit to U.S. Provisional Application Nos. 61/105,654, filed October 15, 2008, and 61/171,010, filed April 20, 2009, each of which is hereby incorporated by reference.

Field of the Invention

The invention relates to improvements in the field of drug delivery. More particularly, the invention relates to polypeptides having a hydrolyzable covalent bond to a therapeutic agent such as a podophyllotoxin derivative (e.g., etoposide or an etoposide derivative such as etoposide 4'-dimethylglycine) or to doxorubicin or a doxorubicin derivative. These polypeptide conjugates can be used as vectors to transport the therapeutic agent across the blood brain barrier (BBB) or into particular cell types such as ovary, liver, lung, or kidney. These conjugates can show improved physicochemical (e.g., increased solubility) and pharmaceutical properties (e.g., enhanced targeting that allows for subtherapeutic doses or reduced toxicity that allows for supertherapeutic doses) relative to the unconjugated therapeutic agent. The invention also relates to pharmaceutical compositions that include the compounds of the invention and to uses thereof in methods of treatment.

Background of the Invention

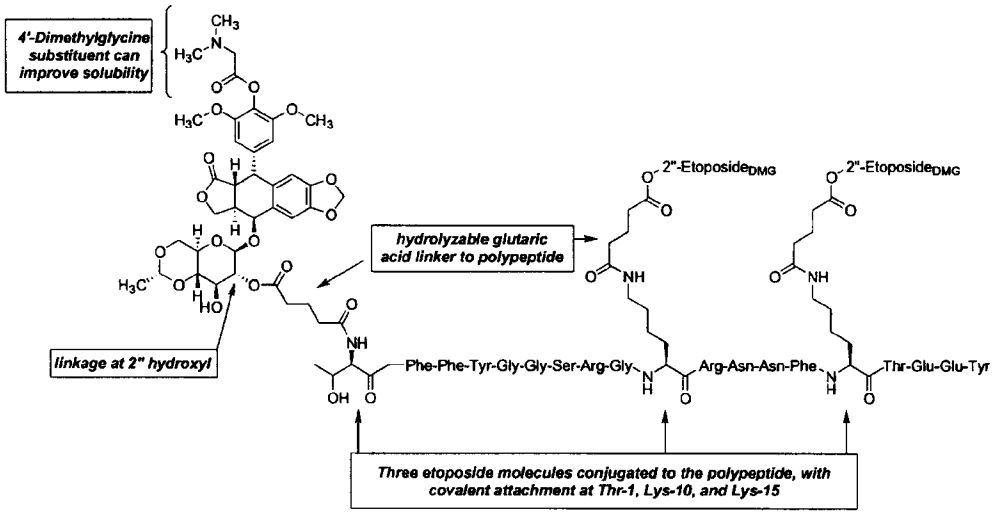
Many therapeutic agents for such diseases have undesirable side effects (e.g., chemotherapeutic agents) or, for reasons such as in vivo stability, transport, or other pharmacokinetic properties, are difficult to provide at a sufficiently high concentration in the target tissue or for a sufficiently long duration to allow maximal therapeutic effect in the target tissue. Accordingly,

there is a need for methods and compositions that increase concentrations of therapeutic and diagnostic agents in target organs or tissues such as the brain, ovary, liver, or lung.

Summary of the Invention

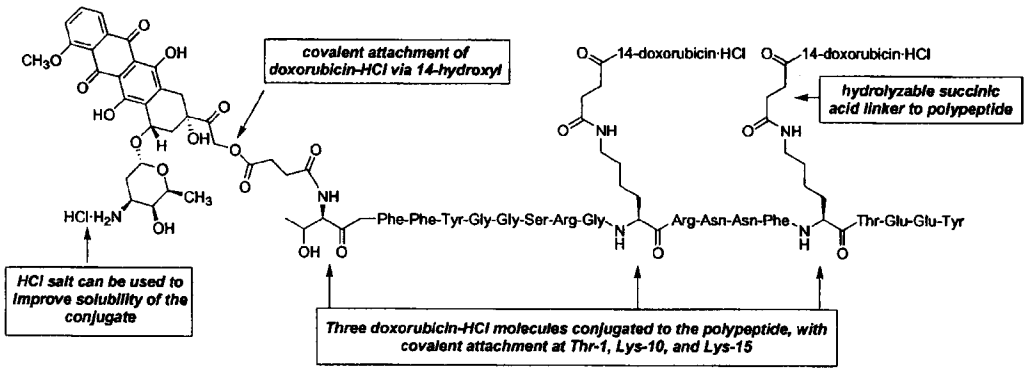
We have developed peptide-therapeutic conjugates, and pharmaceutically acceptable salts thereof, where etoposide is covalently attached to the Angiopep-2 polypeptide (SEQ ID NO:97) at the 2" hydroxyl via a hydrolyzable glutaric acid linker (e.g., Compound (1) shown in **Scheme 1**). A related peptide-therapeutic conjugate has also been prepared in which etoposide 4'-dimethylglycine is used instead of etoposide, and improved properties (e.g., solubility) are observed.

Scheme 1



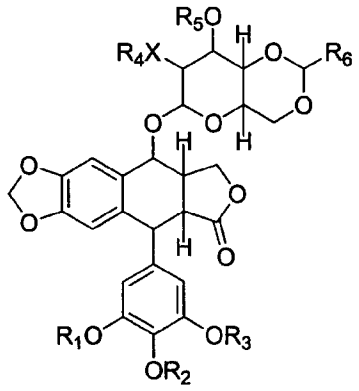
Doxorubicin has also been covalently attached at the 14-hydroxyl to the Angiopep-2 polypeptide using a succinic acid linker (e.g., the trihydrochloride salt of Compound (2) shown in **Scheme 2**). Covalent attachment of the doxorubicin hydrochloride salt can also afford improved properties (e.g., solubility).

Scheme 2



These conjugates can show improved properties relative to the corresponding unconjugated therapeutic agent such as improved physicochemical (e.g., increased solubility) and pharmaceutical (e.g., enhanced targeting that allows for subtherapeutic doses or reduced toxicity that allows for supertherapeutic doses) properties. The solubility of the etoposide$_{DMG}$ and the doxorubicin hydrochloride conjugates can also be useful in adjusting dosing regimens. The invention therefore features these compounds, as well as related compounds. Methods of making and using these compounds are also provided.

Accordingly, in one aspect, the invention features a compound, or a pharmaceutically acceptable salt thereof, that includes an amino acid sequence substantially identical to an amino acid sequence selected from the group consisting of SEQ ID NOS:1-105 and 107-116, or a functional derivative thereof, where the amino acid sequence includes a covalent bond from an amino acid of the amino acid sequence to a podophyllotoxin derivative. In some embodiments, the podophyllotoxin derivative is a compound having a structure according to Formula (I):



(I),

or a stereoisomer or pharmaceutically acceptable salt thereof, where

each R_1, R_2, and R_3 is selected, independently, from H, optionally substituted C_{1-6} alkyl, $C(O)R_8$, $P(O)(OR_9)(OR_{10})$, $S(O)_2(OR_9)$, or a hydrolyzable linker Y that comprises a covalent bond to an amino acid of the polypeptide;

X is O or NR_7;

each R_4, R_5, and R_7 is selected, independently, from H, optionally substituted C_{1-6} alkyl, $C(O)R_8$, or a hydrolyzable linker Y that comprises a covalent bond to an amino acid of the polypeptide;

R_6 is H, optionally substituted C_{1-6} alkyl, optionally substituted aryl, optionally substituted heteroaryl,

R_8 is selected from optionally substituted C_{1-6} alkyl or optionally substituted aryl;

each R_9 and R_{10} is selected, independently, from H, optionally substituted C_{1-6} alkyl, or optionally substituted aryl; and

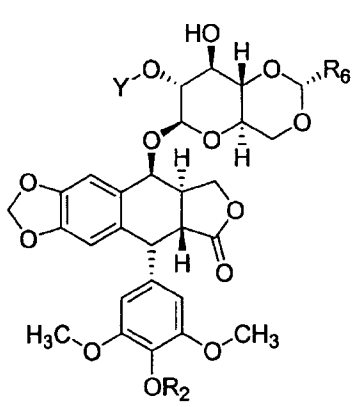
n is 1, 2, 3, 4, 5, 6, 7, or 8;

where one of R_1, R_2, R_3, R_4, R_5, and R_7 is Y and no more than one of R_1, R_2, R_3, R_4, R_5, and R_7 is Y.

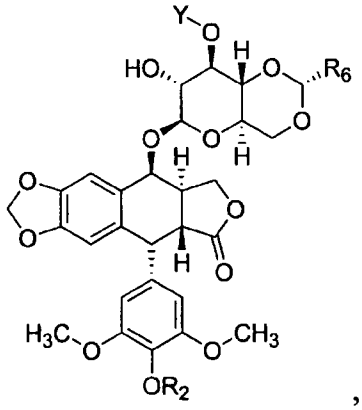
In some embodiments, Y is $-C(O)(CH_2)_nC(O)-$ and n is 2, 3, or 4. In certain embodiments, n is 3.

In some embodiments, the pharmaceutically acceptable salt of the compound is the mono-, di-, or tri-acid addition salt (e.g., the mono-, di-, or trihydrochloride salt).

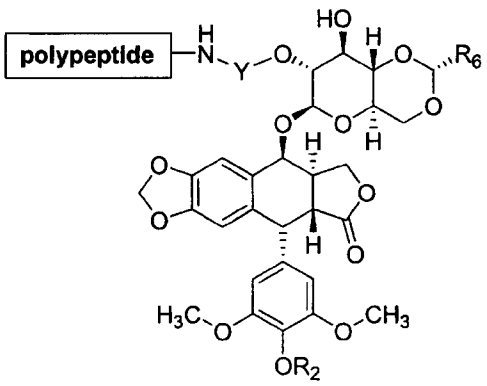
In some embodiments, each compound of Formula (I) is selected, independently, from:



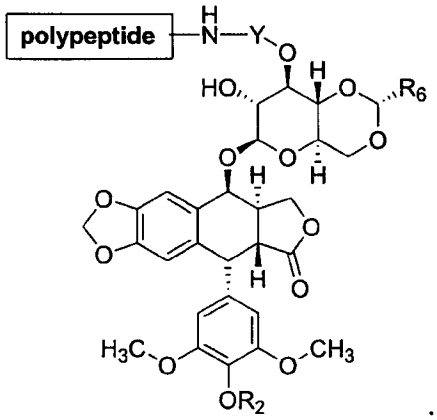
and



, where each R_2 is, independently, H, $P(O)(OH)_2$, or $C(O)CH_2N(CH_3)_2$; each R_6 is, independently, CH_3 or 2-thiophene; each Y is selected from $-C(O)(CH_2)_nC(O)-$; $-[C(O)\{OCH_2CH_2\}_nOC(O)]-$; $-S(O)_2(CH_2)_n\,S(O)_2-$; $-[S(O)_2\{OCH_2CH_2\}_nOS(O)_2]-$; $-[\{P(O)(OR_9)\}(CH_2)_n\{P(O)(OR_9)\}]-$; and $-[\{P(O)(OR_9)\}(OCH_2CH_2)_n\,O\{P(O)(OR_9)\}]-$; each n is, independently, 1, 2, 3, 4, 5, or 6; and where each Y is covalently bound to an amino acid. In some embodiments, each Y is $-C(O)(CH_2)_nC(O)-$ or $-[C(O)\{OCH_2CH_2\}_nOC(O)]-$ and n is 2, 3, or 4. In some embodiments, each R_2 is $C(O)CH_2N(CH_3)_2$. In some embodiments, each compound of Formula (I) is:



or



.

In some embodiments, the compound of the invention has the following structure

T F F Y G G S R G K R N N F K T E E Y
| | |
Formula (I) Formula (I) Formula (I) ,

where each (—(Formula(I)) group represents an optional covalent bond between the indicated amino acid and a compound of Formula (I), and where there is at least one covalent bond between an amino acid of the polypeptide and said compound of Formula (I). In some embodiments, two compounds of Formula (I) are attached to the amino acid sequence. In other embodiments, the threonine at position 1 and the lysines at positions 10 and 15 of the polypeptide each include a covalent bond to a compound having a structure according to Formula (I).

In some embodiments, R_2 is H or $-C(O)CH_2N(CH_3)_2$ (i.e., C-linked N,N-dimethylglycine). In other embodiments, each R_2 is H. In still other embodiments, each R_2 is $-C(O)CH_2N(CH_3)_2$.

In some embodiments, the optionally substituted C_{1-6} alkyl is selected, independently, from methyl, ethyl, n–propyl, isopropyl, n–butyl, iso–butyl, sec–butyl, sec–pentyl, iso–pentyl, tert–butyl, n–pentyl, neopentyl, n–hexyl, or sec–hexyl. In some embodiments, the C_{1-6} alkyl is substituted with at least one optionally substituted amino group (e.g., NH_2 or $N(CH_3)_2$) at any carbon.

In some embodiments, the optionally substituted C_{3-10} cycloalkyl is selected, independently, from cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and cycloheptyl.

In some embodiments, the optionally substituted aryl group is selected, independently, from phenyl, naphthyl, tetrahydronaphthyl, indanyl, or indenyl.

In some embodiments, the optionally substituted heterocyclyl group is selected, independently, from azacyclopropanyl, azacyclobutanyl, 1,3–diazatidinyl, pyrrolidinyl, piperidinyl, piperazinyl, thiranyl, thietanyl, tetrahydrothiophenyl, dithiolanyl, tetrahydrothiopyranyl, oxiranyl, oxetanyl,

tetrahydrofuranyl, tetrahydropyranyl, dioxanyl, oxathiolanyl, morpholinyl, thiomorpholinyl, thioxanyl, and quinuclidinyl.

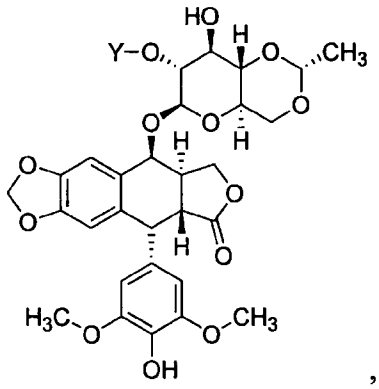
In some embodiments, the optionally substituted heterocyclyl group is selected from pyrrolyl, pyrazolyl, imadazolyl, pyridinyl, pyrimidinyl, pyrazinyl, pyridazinyl, triazinyl, tetrazinyl, pryyrolizinyl, indolyl, quinolinyl, isoquinolynyl, benzimidazolyl, indazolyl, quinolizinyl, cinnolinyl, quinazolinyl, phthalazinyl, napthyridinyl, quinoxalinyl, thiophenyl, thiepinyl, furanyl, benzofuranyl, thiazolyl, isothiazolyl, thiadiazolyl, oxazolyl, isoxazolyl, and oxadiazolyl.

In some embodiments, a substituted alkyl, cycloalkyl, aryl, heterocyclyl, or heteroaryl is substituted with 1, 2, 3, 4, 5, or 6 substituents selected from: C_{1-6} alkyl; halogen; azido($-N_3$), nitro ($-NO_2$), cyano (-CN), acyloxy, acyl (-C(O)R), (-OC(O)R), alkoxy (-OR), amido (-NRC(O)R' or –C(O)NRR'), amino (-NRR'), aryl, carboxylic acid ($-CO_2H$), carboxylic ester ($-CO_2R$), carbamoyl (-OC(O)NRR' or -NRC(O)OR'), cycloalkyl, heterocyclyl, hydroxy (-OH), isocyano (-NC), phosphate (-P(O)(OR)(OR')), sulfonate ($-SO_2OR$), or sulfonyl ($-SO_2R$), where each R or R' is selected, independently, from H, C_{1-6} alkyl, cycloalkyl, heterocyclyl, aryl, or heteroaryl, as defined herein. In some embodiments, these substituents are not further substituted. In other embodiments, the substituents may themselves be further substituted with 1, 2, 3, 4, 5, or 6 substituent groups.

In some embodiments, R_4 is Y. In other embodiments, R_5 is Y.

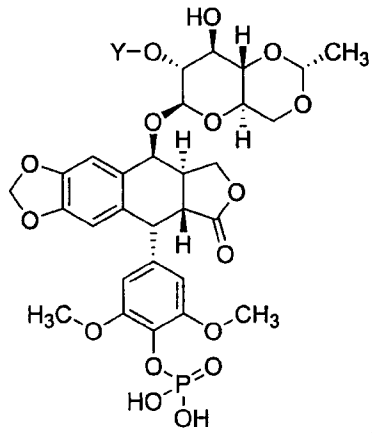
In other embodiments, the amino acid sequence is covalently bonded to additional podophyllotoxin derivatives through a second, third, fourth, or fifth amino acid of said amino acid sequence. In some embodiments, the podophyllotoxin derivative is a compound of Formula (I).

In certain embodiments, the compound of Formula I has the structure:



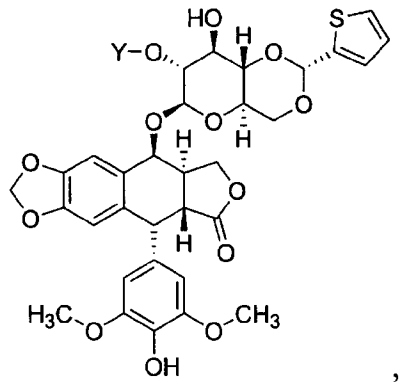
, where Y is–C(O)$(CH_2)_nC(O)$– or –[C(O){OCH_2CH_2}$_n$OC(O)]– and n is 2, 3, or 4.

In other embodiments, the compound of Formula (I) has the following structure:



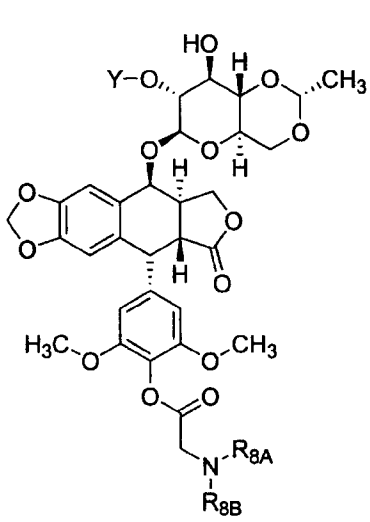
, where Y is–C(O)$(CH_2)_nC(O)$– or –[C(O){OCH_2CH_2}$_n$OC(O)]– and n is 2, 3, or 4.

In still other embodiments, the compound of Formula (I) has the following structure:

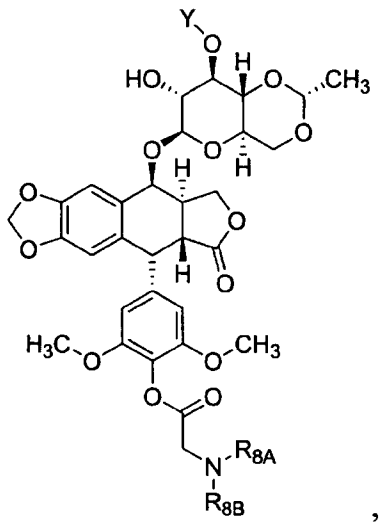


, where Y is–C(O)$(CH_2)_nC(O)$– or –[C(O){OCH_2CH_2}$_n$OC(O)]– and n is 2, 3, or 4.

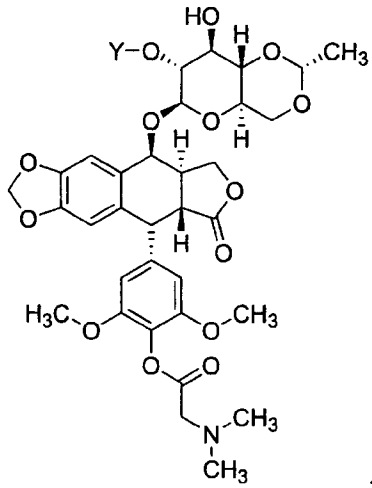
In other embodiments, each compound of Formula (I) is selected, independently, from:



or



, where each R_{8A} and R_{8B} is, independently, H or optionally substituted C_{1-6} alkyl, or R_{8A} and R_{8B} combine to form an optionally substituted 3-7 membered ring. In some embodiments, each R_{8A} and R_{8B} is optionally substituted C_{1-6} alkyl. In other embodiments, each compound of Formula (I) has the following structure:



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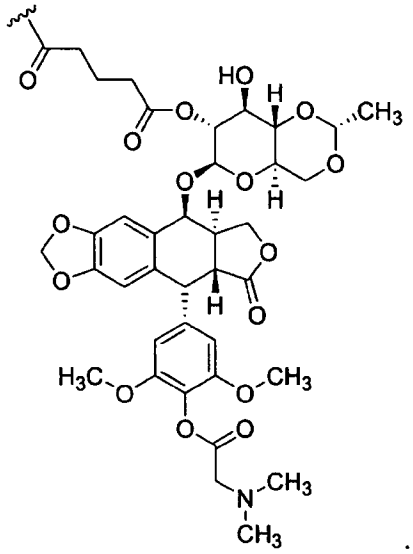
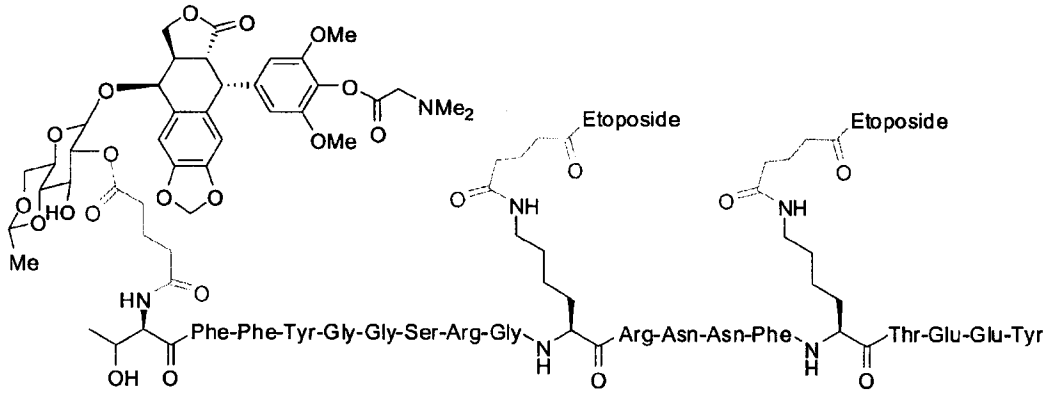
In further embodiments, the compound the following structure:



In particular embodiments, the compound has the structure:

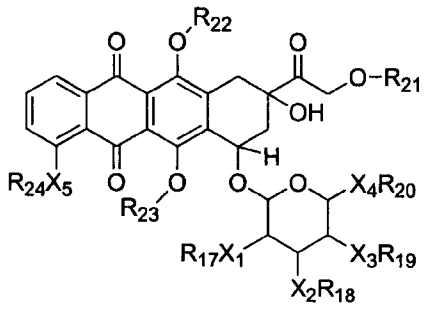


(1), or a pharmaceutically acceptable salt thereof (e.g., the trihydrochloride salt), where in Compound (I), etoposide refers to etoposide 4'-dimethylglycine.

In certain embodiments, each amino acid that is covalently bonded to the hydrolyzable linker Y is attached via an amino-, a guanidino-, a hydroxyl-, a phenol-, or a thiol functional group of said amino acid. In some embodiments, the amino acid that is covalently bonded to the hydrolyzable linker Y is lysine, tyrosine, serine, threonine, or arginine.

In a second aspect, the invention features a compound, or a pharmaceutically acceptable salt thereof, that includes an amino acid sequence substantially identical to an amino acid sequence selected from the group

consisting of SEQ ID NOS:1-105 and 107-116, or a functional derivative thereof, wherein said amino acid sequence includes a covalent bond from an amino acid of said amino acid sequence to a doxorubicin derivative, and wherein said doxorubicin derivative is a compound having a structure according to Formula (II):



(II), or a stereoisomer or a pharmaceutically acceptable salt thereof, wherein

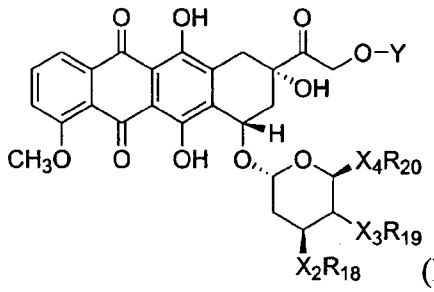
each X_1, X_2, X_3, X_4, and X_5 is selected, independently, from a covalent bond, O, or NR_{25};

each R_{17}, R_{18}, R_{19}, R_{20}, R_{20}, R_{21}, R_{22}, R_{23}, R_{24}, and R_{25} is selected, independently, from H, optionally substituted C_{1-6} alkyl, optionally substituted C_{2-6} alkenyl, optionally substituted C_{2-6} alkynyl, optionally substituted cycloalkyl, optionally substituted heterocyclyl, or is a hydrolyzable linker Y; and

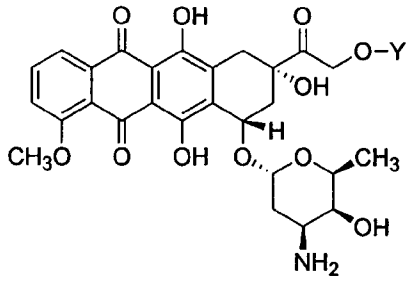
wherein one and only one of R_{17}, R_{18}, R_{19}, R_{20}, R_{20}, R_{21}, R_{22}, R_{23}, R_{24}, and R_{25} is Y.

In some embodiments, the pharmaceutically acceptable salt of the compound is the mono-, di-, or tri-acid addition salt (e.g., the mono-, di-, or trihydrochloride salt).

In certain embodiments, the compound of Formula (II) has the following structure:

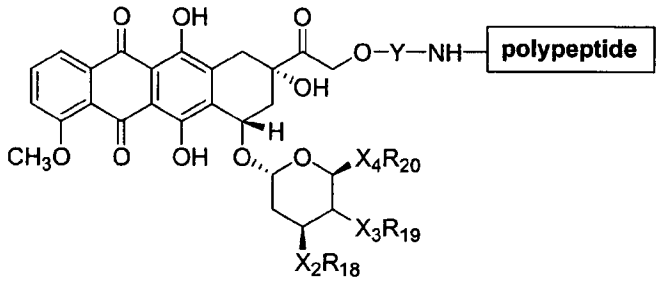


(II-A), where X_2R_{18} is H or NH_2; X_3R_{19} is H or OH; X_4R_{20} is H or optionally substituted C_{1-3} alkyl; and Y is a hydrolyzable linker as described herein. In further embodiments, the compound of Formula (II) has the following structure:

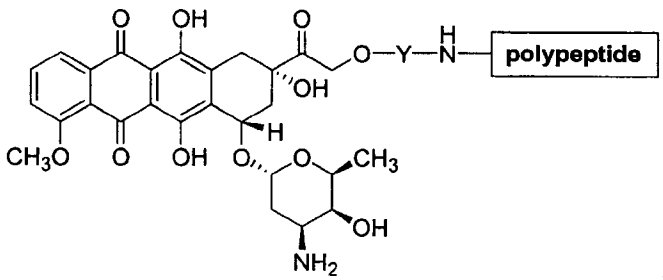


(II-A), or a pharmaceutically acceptable salt thereof.

In further embodiments, the compound of Formula (II) has the following structure:



or



.

In certain embodiments, the compound has the following structure:

T F F Y G G S R G K R N N F K T E E Y

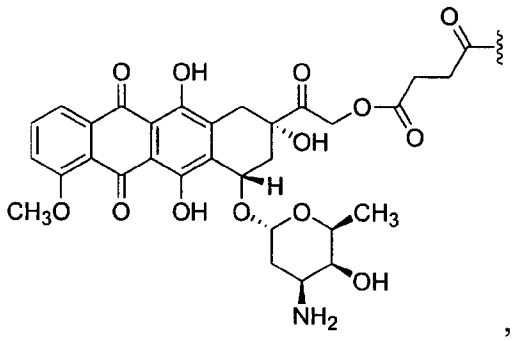
| | |

Formula (II) Formula (II) Formula (II)

wherein each (—(Formula(II)) represents an optional covalent bond between the indicated amino acid and a compound of Formula (II), and wherein there is at least one covalent bond between an amino acid of the polypeptide and said compound of Formula (II). In some embodiments, the threonine at position 1 and the lysines at positions 10 and 15 of the polypeptide each comprise a covalent bond to a compound having a structure according to Formula (II).

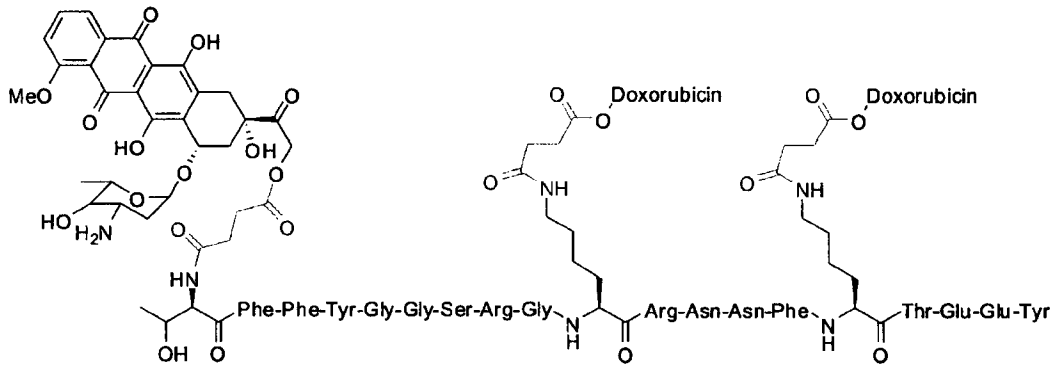
In some embodiments, Y is $-C(O)(CH_2)_nC(O)-$, and n is 2, 3, or 4. In certain embodiments, n is 2. In other embodiments, the amino acid sequence is covalently bonded to a compound having a structure according to Formula (II) through a second, third, fourth, or fifth amino acid of the amino acid sequence. In another embodiment, each amino acid covalently bonded to said hydrolyzable linker Y is attached via an amino-, a guanidino-, a hydroxyl-, a phenol-, or a thiol functional group of said amino acid. In certain embodiments, the amino acid is lysine or threonine.

In some embodiments, the compound of Formula (II) has the following structure:



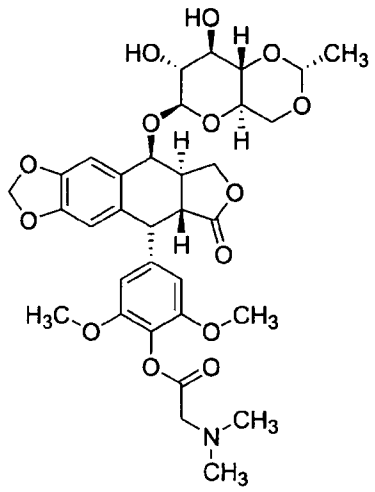
, or a pharmaceutically acceptable salt thereof.

In particular embodiments, the compound has the structure:



(2), or a pharmaceutically acceptable salt thereof (e.g., the trihydrochloride salt).

In another aspect, the invention features the following compound,



("etoposide 4'-dimethylglycine" or "$etoposide_{DMG}$"), or any stereoisomer, or any pharmaceutically acceptable salt or solvent thereof.

In any of the above aspects, the amino acid sequence may be substantially identical to any of the sequences set forth in Table 1, or a fragment thereof or a pharmaceutically acceptable salt thereof. In certain embodiments, the amino acid sequence has a sequence of Angiopep-1 (SEQ ID NO:67), Angiopep-2 (SEQ ID NO:97), Angiopep-3 (SEQ ID NO:107), Angiopep-4a

(SEQ ID NO:108), Angiopep-4b (SEQ ID NO:109), Angiopep-5 (SEQ ID NO:110), Angiopep-6 (SEQ ID NO:111), or Angiopep-7 (SEQ ID NO:112)). The amino acid sequence or the compounds of the invention may be efficiently transported into a particular cell type (e.g., any one, two, three, four, or five of liver, ovary, lung, kidney, spleen, and muscle) or may cross the mammalian BBB efficiently (e.g., Angiopep-1, -2, -3, -4a, -4b, -5, and -6). In some embodiments, the cells are ovary cells. In another embodiment, the conjugate is able to enter a particular cell type (e.g., any one, two, three, four, or five of liver, ovary, lung, kidney, spleen, and muscle) but does not cross the BBB efficiently (e.g., a conjugate including Angiopep-7). In some embodiments, the cells are ovary cells. The polypeptide may be of any length, for example, at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 25, 35, 50, 75, 100, 200, or 500 amino acids. In certain embodiments, the polypeptide is 10 to 50 amino acids in length. The conjugate may be substantially pure. The polypeptide may be produced by recombinant genetic technology or chemical synthesis. The conjugate can be formulated with a pharmaceutically acceptable carrier.

Table 1: Exemplary Polypeptides

SEQ ID NO:																			
1	T	F	V	Y	G	G	C	R	A	K	R	N	N	F	K	S	A	E	D
2	T	F	Q	Y	G	G	C	M	G	N	G	N	N	F	V	T	E	K	E
3	P	F	F	Y	G	G	C	G	G	N	R	N	N	F	D	T	E	E	Y
4	S	F	Y	Y	G	G	C	L	G	N	K	N	N	Y	L	R	E	E	E
5	T	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	K	Y
6	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	R	A	K	Y
7	T	F	F	Y	G	G	C	R	A	K	K	N	N	Y	K	R	A	K	Y
8	T	F	F	Y	G	G	C	R	G	K	K	N	N	F	K	R	A	K	Y
9	T	F	Q	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	K	Y
10	T	F	Q	Y	G	G	C	R	G	K	K	N	N	F	K	R	A	K	Y
11	T	F	F	Y	G	G	C	L	G	K	R	N	N	F	K	R	A	K	Y
12	T	F	F	Y	G	G	S	L	G	K	R	N	N	F	K	R	A	K	Y

13	P	F	F	Y	G	G	C	G	G	K	K	N	N	F	K	R	A	K	Y
14	T	F	F	Y	G	G	C	R	G	K	G	N	N	Y	K	R	A	K	Y
15	P	F	F	Y	G	G	C	R	G	K	R	N	N	F	L	R	A	K	Y
16	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	R	E	K	Y
17	P	F	F	Y	G	G	C	R	A	K	K	N	N	F	K	R	A	K	E
18	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	R	A	K	D
19	T	F	F	Y	G	G	C	R	A	K	R	N	N	F	D	R	A	K	Y
20	T	F	F	Y	G	G	C	R	G	K	K	N	N	F	K	R	A	E	Y
21	P	F	F	Y	G	G	C	G	A	N	R	N	N	F	K	R	A	K	Y
22	T	F	F	Y	G	G	C	G	G	K	K	N	N	F	K	T	A	K	Y
23	T	F	F	Y	G	G	C	R	G	N	R	N	N	F	L	R	A	K	Y
24	T	F	F	Y	G	G	C	R	G	N	R	N	N	F	K	T	A	K	Y
25	T	F	F	Y	G	G	S	R	G	N	R	N	N	F	K	T	A	K	Y
26	T	F	F	Y	G	G	C	L	G	N	G	N	N	F	K	R	A	K	Y
27	T	F	F	Y	G	G	C	L	G	N	R	N	N	F	L	R	A	K	Y
28	T	F	F	Y	G	G	C	L	G	N	R	N	N	F	K	T	A	K	Y
29	T	F	F	Y	G	G	C	R	G	N	G	N	N	F	K	S	A	K	Y
30	T	F	F	Y	G	G	C	R	G	K	K	N	N	F	D	R	E	K	Y
31	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	L	R	E	K	E
32	T	F	F	Y	G	G	C	R	G	K	G	N	N	F	D	R	A	K	Y
33	T	F	F	Y	G	G	S	R	G	K	G	N	N	F	D	R	A	K	Y
34	T	F	F	Y	G	G	C	R	G	N	G	N	N	F	V	T	A	K	Y
35	P	F	F	Y	G	G	C	G	G	K	G	N	N	Y	V	T	A	K	Y
36	T	F	F	Y	G	G	C	L	G	K	G	N	N	F	L	T	A	K	Y
37	S	F	F	Y	G	G	C	L	G	N	K	N	N	F	L	T	A	K	Y
38	T	F	F	Y	G	G	C	G	G	N	K	N	N	F	V	R	E	K	Y
39	T	F	F	Y	G	G	C	M	G	N	K	N	N	F	V	R	E	K	Y
40	T	F	F	Y	G	G	S	M	G	N	K	N	N	F	V	R	E	K	Y
41	P	F	F	Y	G	G	C	L	G	N	R	N	N	Y	V	R	E	K	Y
42	T	F	F	Y	G	G	C	L	G	N	R	N	N	F	V	R	E	K	Y
43	T	F	F	Y	G	G	C	L	G	N	K	N	N	Y	V	R	E	K	Y
44	T	F	F	Y	G	G	C	G	G	N	G	N	N	F	L	T	A	K	Y
45	T	F	F	Y	G	G	C	R	G	N	R	N	N	F	L	T	A	E	Y
46	T	F	F	Y	G	G	C	R	G	N	G	N	N	F	K	S	A	E	Y
47	P	F	F	Y	G	G	C	L	G	N	K	N	N	F	K	T	A	E	Y
48	T	F	F	Y	G	G	C	R	G	N	R	N	N	F	K	T	E	E	Y

49	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	E	E	D	
50	P	F	F	Y	G	G	C	G	G	N	G	N	N	F	V	R	E	K	Y	
51	S	F	F	Y	G	G	C	M	G	N	G	N	N	F	V	R	E	K	Y	
52	P	F	F	Y	G	G	C	G	G	N	G	N	N	F	L	R	E	K	Y	
53	T	F	F	Y	G	G	C	L	G	N	G	N	N	F	V	R	E	K	Y	
54	S	F	F	Y	G	G	C	L	G	N	G	N	N	Y	L	R	E	K	Y	
55	T	F	F	Y	G	G	S	L	G	N	G	N	N	F	V	R	E	K	Y	
56	T	F	F	Y	G	G	C	R	G	N	G	N	N	F	V	T	A	E	Y	
57	T	F	F	Y	G	G	C	L	G	K	G	N	N	F	V	S	A	E	Y	
58	T	F	F	Y	G	G	C	L	G	N	R	N	N	F	D	R	A	E	Y	
59	T	F	F	Y	G	G	C	L	G	N	R	N	N	F	L	R	E	E	Y	
60	T	F	F	Y	G	G	C	L	G	N	K	N	N	Y	L	R	E	E	Y	
61	P	F	F	Y	G	G	C	G	G	N	R	N	N	Y	L	R	E	E	Y	
62	P	F	F	Y	G	G	S	G	G	N	R	N	N	Y	L	R	E	E	Y	
63	M	R	P	D	F	C	L	E	P	P	Y	T	G	P	C	V	A	R	I	
64	A	R	I	I	R	Y	F	Y	N	A	K	A	G	L	C	Q	T	F	V	Y	G
65	Y	G	G	C	R	A	K	R	N	N	Y	K	S	A	E	D	C	M	R	T	C	G
66	P	D	F	C	L	E	P	P	Y	T	G	P	C	V	A	R	I	I	R	Y	F	Y
67	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	E	E	Y	
68	K	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	E	E	Y	
69	T	F	Y	Y	G	G	C	R	G	K	R	N	N	Y	K	T	E	E	Y	
70	T	F	F	Y	G	G	S	R	G	K	R	N	N	F	K	T	E	E	Y	
71	C	T	F	F	Y	G	C	C	R	G	K	R	N	N	F	K	T	E	E	Y
72	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	E	E	Y	C
73	C	T	F	F	Y	G	S	C	R	G	K	R	N	N	F	K	T	E	E	Y
74	T	F	F	Y	G	G	S	R	G	K	R	N	N	F	K	T	E	E	Y	C
75	P	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	E	E	Y	
76	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	K	E	Y	
77	T	F	F	Y	G	G	K	R	G	K	R	N	N	F	K	T	E	E	Y	
78	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	K	R	Y	
79	T	F	F	Y	G	G	K	R	G	K	R	N	N	F	K	T	A	E	Y	
80	T	F	F	Y	G	G	K	R	G	K	R	N	N	F	K	T	A	G	Y	
81	T	F	F	Y	G	G	K	R	G	K	R	N	N	F	K	R	E	K	Y	
82	T	F	F	Y	G	G	K	R	G	K	R	N	N	F	K	R	A	K	Y	
83	T	F	F	Y	G	G	C	L	G	N	R	N	N	F	K	T	E	E	Y	
84	T	F	F	Y	G	C	G	R	G	K	R	N	N	F	K	T	E	E	Y	

85 T F F Y G G R C G K R N N F K T E E Y

86 T F F Y G G C L G N G N N F D T E E E

87 T F Q Y G G C R G K R N N F K T E E Y

88 Y N K E F G T F N T K G C E R G Y R F

89 R F K Y G G C L G N M N N F E T L E E

90 R F K Y G G C L G N K N N F L R L K Y

91 R F K Y G G C L G N K N N Y L R L K Y

92 K T K R K R K K Q R V K I A Y E E I F K N Y

93 K T K R K R K K Q R V K I A Y

94 R G G R L S Y S R R F S T S T G R

95 R R L S Y S R R R F

96 R Q I K I W F Q N R R M K W K K

97 T F F Y G G S R G K R N N F K T E E Y

98 M R P D F C L E P P Y T G P C V A R I

I R Y F Y N A K A G L C Q T F V Y G G

C R A K R N N F K S A E D C M R T C G G A

99 T F F Y G G C R G K R N N F K T K E Y

100 R F K Y G G C L G N K N N Y L R L K Y

101 T F F Y G G C R A K R N N F K R A K Y

102 N A K A G L C Q T F V Y G G C L A K R N N F

E S A E D C M R T C G G A

103 Y G G C R A K R N N F K S A E D C M R T C G

G A

104 G L C Q T F V Y G G C R A K R N N F K S A E

105 L C Q T F V Y G G C E A K R N N F K S A

107 T F F Y G G S R G K R N N F K T E E Y

108 R F F Y G G S R G K R N N F K T E E Y

109 R F F Y G G S R G K R N N F K T E E Y

110 R F F Y G G S R G K R N N F R T E E Y

111 T F F Y G G S R G K R N N F R T E E Y

112 T F F Y G G S R G R R N N F R T E E Y

113 C T F F Y G G S R G K R N N F K T E E Y

114 T F F Y G G S R G K R N N F K T E E Y C

115 C T F F Y G G S R G R R N N F R T E E Y

116 T F F Y G G S R G R R N N F R T E E Y C

Polypeptides Nos. 5, 67, 76, and 91, include the sequences of SEQ ID NOS:5, 67, 76, and 91, respectively, and are amidated at the C-terminus.
Polypeptides Nos. 107, 109, and 110 include the sequences of SEQ ID NOS:97, 109, and 110, respectively, and are acetylated at the N-terminus.

In any of the above aspects, the polypeptide may include an amino acid sequence having the formula:

X1-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-X15-X16-X17-X18-X19

where each of X1-X19 (e.g., X1-X6, X8, X9, X11-X14, and X16-X19) is, independently, any amino acid (e.g., a naturally occurring amino acid such as Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val) or absent and at least one (e.g., 2 or 3) of X1, X10, and X15 is arginine. In some embodiments, X7 is Ser or Cys; or X10 and X15 each are independently Arg or Lys. In some embodiments, the residues from X1 through X19, inclusive, are substantially identical to any of the amino acid sequences of any one of SEQ ID NOS:1-105 and 107-116 (e.g., Angiopep-1, Angiopep-2, Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7). In some embodiments, at least one (e.g., 2, 3, 4, or 5) of the amino acids X1-X19 is Arg. In some embodiments, the polypeptide has one or more additional cysteine residues at the N-terminal of the polypeptide, the C-terminal of the polypeptide, or both.

In certain embodiments of any of the above aspects, the polypeptide is modified (e.g., as described herein). The polypeptide may be amidated, acetylated, or both. Such modifications to polypeptides may be at the amino or carboxy terminus of the polypeptide. The conjugates of the invention may also include peptidomimetics (e.g., those described herein) of any of the polypeptides described herein. The polypeptide may be in a multimeric form, for example, dimeric form (e.g., formed by disulfide bonding through cysteine residues).

In certain embodiments, the polypeptide has an amino acid sequence described herein with at least one amino acid substitution (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 substitutions). The polypeptide may contain, for example, 1 to 12, 1 to 10, 1 to 5, or 1 to 3 amino acid substitutions, for example, 1 to 10 (e.g., to 9, 8, 7, 6, 5, 4, 3, 2) amino acid substitutions. The amino acid substitution(s) may be conservative or non-conservative. For example, the polypeptide may gave an arginine at one, two, or three of the positions corresponding to positions 1, 10, and 15 of the amino acid sequence of any of SEQ ID NO:1, Angiopep-1, Angiopep-2, Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7.

In any of the above aspects, the conjugate may specifically exclude a polypeptide including or consisting of any of SEQ ID NOS:1-105 and 107-116 (e.g., Angiopep-1, Angiopep-2, Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7). In some embodiments, the polypeptides and conjugates of the invention exclude the polypeptides of SEQ ID NOs:102, 103, 104, and 105.

In some embodiments, the amino acid sequence has at least 35%, 40%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity to an amino acid sequence selected from the group consisting of SEQ ID NOS:1-105 and 107-116, or a functional derivative thereof. In certain embodiments, the amino acid sequence has at least 35%, 40%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity to an amino acid sequence selected from the group consisting of Angiopep-2 (SEQ ID NO:97), Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7 (SEQ ID NOS:109-112). In still other embodiments, the amino acid sequence has at least 35%, 40%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity to an amino acid sequence of Angiopep-2 (SEQ ID NO:97).

In some embodiments, the amino acid sequence comprises the amino acid sequence selected from the group consisting of SEQ ID NOS:1-105 and 107-116, or a functional derivative thereof. In certain embodiments, the amino

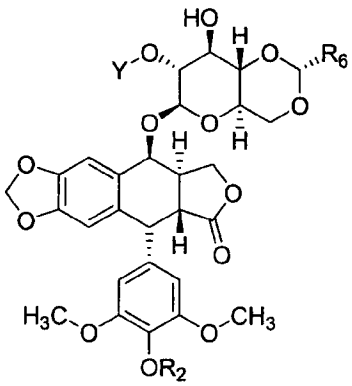
acid sequence is that of Angiopep-2 (SEQ ID NO:97), Angiopep-4b, Angiopep-5, Angiopep-6, or Angiopep-7 (SEQ ID NOS:109-112).

In still other embodiments, the amino acid sequence consists of the amino acid sequence selected from the group consisting of SEQ ID NOS:1-105 and 107-116, or a functional derivative thereof. In certain embodiments, the amino acid sequence is that of Angiopep-2 (SEQ ID NO:97), Angiopep-4b, Angiopep-5, Angiopep-6, or Angiopep-7 (SEQ ID NOS:109-112).

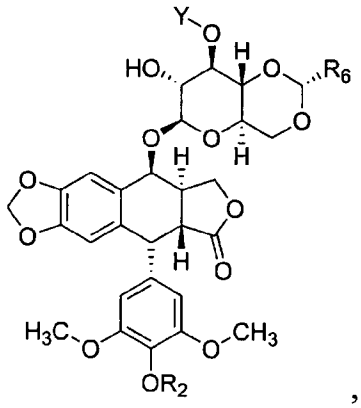
In some embodiments, the compounds of the invention can alter the accumulation of a biologically active agent (e.g., podophyllotoxin derivatives such as the compounds of Formula (I) or doxorubicin derivatives such as the compounds of Formula (II)) in a target cell type or tissue relative to the corresponding unconjugated biologically active agent. In still other embodiments, the compound of the invention promotes accumulation of the biologically active agent in a target cell type or tissue. In certain embodiments, the concentration of the biologically active agent increases by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, 350%, 400%, 450%, 500%, 550%, 600%, 650%, 700%, 750%, 800%, 850%, 900%, 950%, 1,000%, 2,000%, 3,000%, 4,000%, 5,000%, 6,000%, 7,000%, 8,000%, 9,000%, 10,000%, 12500%, 15,000%, 17,500%, or 20,000% relative to that observed with the unconjugated biologically active agent. In some embodiments, the target cell type or tissue is the brain, ovary, liver, lung, kidney, spleen, or muscle. In some embodiments, the target cell type is the brain or the ovary. In certain embodiments, the biologically active agent is selected from etoposide, etoposide phosphate, etoposide$_{DMG}$, teniposide, doxorubicin, or epirubicin. In other embodiments, the compound of the invention includes the amino acid sequence of Angiopep-2 (SEQ ID NO:97), Angiopep-4b, Angiopep-5, Angiopep-6, or Angiopep-7 (SEQ ID NOS:109-112), or a functional derivative thereof.

In a third aspect, the invention features a pharmaceutical composition that includes any compound of the invention as described herein (e.g., a

compound that includes an amino acid sequence substantially identical to an amino acid sequence selected from the group consisting of SEQ ID NOS:1-105 and 107-116, or a functional derivative or pharmaceutically acceptable salt thereof, where the amino acid sequence includes a covalent bond from an amino acid of the amino acid sequence to a compound of Formulas (I) or (II) (e.g., Compound (1) or (2)) and a pharmaceutically acceptable carrier. In a fourth aspect, the invention features a method of treating or treating prophylactically a cancer, where the method includes administering to a patient a therapeutically effective amount of any compound of the invention as described herein (e.g., a compound that includes an amino acid sequence substantially identical to an amino acid sequence selected from the group consisting of SEQ ID NOS:1-105 and 107-116 , or a functional derivative thereof, where the amino acid sequence includes a covalent bond from an amino acid of the amino acid sequence to a compound of Formulas (I) or (II)). In some embodiments ,the compound is Compound (1) or (2). In some embodiments, the podophyllotoxin derivative is selected from



and

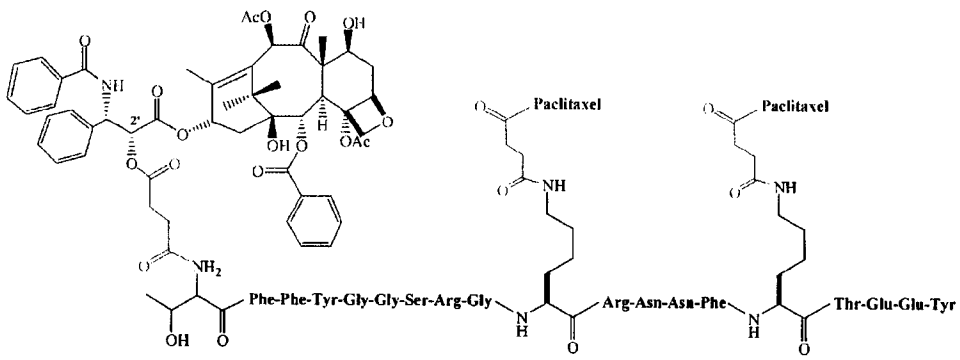


, where

Y is H; each R_2 is, independently, H or $P(O)(OH)_2$, or $-C(O)R_8$; each R_6 is, independently, CH_3 or 2-thiophene; each Y is $-C(O)(CH_2)_nC(O)-$; each R_8 is, independently, optionally substituted C_{1-6} alkyl; and each n is, independently, 2, 3, or 4. In some embodiments, n is 3. In some embodiments, each R_2 is $-C(O)R_8$. In some embodiments, R_8 is a C_{1-6} alkyl that includes an least one optionally substituted amino group (e.g., NH_2 or $N(CH_3)_2$). In certain

embodiments -C(O)R_8 is a C-linked amino acid. In some embodiments, the podophyllotoxin derivative is etoposide, etoposide phosphate, etoposide 4'-dimethylglycine (etoposide$_{DMG}$), or teniposide. In still other embodiments, the compound is doxorubicin or any of the doxorubicin derivatives (e.g., a compound of Formula (II)) described herein.

In some embodiments, the method also includes the administration of a second agent. In still other embodiments, the agent is a therapeutic agent. In certain embodiments, the second therapeutic agent is also covalently bonded to the compound of the invention. In still other embodiments, the second therapeutic agent is not covalently bonded to the compounds of the invention. In some embodiments, the therapeutic agent is drug, a medicine, an agent emitting radiation, a cellular toxin, a biologically active fragment thereof, or a mixture thereof to treat a disease. In other embodiments, the administering is concurrent with another therapeutic regime. In some embodiments, the therapeutic regime is radiation therapy, chemotherapy, stem cell transplantation, bone marrow transplant, surgery, or hyperthermia treatment. In some embodiments, the second therapeutic agent is a polypeptide that includes or that consists of the sequence of Angiopep-2 (SEQ ID NO:97), preferably where the Angiopep-2 is conjugated to an anticancer agent (e.g., paclitaxel), e.g., ANG1005, which has the following structure:



ANG1005:
TxlAn2 (3:1 conjugate)

Still other exemplary second therapeutic agents are described in U.S. Patent No. 7,557,182, herein incorporated by reference.

In some embodiments, the cancer is cancer of the brain. In other embodiments, the cancer of the brain is glioblastoma, a glioma, an acoustic neuroma, an adenoma, an astrocytoma, a choroid plexus papilloma, CNS lymphoma, ependymoma, a gangliocytoma, a ganglioglioma, a medulloblastoma (mdl), an anaplastic (malignant) meningioma, or neurofibromatosis. In still other embodiments, the cancer is acute lymphocytic leukemia, acute myeloblastic leukemia, adrenocortical cancer, intravenous and intravesical bladder cancer, bone sarcoma, breast cancer, carcinoid syndrome (small bowel), endometrial cancer, Ewing's sarcoma, gynecological sarcoma, head and neck cancer (squamous cell), hepatic cancer, Hodgkin's disease, islet cell cancer, leukemia, lung cancer, malignant lymphoma, multiple myeloma, neuroblastoma, non-Hodgkin's lymphoma, pancreatic cancer, prostate cancer, osteogenic sarcoma, ovarian cancer, retinoblastoma, rhabdomyosarcoma, stomach cancer, testicular cancer, thyroid cancer, transitional cell bladder carcinoma, soft tissue sarcoma, or Wilms' tumor.

In any of therapeutic methods described herein, the compound of the invention (e.g., Compound (1) or (2)), or a pharmaceutically acceptable salt thereof, can be administered to a patient as a subtherapeutic dose or a supertherapeutic dose relative to the unconjugated therapeutic agent (e.g., etoposide, etoposide phosphate, etoposide 4-dimethylglycine, or doxorubicin).

In another aspect, the invention features a method of making any of the compounds of the invention described herein, where the method includes the step of covalently binding a podophyllotoxin derivative to any amino acid sequence described herein, or functional derivative thereof, using a difunctional hydrolyzable linking group. In some embodiments, the amino acid sequence is selected from SEQ ID NOS:1-105 and 107-116, or a functional derivative thereof. In other embodiments, the amino acid sequence includes the amino

acid sequence of Angiopep-2 (SEQ ID NO:97), Angiopep-4b, Angiopep-5, Angiopep-6, or Angiopep-7 (SEQ ID NOS:109-112).

In some embodiments, the method of making any of the compounds of the invention includes the steps of

(a) combining said compound of Formula (I) with said difunctional hydrolyzable linking group to form a covalent adduct; and

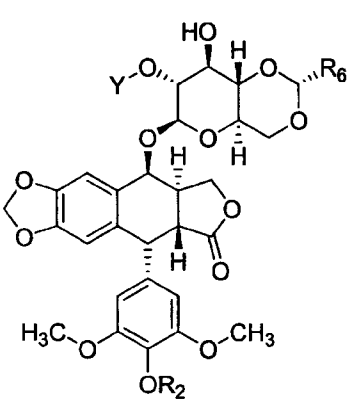
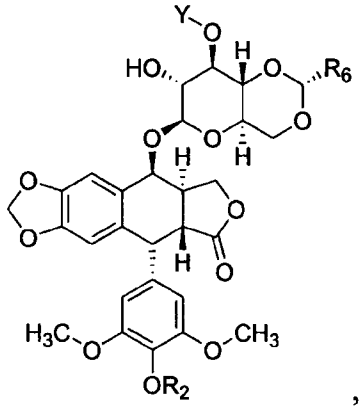
(b) combining the adduct of (a) with said amino acid sequence; and

where the adduct of (a) may be optionally purified prior to use in (b).

In some embodiments, 1.0-10.0 equivalents of the difunctional hydrolyzable linking group is used relative to the compound of Formula (I). For example, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0. 2.1, 2.2., 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, or 3.0 equivalents can be used. In other embodiments, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0. 7.5, 8.0, 8.5, 9.0, 9.5, or 10.0 equivalents the difunctional hydrolyzable linking group is used. In certain embodiments, the method includes the use of a peptide coupling agent. In some embodiments, peptide coupling agent is *N,N,N',N'*-Tetramethyl-O-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU).

In some embodiments, the hydrolyzable difunctional linking group is selected from dicarboxylic acids, dicarbonates, carboxylic anhydrides, diisocyanates, or diphosphonic acids. In certain embodiments, the hydrolyzable difunctional linking group is selected from succinic acid, glutaric acid, glutaric anhydride, or butaric acid.

In some embodiments, the podophyllotoxin derivative is selected from:

and , where

Y is H; each R_2 is, independently, H or $P(O)(OH)_2$, or $-C(O)R_8$; each R_6 is, independently, CH_3 or 2-thiophene; each Y is $-C(O)(CH_2)_nC(O)-$; each R_8 is, independently, optionally substituted C_{1-6} alkyl; and each n is, independently, 2, 3, or 4. In some embodiments, n is 3. In some embodiments, each R_2 is $-C(O)R_8$. In some embodiments, R_8 is a C_{1-6} alkyl that includes an least one optionally substituted amino group (e.g., NH_2 or $N(CH_3)_2$). In certain embodiments $-C(O)R_8$ is a C-linked amino acid. In some embodiments, the podophyllotoxin derivative is etoposide, etoposide phosphate, $etoposide_{DMG}$, or teniposide.

In any of the methods or compositions described herein, the pharmaceutically acceptable salt of the compound can be the mono-, di-, tri-, or tetra acid addition salt (e.g., the trihydrochloride salt). In any of the embodiments described herein, any of the compounds of Formula (I) or (II) (e.g., etoposide, $etoposide_{DMG}$, or doxorubicin) that is covalently bonded to the polypeptide is the site of protonation. For example, in Compound (1), 1, 2, or 3 of the $etoposide_{DMG}$ moieties is protonated, or in Compound (2), 1, 2, or 3 of the doxorubicin moieties, is protonated to form the acid addition salt (e.g., the mono-, di-, or trihydrochloride salt).

The term "C_{1-6} alkyl" or "alkyl" as used herein refers to an optionally substituted C_{1-6} saturated hydrocarbon group. An alkyl group may be linear or branched. Examples of alkyl radicals include, but are not limited to, methyl,

ethyl, n–propyl, isopropyl, n–butyl, iso–butyl, sec–butyl, sec–pentyl, iso–pentyl, tert–butyl, n–pentyl, neopentyl, n–hexyl, sec–hexyl, n–heptyl, n–octyl, n–decyl, n–undecyl, dodecyl, and the like, which may bear one or more substituents. For example, substituted alkyl groups may have 1, 2, 3, 4, 5, or 6 substituents.

The term "aryl" as used herein refers to an optionally substituted mono– or polycyclic, aromatic all–carbon moiety having 5–14 carbon atoms. In certain embodiments of the present invention, "aryl" refers to a substituted or unsubstituted monocyclic or bicyclic group. Exemplary aryl groups include, but are not limited to, phenyl, naphthyl, tetrahydronaphthyl, indanyl, indenyl, and the like, which may bear one or more substituents. Aryls also include heteroaryls.

The term "C-linked amino acid" as used herein refers to an amino acid that is covalently attached to another compound (e.g., any of the podophyllotoxin derivatives described herein) by the C-terminus of the amino acid.

The term "C_{3-10} cycloalkyl" or "cycloalkyl" as used herein refers to an optionally substituted saturated 3– to 10–membered monocyclic or bicyclic hydrocarbon ring system. Exemplary cycloalkyls include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and cycloheptyl. A substituted cycloalkyl can have, for example, 1, 2, 3, 4, 5, 6, or 7 substituents.

The term "heteroaryl" as used herein refers to a substituted or unsubstituted mono– or polycyclic, aromatic moiety having 5–14 ring atoms of which one, two, three, or four ring atoms may be selected from S, O, and N and the remaining ring atoms are carbon. Exemplary heteroaryl groups include, but are not limited to, pyrrolyl, pyrazolyl, imadazolyl, pyridinyl, pyrimidinyl, pyrazinyl, pyridazinyl, triazinyl, tetrazinyl, pryyrolizinyl, indolyl, quinolinyl, isoquinolynyl, benzimidazolyl, indazolyl, quinolizinyl, cinnolinyl, quinazolinyl, phthalazinyl, napthyridinyl, quinoxalinyl, thiophenyl, thiepinyl, furanyl, benzofuranyl, thiazolyl, isothiazolyl, thiadiazolyl, oxazolyl, isoxazolyl, oxadiazolyl, and the like, which may bear one or more substituents.

The term "heterocyclic," or "heterocyclyl," as used herein, refers to an optionally substituted non–aromatic, partially unsaturated or fully saturated, 3– to 10–membered ring system, which includes single rings of 3 to 8 atoms in size, and bi– and tri–cyclic ring systems which may include aromatic five– or six–membered aryl or heteroaryl groups fused to a non–aromatic ring. These heterocyclic rings include those having from one to three heteroatoms independently selected from oxygen, sulfur, and nitrogen, in which the nitrogen and sulfur heteroatoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized or substituted. In certain embodiments, the term heterocyclic refers to a non–aromatic 5–, 6–, or 7–membered monocyclic ring wherein at least one ring atom is a heteroatom selected from O, S, and N (wherein the nitrogen and sulfur heteroatoms may be optionally oxidized), and the remaining ring atoms are carbon, the radical being joined to the rest of the molecule via any of the ring atoms. Exemplary heterocyclics include, but are not limited to, azacyclopropanyl, azacyclobutanyl, 1,3–diazatidinyl, pyrrolidinyl, piperidinyl, piperazinyl, thiranyl, thietanyl, tetrahydrothiophenyl, dithiolanyl, tetrahydrothiopyranyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, dioxanyl, oxathiolanyl, morpholinyl, thiomorpholinyl, thioxanyl, quinuclidinyl, and the like, which may bear one or more substituents.

The term "pharmaceutically acceptable salt," as use herein, represents those acid addition salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and animals without undue toxicity, irritation, allergic response and the like and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well known in the art. For example, pharmaceutically acceptable salts are described in: Berge et al., *J. Pharmaceutical Sciences* 66:1-19, 1977 and in *Pharmaceutical Salts: Properties, Selection, and Use*, (Eds. P.H. Stahl and C.G. Wermuth), Wiley-VCH, 2008. The salts can be prepared in situ during the final isolation and purification of the compounds described herein or separately by reacting a compound having one or basic

groups (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) with the desired equivalents of a suitable organic or inorganic acid. Representative acid addition salts include acetate, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, trifluoroacetate, trifluoromethylsulfonate, undecanoate, and valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine and the like. Desirably, the "pharmaceutically acceptable acid addition salt" is the mono-, bis-, tri-, or tetra acid addition salt of any of the compounds described herein (e.g., a mono-, bis-, tris-, or tetrahydrochloride salt of any of the compounds described herein).

The term "phosphate" as used herein refers to a pentavalent phosphorous group having the formula –OP(=O)(OR')(OR"), where each R' and R" is selected, independently, from hydrogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-10} cycloalkyl, heterocyclyl, aryl, or heteroaryl.

Where a group is described as "optionally substituted," the optional substituents may be selected, independently, from groups that include, but are not limited to: C_{1-6} alkyl; halogen; azido(-N_3), nitro (-NO_2), cyano (-CN), acyloxy, acyl (-C(O)R), (-OC(O)R), alkoxy (-OR), amido (-NRC(O)R' or –C(O)NRR'), amino (-NRR'), aryl, carboxylic acid (-CO_2H), carboxylic ester (-

CO_2R), carbamoyl (-OC(O)NRR' or -NRC(O)OR'), cycloalkyl, heterocyclyl, hydroxy (-OH), isocyano (-NC), phosphate (-P(O)(OR)(OR')), sulfonate ($-SO_2OR$), or sulfonyl ($-SO_2R$), where each R or R' is selected, independently, from H, C_{1-6} alkyl, cycloalkyl, heterocyclyl, aryl, or heteroaryl . A substituted group may have, for example, 1, 2, 3, 4, 5, 6, 7, 8, or 9 substituents. In some embodiments, a substituent group may itself be further substituted by replacing a hydrogen with a substituent group such as those described herein.

By "vector" is meant a compound or molecule such as a polypeptide that is able to be transported into a particular cell type (e.g., liver, ovary, lungs, kidney, spleen, or muscle) or across the BBB. The vector may be attached to (covalently or not) or conjugated to an agent and thereby may be able to transport the agent into a particular cell type or across the BBB. In certain embodiments, the vector may bind to receptors present on cancer cells or brain endothelial cells and thereby be transported into the cancer cell or across the BBB by transcytosis. The vector may be a molecule for which high levels of transendothelial transport may be obtained, without affecting the cell or BBB integrity. The vector may be a polypeptide or a peptidomimetic and may be naturally occurring or produced by chemical synthesis or recombinant genetic technology.

By "conjugate" is meant a vector linked to an agent. The conjugation may be chemical in nature, such as via a linker, or genetic in nature for example by recombinant genetic technology, such as in a fusion protein with for example a reporter molecule (e.g., green fluorescent protein, β-galactosidase, Histag, etc.).

By a vector which is "efficiently transported across the BBB" is meant a vector that is able to cross the BBB at least as efficiently as AngioPep-6 (i.e., greater than 38.5% that of AngioPep-1 (250 nM) in the in situ brain perfusion assay described herein). Accordingly, a vector or conjugate which is "not efficiently transported across the BBB" is transported to the brain at lower levels (e.g., transported less efficiently than AngioPep-6).

By a vector or conjugate which is "efficiently transported to a particular cell type" is meant a vector or conjugate that is able to accumulate (e.g., either due to increased transport into the cell, decreased efflux from the cell, or a combination thereof) in that cell type at least 10% (e.g., 25%, 50%, 100%, 200%, 500%, 1,000%, 5,000%, or 10,000%) greater extent than either a control substance, or, in the case of a conjugate, as compared to the unconjugated agent.

By "substantially pure" or "isolated" is meant a compound (e.g., a polypeptide or conjugate) that has been separated from other chemical components. Typically, the compound is substantially pure when it is at least 30%, by weight, free from other components. In certain embodiments, the preparation is at least 50%, 60%, 75%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% by weight, free from other components. A purified polypeptide may be obtained, for example, by expression of a recombinant polynucleotide encoding such a polypeptide or by chemically synthesizing the polypeptide. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By "analogue" is meant a polypeptide originating from an original sequence or from a portion of an original sequence and which may include one or more modification; for example, one or more modification in the amino acid sequence (e.g., an amino acid addition, deletion, insertion, or substitution), one or more modification in the backbone or side-chain of one or more amino acid, or an addition of a group or another molecule to one or more amino acids (side-chains or backbone). An analogue may have one or more amino acid insertions, at either or at both of the ends of the polypeptide or inside the amino acid sequence of the polypeptide. An analogue may have sequence similarity and/or sequence identity (e.g., may be substantially identical) with that of an original sequence or a portion of an original sequence. Analogues may include a modification of its structure, e.g., as described herein. The degree of similarity between two sequences is base upon the percentage of identities

(identical amino acids) and of conservative substitution. An analogue may have at least 35%, 50 %, 60%, 70%, 80%, 90%, or 95% (e.g., 96%, 97%, 98%, 99%, and 100%) sequence similarity to an original sequence with a combination of one or more modifications in a backbone or side-chain of an amino acid, or an addition of a group or another molecule. Exemplary amino acids which are intended to be similar (a conservative amino acid) to others are known in the art and include, for example, those listed in Table 3.

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 35%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 85%, 90%, 95%, or even 99% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 4 (e.g., at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 50, or 100) amino acids. For nucleic acids, the length of comparison sequences will generally be at least 60 nucleotides, preferably at least 90 nucleotides, and more preferably at least 120 nucleotides, or full length. It is to be understood herein that gaps may be found between the amino acids of an analogs which are identical or similar to amino acids of the original polypeptide. The gaps may include no amino acids, one or more amino acids which are not identical or similar to the original polypeptide. Biologically active analogs of the vectors (polypeptides) of the invention are encompassed herewith. Percent identity may be determined, for example, with n algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

By "functional derivative" is meant a "chemical derivative," "fragment," or "variant" biologically active sequence or portion of a vector or agent or conjugate and a salt thereof of the invention. A vector functional derivative may be able to be attached to or conjugated to an agent and enter a particular cell type, thereby transporting the agent into that cell.

By "chemical derivative" is meant a vector, an agent, or a conjugate of the invention, which contains additional chemical moieties not a part of the

vector, agent or vector-agent conjugate, including covalent modifications. A chemical derivative may be prepared by direct chemical synthesis using methods known in the art. Such modifications may be introduced into a protein or peptide vector, agent, or vector-agent conjugate by reacting targeted amino acid residues with an organic derivatizing agent capable of reacting with selected side chains or terminal residues. A vector chemical derivative may be able to cross the BBB or to enter or accumulate in a particular cell type (e.g., those described herein such as the ovary). In a preferred embodiment, very high levels of transendothelial transport across the BBB are obtained without effecting BBB integrity.

By "fragment" is meant a polypeptide originating from a portion of an original or parent sequence or from an analogue of said parent sequence. Fragments encompass polypeptides having truncations of one or more amino acids, wherein the truncation may originate from the amino terminus (N-terminus), carboxy terminus (C-terminus), or from the interior of the protein. A fragment may include the same sequence as the corresponding portion of the original sequence. Functional fragments of the vector (polypeptide) described herein are encompassed by the invention. Fragments may be at least 5 (e.g., at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 28, 30, 35, 40, 45, 50, 60, 75, 100, or 150) amino acids. Fragments of the invention may include, for example, a polypeptide of 7, 8, 9 or 10 amino acids to 18 amino acids. Fragments may contain any of the modifications described herein (e.g., acetylation, amidation, amino acid substitutions)

A "non-naturally occurring amino acid" is an amino acid which is not naturally produced or found in a mammal.

By "agent" is meant, any compound, for example, an antibody, or a therapeutic agent, a marker, a tracer, or an imaging compound.

By "therapeutic agent" is meant an agent having a biological activity. In some cases, the therapeutic agent is used to treat the symptoms of a disease, a physical or mental condition, an injury, or an infection and includes anti-cancer

agents, antibiotics, anti-angiogenic agents, and molecules active at the level of the central nervous system.

By "small molecule drug" is meant a drug having a molecular weight of 1000 g/mol or less (e.g., less than 800, 600, 500, 400, or 200 g/mol).

By "subject" is meant a human or non-human animal (e.g., a mammal).

By "treating" a disease, disorder, or condition in a subject is meant reducing at least one symptom of the disease, disorder, or condition by administrating a therapeutic agent to the subject.

By "treating prophylactically" a disease, disorder, or condition in a subject is meant reducing the frequency of occurrence of (e.g., preventing) a disease, disorder or condition by administering a therapeutic agent to the subject.

By "cancer" is meant any cellular proliferation whose unique trait is the loss of normal controls which can result in unregulated growth, lack of differentiation, or ability to invade tissues and metastasize. Cancer can develop in any tissue or in any organ. Cancer is intended to include, without limitation, cancer of the brain, ovary, liver, lungs, kidney, or spleen. Additional cancers are described herein.

By "providing" is meant, in the context of a vector or conjugate of the invention, to bring the vector or conjugate into contact with a target cell or tissue either in vivo or in vitro. A vector or conjugate may be provided by administering the vector or conjugate to a subject.

By "administering" and "administration" is meant a mode of delivery including, without limitation, intra-arterially, intra-nasally, intraperitoneally, intravenously, intramuscularly, subcutaneously, transdermally or *per os*. A daily dosage can be divided into one, two or more doses in a suitable form to be administered at one, two or more times throughout a time period.

By "therapeutically effective" or "effective amount" is meant an amount of a therapeutic agent sufficient to improve, decrease, prevent, delay, suppress, or arrest any symptom of the disease or condition being treated. A

therapeutically effective amount of an agent need not cure a disease or condition but will provide a treatment for a disease or condition such that the onset of the disease or condition is delayed, hindered, or prevented, or the disease or condition symptoms are ameliorated, or the term of the disease or condition is changed or, for example, is less severe or recovery is accelerated in an individual. A "subtherapeutic dose" is a dose less than the minimum effective amount of a therapeutic agent that has been approved for clinical use by a patient. A "supertherapeutic dose" is a dose greater than the maximum effective amount of a therapeutic agent that has been approved for clinical use by a patient. The amount of a subtherapeutic dose or a supertherapeutic dose may vary according to the patient demographics (e.g., adult, pediatric, or geriatric) or when used in conjunction with the administration of additional therapeutic agents (e.g., when administered concurrently with other therapeutic agents or treatment regimes such as, for example, in cancer chemotherapy).

By "condition" is meant any situation causing pain, discomfort, sickness, disease or disability (mental or physical) to or in an individual, including neurological disease, injury, infection, or chronic or acute pain. Neurological diseases include brain tumors, brain metastases, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, and stroke.

By "pharmaceutical composition" is meant a therapeutically effective amount of an agent together with a pharmaceutically acceptable diluents, preservative, solubilizer, emulsifier, or adjuvant, for example, any of those described herein.

By "therapeutic dose" is meant the dosage of a agent such as a drug (without the vector) acceptable for use clinically with respect to its toxicity or efficacy. By conjugation of an agent to a vector of the invention, it may be possible to administer the agent at a dosage either lower or higher dosage than the therapeutic dose.

If a "range" or "group of substances" is mentioned with respect to a particular characteristic (e.g., temperature, concentration, time and the like), the

invention relates to and explicitly incorporates herein each and every specific member and combination of sub-ranges or sub-groups therein. Thus, for example, with respect to a length of from 9 to 18 amino acids, is to be understood as specifically incorporating herein each and every individual length, e.g., a length of 18, 17, 15, 10, 9, and any number there between. Therefore, unless specifically mentioned, every range mentioned herein is to be understood as being inclusive. For example, in the expression from 5 to 19 amino acids long is to be as including 5 and 19. This similarly applies with respect to other parameters such as sequences, length, concentrations, elements, and the like.

The sequences, regions, portions defined herein each include each and every individual sequence, region, and portion described thereby as well as each and every possible sub-sequence, sub-region, and sub-portion whether such sub-sequences, sub-regions, and sub-portions are defined as positively including particular possibilities, as excluding particular possibilities or a combination thereof. For example, an exclusionary definition for a region may read as follows: "provided that said polypeptide is no shorter than 4, 5, 6, 7, 8 or 9 amino acids. A further example of a negative limitation is the following; a sequence including SEQ ID NO:X with the exclusion of a polypeptide of SEQ ID NO:Y; etc. An additional example of a negative limitation is the following; provided that said polypeptide is not (does not include or consist of) SEQ ID NO:Z.

Other features and advantages of the invention will be apparent from the following Detailed Description, the drawings, and the claims.

Brief Description of the Drawings

Fig. 1 shows inhibition of subcutaneous U87 (s.c. U87) xenograft tumor growth using the doxorubicin-An2(3:1) ("Doxorubicin-An2(3:1)") conjugate.

Fig. 2A shows the brain uptake of the 3:1 Etoposide:Angiopep-2 conjugate ("Etop-An2(3:1)") measured by in situ brain perfusion.

Fig. 2B shows the brain uptake of the 3:1 etoposide 4'-dimethylglycine:Angiopep-2 conjugate ("$Etop_{DMG}$-An2(3:1)") measured by in situ brain perfusion.

Fig. 2C shows the brain uptake of the Doxorubicin-An2(3:1) measured by in situ brain perfusion.

Fig. 3 shows the in situ perfusion of Etop-An2(3:1).

Fig. 4A shows the parenchymal uptake of unconjugated etoposide compared to Etop-An2(3:1).

Fig. 4B shows the brain repartition of Etop-An2(3:1) following brain capillary depletion.

Fig. 5 shows the in situ brain perfusion of Etop-An2(3:1) compared to unconjugated etoposide in CD-1 versus P-gp knock-out mice.

Fig. 6 shows the inhibition of brain uptake of Etop-An2(3:1) by Angiopep-2.

Fig. 7 shows the in situ brain perfusion of $Etop_{DMG}$-An2 compared to unconjugated $Etop_{DMG}$ in CD-1 versus P-gp knock-out mice.

Fig. 8 shows data on the data on plasma kinetics of Etop-An2(3:1).

Fig. 9 shows the brain distribution of $Etop_{DMG}$-An2 following IV bolus administration in mice.

Fig. 10 shows the brain distribution of Etop-An2(3:1).

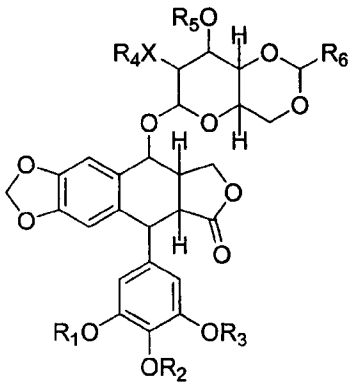
Fig. 11 shows the brain distribution of Etop-An2(3:1) compared to unconjugated etoposide thirty minutes after IV bolus administration.

Fig. 12 shows the tissue distribution of Etop-An2(3:1) compared to unconjugated etoposide thirty minutes after IV bolus administration.

Figs. 13A and **Fig. 13B** each show the *in vivo* effect of $(DoxSu)_3$-An2 in mice that have been intracranially injected with U87 glioblastoma cells. **Fig. 13A** shows the results obtained in the first trial (Trial 1). **Fig. 13B** shows the results obtained in the second trial (Trial 2) showing that the administration of Compound (2) results in a statistically significant extension of mean survival time.

Detailed Description of the Invention

The invention features compounds, or any pharmaceutically acceptable salt thereof, that include an amino acid sequence substantially identical to an amino acid sequence selected from the amino acid sequences described herein (e.g., SEQ ID NOS:1-105 and 107-116), or a functional derivative thereof, where said amino acid sequence includes a covalent bond from an amino acid of the amino acid sequence to an anti-cancer agent (e.g., podophyllotoxin derivatives, doxorubicin, or doxorubicin derivatives). Exemplary podophyllotoxin derivatives include, for example, a compound having a structure according to Formula (I):



(I), or a stereoisomer or pharmaceutically acceptable salt thereof, where

each R_1, R_2, and R_3 is selected, independently, from H, optionally substituted C_{1-6} alkyl, $C(O)R_8$ (e.g., $C(O)CH_2N(CH_3)_2$), $P(O)(OR_9)(OR_{10})$, $S(O)_2(OR_9)$, or a hydrolyzable linker Y that includes a covalent bond to an amino acid of the polypeptide;

X is O or NR_7;

each R_4, R_5, and R_7 is selected, independently, from H, optionally substituted C_{1-6} alkyl, $C(O)R_8$, or a hydrolyzable linker Y that includes a covalent bond to an amino acid of the polypeptide;

R_6 is H, optionally substituted C_{1-6} alkyl, optionally substituted aryl, optionally substituted heteroaryl,

R_8 is selected from optionally substituted C_{1-6} alkyl (e.g., $CH_2N(CH_3)_2$) or optionally substituted aryl;

each R_9 and R_{10} is selected, independently, from H, optionally substituted C_{1-6} alkyl, or optionally substituted aryl; and

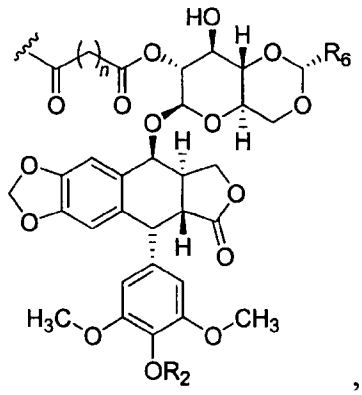
n is 2, 3, or 4; and

where one of R_1, R_2, R_3, R_4, R_5, and R_7 is Y. In some embodiments, no more than one of R_1, R_2, R_3, R_4, R_5, and R_7 is Y. In some embodiments, Y is $-C(O)(CH_2)_nC(O)-$. In some embodiments, each R_2 is H or $C(O)CH_2N(CH_3)_2$. In certain embodiments, the polypeptide may have at least 35%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or even 100% identity to a polypeptide described herein. The polypeptide may have one or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15) substitutions relative to one of the sequences described herein. In certain embodiments, the amino acid sequence is covalently bonded to additional podophyllotoxin derivatives (e.g., a compound of Formula (I)) through a second, third, fourth, fifth, or even sixth amino acid of said amino acid sequence and at any position of the amino acid sequence.

Exemplary compounds of the invention include, but are not limited to, those having a polypeptide sequence according to (SEQ ID NO:97). In some embodiments, the compounds have the following structure:

T F F Y G G S R G K R N N F K T E E Y
| | | |
Formula (I) Formula (I) Formula (I)

wherein each (—(Formula(I)) represents a covalent bond between the indicated amino acid and a compound of Formula (I). In certain embodiments, the compounds of Formula (I) have the following structure:



, where n is 1, 2, or 3, R_6 is CH_3 or 2-thienyl, and R_2 is H, $-OP(O)(OH)_2$, or $-C(O)CH_2N(CH_3)_2$, or any pharmaceutically acceptable salts thereof. In some embodiments, n is 3, R_6 is CH_3, and R_2 is H. In other embodiments, n is 3, R_6 is CH_3, and R_2 is $-C(O)CH_2N(CH_3)_2$.

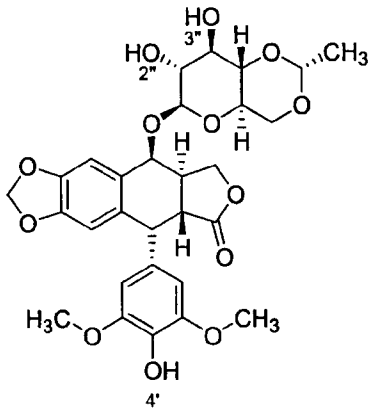
Other embodiments are described in greater detail below.

Podophyllotoxin Derivatives

Podophyllotoxin derivatives include compounds such as those described by Formula (I), e.g., etoposide, teniposide, and derivatives thereof, or a pharmaceutically acceptable salt thereof. Podophyllotoxin derivatives are exemplary therapeutic agents and can be covalently bonded to an amino acid in any of the polypeptides described herein (e.g., Angiopep-2). These compounds can have, for example, antineoplastic activity, inhibit the activity of topoisomerase II, or have antiviral activity.

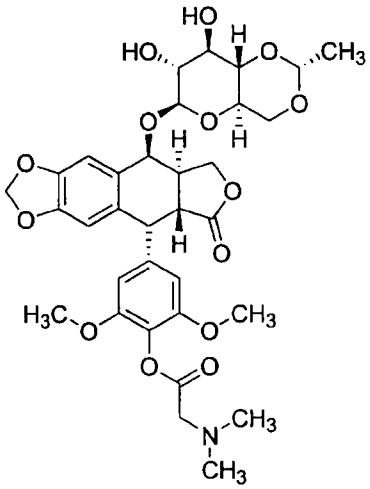
Etoposide and Etoposide Derivatives

Etoposide (also known as Toposar, Vepesid, or VP16) is a podophyllotoxin derivative having the following structure



The chemical structure of etoposide can be varied to afford etoposide derivatives. An exemplary derivative of etoposide is etoposide phosphate (ETOPOPHOS®), where the phenolic -OH is replaced with $-OP(O)(OH)_2$, or any pharmaceutically acceptable salt thereof (e.g., $-OP(O)(ONa)_2$). Etoposide phosphate has improved water solubility compared to etoposide.

Other etoposide derivatives include those where the phenolic -OH is replaced with an acyloxy group (e.g., $-OC(O)R_8$, as described herein) such as the following compound:

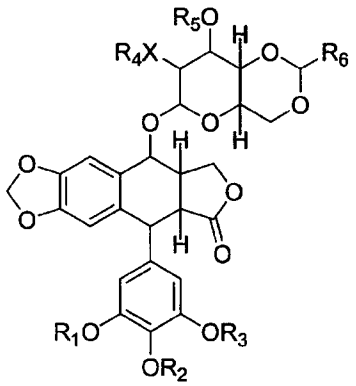


("etoposide 4'-dimethylglycine" or "$etoposide_{DMG}$").

These acylated etoposide derivatives can also show improved water solubility relative to etoposide when covalently attached to any of the polypeptides described herein.

Etoposide, etoposide phosphate, etoposide$_{DMG}$, or derivatives thereof, can be covalently attached to an amino acid in a polypeptide by attaching a hydrolyzable covalent linker Y to, for example, the 2" hydroxyl or the 3" hydroxyl of the molecule. Exemplary linkers may be derived, for example, from dicarboxylic acids such as succinic, glutaric, and butaric acids, or any anhydrides thereof. Additionally, a covalent linker can be attached to etoposide, or derivatives thereof, at the phenol –OH group.

Etoposide derivatives can be described generally by the following formula:



(I-A), or any stereoisomer thereof, wherein

each R_1, R_2, and R_3 is selected, independently, from H, optionally substituted C_{1-6} alkyl, $C(O)R_8$, $P(O)(OR_9)(OR_{10})$, or $S(O)_2(OR_9)$;

X is O or NR_7;

each R_4, R_5, and R_7 is selected, independently, from H, optionally substituted C_{1-6} alkyl, or $C(O)R_8$;

R_6 is H, optionally substituted C_{1-6} alkyl, optionally substituted aryl, optionally substituted heteroaryl,

R_8 is selected from optionally substituted C_{1-6} alkyl or optionally substituted aryl; and

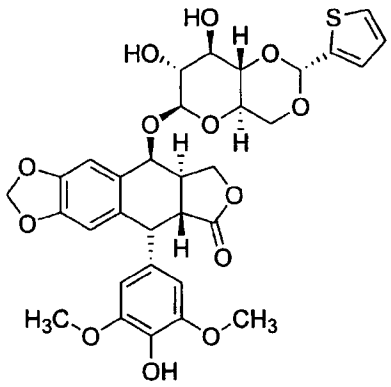
each R_9 and R_{10} is selected, independently, from H, optionally substituted C_{1-6} alkyl, or optionally substituted aryl.

When the compounds of the invention includes an etoposide derivative according to Formula (I), one of R_1-R_6 includes a hydrolyzable linker Y as described herein. In some embodiments, Y is $–C(O)(CH_2)_nC(O)–$ and n is 2, 3,

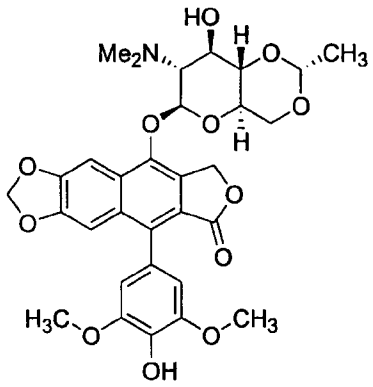
or 4. In exemplary, non-limiting embodiments where R_2 is $C(O)R_8$, R_8 can be C_{1-6} alkyl including an amino substituent and having optional additional substituents. In some embodiments $C(O)R_8$ is a C-linked α-amino acid. The C-linked α-amino acid can be a natural or an unnatural amino acid.

Other exemplary podophyllotoxin derivatives of Formula (I) that can be covalently attached to any of the polypeptides described herein include teniposide and NK611(**Scheme 3**).

Scheme 3



TENIPOSIDE

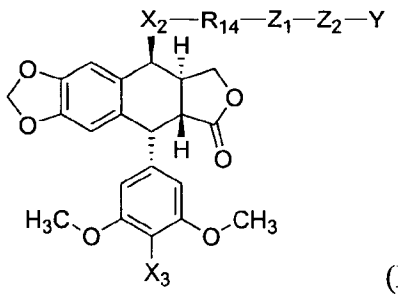


NK 611

Additional Podophyllotoxin Derivatives

Still other podophyllotoxin derivatives suitable for use in the invention are described in U.S. Patent Nos. 4,567,253; 4,609,644; 4,900,814; 4,958,010; 5,489,698; 5,536,847; 5,571,914; 6,051,721; 6,107,284; 6,475,486; 6,610,299; 6,878,746; 6,894,075; 7,087,641; 7,176,236; 7,241,595; 7,342,114; and 7,378,419; and in U.S. Patent Publication Nos. 20030064482, 20030162722, 20040044058, 20060148728, and 20070249651, each of which is hereby incorporated by reference.

For example, the etoposide derivatives described in U.S. Patent No. 7,176,236 can be covalently bonded to an amino acid in any of the polypeptides described herein (e.g., Angiopep-2). Accordingly, in one embodiment, the compounds of the invention include a structure according to Formula (I)



(I-B), wherein

R_2 and Y are as described for Formula (I);

X_2 is –O-, -S-, -NH-, -CO-, -CH=N-, or -CH_2NH-;

X_3 is OR_2 or $N(R_2)_2$;

Z_1 is a covalent bond, -NHCO-, -CONH-, -OCO-, or –COO-;

Z_2 is a covalent $-(CH_2)_oR_{15}$, or $-(CH_2)_o$ is incorporated into Z_2 as a 5-8 membered ring;

R_{14} is a covalent bond or optionally substituted alkyl, alkenyl, or phenyl; and

R_{15} is substituted alkyl, substituted alkenyl, or substituted aryl, wherein the substituted group includes at least one amino group.

In some embodiments, X_3 is -OH, $-OC(O)CH_2NH_2$, $-OC(O)CH_2NHCH_3$,or $-OC(O)CH_2N(CH_3)_2$. In other embodiments, X is -NH-. In some embodiments, $-R_{14}-Z_1-Z_2-$ is $-(p-C_6H_4-R_{16})-$, where R_{16} is $-NO_2$, -F, $-CONHCH_2CH_2C_6H_5$, or $-CONHCH_2CH_2(p-C_6H_4OH)$.

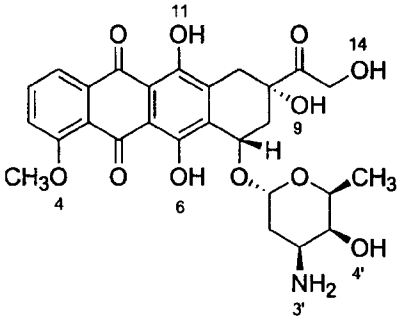
In any compounds of Formulas (I) or (I-A), the group OR_2 may be $-OC(O)R_8$.

In some embodiments, the compound of Formula (I), or a pharmaceutically acceptable salt thereof, that is used can allow for improved physicochemical (e.g., solubility properties). For example, when increased solubility is desired, the compound of Formula (I) is preferably $Etoposide_{DMG}$.

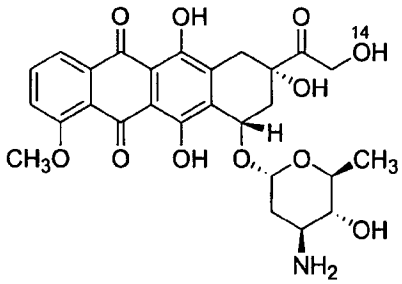
Doxorubicin Derivatives

In some embodiments, the anti-cancer agent is doxorubicin (hydroxydaunorubicin or Adriamycin®) or a doxorubicin derivative such as epirubicin (Ellence® or Pharmorubicin®), or a pharmaceutically acceptable salt thereof. The structures of these exemplary compounds are shown in **Scheme 4**. Doxorubicin and doxorubicin derivatives can be covalently attached to an amino acid in any of the polypeptides described herein through a hydrolyzable covalent linker Y, as defined herein, covalently bonded to, for example, the 14-hydroxyl group.

Scheme 4

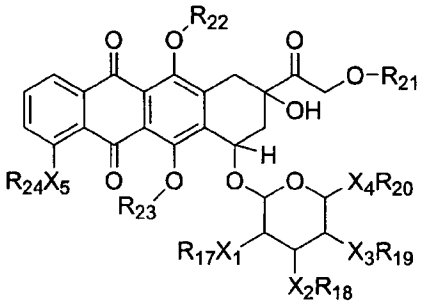


doxorubicin



epirubicin

Doxorubicin derivatives can be described generally by the following Formula (II):

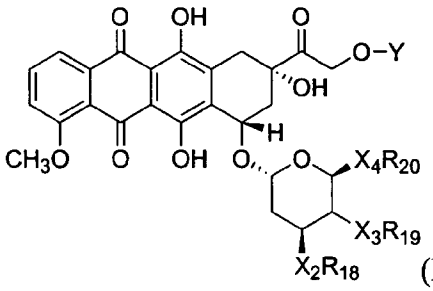


(II), wherein

each X_1, X_2, X_3, X_4, and X_5 is selected, independently, from a covalent bond, O, or NR_{25};

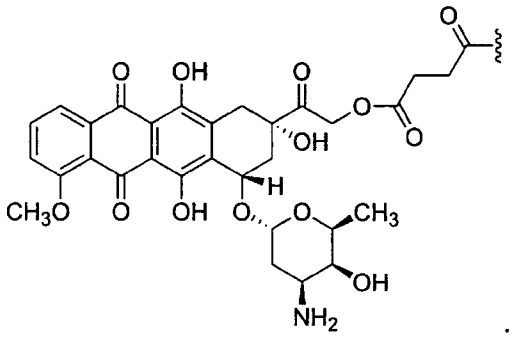
each R_{17}, R_{18}, R_{19}, R_{20}, R_{20}, R_{21}, R_{22}, R_{23}, R_{24}, and R_{25}, is selected, independently, from H, optionally substituted C_{1-6} alkyl, optionally substituted C_{2-6} alkenyl, optionally substituted C_{2-6} alkynyl, optionally substituted cycloalkyl, optionally substituted heterocyclyl, or is a hydrolyzable linker Y as defined herein.

When a compound of Formula (II) is attached to any of the polypeptides described herein, one of R_{17}, R_{18}, R_{19}, R_{20}, R_{20}, R_{21}, R_{22}, R_{23}, R_{24}, and R_{25} is Y. In certain embodiments, R_{21} is Y. Compounds of Formula (II) include compounds having a structure according to Formula (II-A)



(II-A), wherein

Y is a hydrolyzable linker as described herein; X_2R_{18} is H or NH_2; X_3R_{19} is H or OH; and X_4R_{20} is H or optionally substituted C_{1-3} alkyl. In some embodiments, the hydrolyzable linker Y is $-C(O)(CH_2)_nC(O)-$ and n is 2, 3, or 4. In certain embodiments, the compound of Formula (II) is:



Other doxorubicin derivatives can be found in U.S. Patent Nos. 4,098,884, 4,301,277, 4,314,054, 4,464,529, 4,585,859, 4,672,057, 4,684,629, 4,826,964, 5,200,513, 5,304,687, 5,594,158, 5,625,043, and 5,874,412, each of which is hereby incorporated by reference.

In some embodiments, the compound of Formula (II), or a pharmaceutically acceptable salt thereof, that is used can allow for improved physicochemical (e.g., solubility properties). For example, when increased solubility is desired, the compound of Formula (II) is preferably the hydrochloride salt of doxorubicin.

In addition to Angiopep-2, podophyllotoxin derivatives such as etoposide, etoposide phosphate, $etoposide_{DMG}$, teniposide, NK611, and other compounds of Formulas (I) and (I-A), or doxorubicin, epirubicin, and other doxorubicin derivatives (e.g., compounds of Formula (II)) can also be conjugated to any of the polypeptides described herein (e.g., Angiopep-4b,

Angiopep-5, Angiopep-6, or Angiopep-7). Hydrolysable linkers, such as linkers that include ester groups, can be used to covalently bind the anticancer agent (e.g., podophyllotoxin derivatives or doxorubicin derivatives) to a polypeptide (e.g., Example 1 described herein). Etoposide, etoposide phosphate, etoposide$_{DMG}$, other podophyllotoxin derivatives thereof, doxorubicin, epirubicin, and other doxorubicin derivatives have multiple strategic positions (e.g., the 2" and 3" hydroxyls of etoposide, etoposide phosphate, and etoposide$_{DMG}$, and the 14 hydroxyl of doxorubicin and epirubicin). For example, a difunctional group (e.g., a reagent derived from succinic acid, glutaric acid, glutaric anhydride, or butaric acid, or any anhydrides thereof) can be attached to etoposide at the 2" hydroxyl or to doxorubicin at the 14 hydroxyl. These exemplary intermediates can then be activated with a peptide-coupling reagent such as BTTU and treated with a polypeptide. Other peptide coupling agents include carbodiimides (e.g., dicyclohexylcarbodiimide (DCC), diisopropylcarbodiimide (DIC), and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (EDC-HCl)), triazoles (e.g., 1-hydroxy-benzotriazole (HOBt) and 1-hydroxy-7-aza-benzotriazole (HOAt)), related benzotriazole peptide coupling agents such as O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), 2-(1H-9-Azobenzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HATU), Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP reagent), and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), and 3-(Diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one (DEPBT). The conjugate can then be purified. Each intermediate or product of this synthetic procedure is purified and validated using different approaches such as HPLC, thin liquid chromatography, NMR (^{13}C or ^{1}H exchange), melting point, mass spectrometry. The final conjugate is analyzed by mass spectrometry and SDS-polyacrylamide gel electrophoresis. This allows the

determination of the number of molecules (e.g., of etoposide, etoposide phosphate, etoposide$_{DMG}$, doxorubicin, or epirubicin) conjugated to each vector.

Hydrolyzable Linkers

When a compound of Formula (I) is covalently attached by a hydrolyzable linker Y to an amino acid in a polypeptide, the linker can be located at R_1, R_2, R_3, R_4, R_5, or R_7. Similarly, when a compound of Formula (II) is covalently attached by a hydrolyzable linker Y to an amino acid in a polypeptide, the linker can be located at any of R_{17}, R_{18}, R_{19}, R_{20}, R_{20}, R_{21}, R_{22}, R_{23}, R_{24}, and R_{25}. Exemplary, non-limiting hydrolyzable linkers may be prepared from dicarboxylic acids, dicarbonates, carboxylic anhydrides, diisocyanates, or diphosphonic acids. A compound that includes a compound of Formula (I) or (II) that is covalently attached to an amino acid in any of the amino acid sequences described herein may also be described by the following formula

$$D–G–X–G'–A \qquad (III),$$

where each G and G' is a group selected, independently, from -C(O)-, -C(O)O-, -OC(O)-, -S(O)$_2$O-, -OS(O)$_2$-, -S(O)$_2$NH-, -NHS(O)$_2$-, and -OP(O)(OR$_{11}$)O-;

G is covalently bonded to D, where D is a podophyllotoxin derivative (e.g., a compound of Formula (I)) or doxorubicin or a doxorubicin derivative (e.g., a compound of Formula (II);

G' is covalently bonded to A, where A is an amino acid in an amino acid sequence described herein (e.g., the amino acid sequences described in Table 1, or functional derivatives thereof); and

X is -(optionally substituted aryl)-, -(CR$_{12}$R$_{13}$)$_n$-, -O{(CR$_{12}$R$_{13}$)$_2$O}$_n$-, -{(CR$_{12}$R$_{13}$)$_2$O(CR$_{12}$R$_{13}$)$_2$}$_n$-, or -(CR$_{12}$R$_{13}$)$_o$Y(CR$_{12}$R$_{13}$)$_p$-, where each n, o, and p is, independently, an integer between 1-10;

R$_{11}$ is H or lower C$_{1-6}$ alkyl;

R_{12} and R_{13} are each selected, independently, from H, OH, or lower C_{1-6} alkyl; and

Y is O, NH, N(lower C_{1-6} alkyl), or -optionally substituted aryl.

Each n, o, and p may be, independently, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In some embodiments, the G-X-G' moiety in Formula (III) is selected from $-C(O)CH_2C(O)-$, $-C(O)(CH_2)_2C(O)-$, $-C(O)(CH_2)_3C(O)-$, $-C(O)(CH_2)_4C(O)-$, $-C(O)(CH_2)_5C(O)-$, $-C(O)(CH_2)_6C(O)-$, $-C(O)(OCH_2CH_2)OC(O)-$, $-C(O)(OCH_2CH_2)_2OC(O)-$, $-C(O)(OCH_2CH_2)_3OC(O)-$, and $-C(O)(OCH_2CH_2)_4OC(O)-$.

Polypeptides

Exemplary amino acid sequences useful in the compounds of the invention include, but are not limited to, the amino acid sequences described in **Table 1**.

In addition to the amino acid sequences described in Table 1, the invention also features fragments of these amino acid sequences (e.g., a functional fragment). In certain embodiments, the fragments are capable of entering or accumulating in a particular cell type (e.g., ovary, liver, lung, kidney, spleen, or muscle) or capable of crossing the BBB. Truncations of the polypeptide may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more amino acids from either the N-terminus of the polypeptide, the C-terminus of the polypeptide, or a combination thereof. Other fragments include sequences where internal portions of the polypeptide are deleted.

Additional polypeptides of the invention may be identified by using one of the assays or methods described in U.S. Patent Application Publication No. 2006/0189515, which is hereby incorporated by reference, or by any method known in the art. For example, a candidate vector may be produced by conventional polypeptide synthesis and conjugated with, for example, a compound of Formulas (I) or (II), and administered to a laboratory animal. A biologically active vector may be identified, for example, based on its efficacy

to increase survival of an animal injected with tumor cells and treated with the conjugate as compared to a control which has not been treated with a conjugate (e.g., treated with the unconjugated agent).

In another example, a biologically active polypeptide of the invention may be identified based on its location in the parenchyma in an in situ cerebral perfusion assay. In vitro BBB assays, such as the model developed by CELLIAL™ Technologies, may be used to identify such vectors.

Assays to determine accumulation in other tissues may be performed as well and exemplary assays are described herein. Labeled polypeptides of the invention can be administered to an animal, and accumulation in different organs can be measured. For example, a polypeptide conjugated to a detectable label (e.g., a near-IR fluorescence spectroscopy label such as Cy5.5) allows live in vivo visualization. Such a polypeptide can be administered to an animal, and the presence of the polypeptide in an organ can be detected, thus allowing determination of the rate and amount of accumulation of the polypeptide in the desired organ. In other embodiments, the polypeptide of the invention can be labeled with a radioactive isotope (e.g., ^{125}I). The polypeptide is then administered to an animal. After a period of time, the animal is sacrificed, and the animal's organs are extracted. The amount of radioisotope in each organ can then measured using any means known in the art. By comparing the amount of a labeled candidate polypeptide in a particular organ without amount of labeled control, the ability of the candidate polypeptide the rate or amount of accumulation of a candidate polypeptide in a particular tissue can be ascertained. Appropriate negative controls include any polypeptide known not be transported into a particular cell type.

For example, the amine groups of Angiopep-1 (SEQ ID NO:67) and Angiopep-2 (SEQ ID NO:97) can be used as sites for conjugation of agents. To study the role of amine groups in conjugation and their impact in the overall transport capacity of these vectors, other vectors have been developed based on the Angiopep-1 and Angiopep-2 sequence. These vectors variable reactive

amine groups and variable overall charge. These polypeptides are shown in Table 2.

Table 2: Vectors with variable amine group targets

Polypeptide Name	Polypeptide Sequences	Reactive amines (positions)	Charge	SEQ ID No.
Angiopep-3*	Ac[1]-TFFYGGSRGKRNNFKTEEY	2 (10,15)	+1	107
Angiopep-4b	RFFYGGSRGKRNNFKTEEY	3 (1,10,15)	+3	108
Angiopep-4a	Ac[1]-RFFYGGSRGKRNNFKTEEY	2 (10,15)	+2	109
Angiopep-5	Ac[1]-RFFYGGSRGKRNNFRTEEY	1 (10)	+2	110
Angiopep-6	TFFYGGSRGKRNNFRTEEY	2 (1,10)	+2	111
Angiopep-7	TFFYGGSRGRRNNFRTEEY	1 (1)	+2	112

*Angiopep-3 is an acetylated form of Angiopep-2.
[1]Ac represents acetylation.

Modified polypeptides

The invention can also include polypeptides having a modification of an amino acid sequence described herein (e.g., polypeptide having a sequence described in any one of SEQ ID NOS:1-105 and 107-116 such as AngioPep-3, -4a, -4b, -5, -6, or -7) and in which the polypeptide includes an amino acid that is covalently bonded to a compound of Formulas (I) or (II). In certain embodiments, the modification does not destroy significantly a desired biological activity. In some embodiments, the modification may cause a reduction in biological activity (e.g., by at least 5%, 10%, 20%, 25%, 35%, 50%, 60%, 70%, 75%, 80%, 90%, or 95%). In other embodiments, the modification has no effect on the biological activity or may increase (e.g., by at least 5%, 10%, 25%, 50%, 100%, 200%, 500%, or 1000%) the biological activity of the original polypeptide. The modified polypeptide may have or may optimize one or more of the characteristics of a polypeptide of the invention which, in some instance might be needed or desirable. Such characteristics

include in vivo stability, bioavailability, toxicity, immunological activity, or immunological identity.

Polypeptides of the invention may include amino acids or sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques known in the art. Modifications may occur anywhere in a polypeptide including the polypeptide backbone, the amino acid side-chains and the amino- or carboxy-terminus. The same type of modification may be present in the same or varying degrees at several sites in a given polypeptide, and a polypeptide may contain more than one type of modification. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic polypeptides may result from posttranslational natural processes or may be made synthetically. Other modifications include pegylation, acetylation, acylation, addition of acetomidomethyl (Acm) group, ADP-ribosylation, alkylation, amidation, biotinylation, carbamoylation, carboxyethylation, esterification, covalent attachment to fiavin, covalent attachment to a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of drug, covalent attachment of a marker (e.g., fluorescent or radioactive), covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation and ubiquitination.

A modified polypeptide of the invention may further include an amino acid insertion, deletion, or substitution, either conservative or non-conservative (e.g., D-amino acids, desamino acids) in the polypeptide sequence (e.g., where

such changes do not substantially alter the biological activity of the polypeptide).

For example, in some embodiments, the amino acid sequence (e.g., SEQ ID NOS 1-105 or 107-116) is modified by inserting one or more additional cysteine residues at the N-terminal of the peptide, the C-terminal of the peptide, or both. The addition of one or more cysteine residues to the amino or carboxy terminus of any of the amino acid sequences described herein can facilitate conjugation of these polypeptides to nucleic acids (e.g., siRNA molecules) or lipid vectors by, e.g., disulfide bonding. For example, Angiopep-1 (SEQ ID NO:67), Angiopep-2 (SEQ ID NO:97), or Angiopep-7 (SEQ ID NO:112) can be modified to include a single cysteine residue at the amino-terminus (SEQ ID NOS: 71, 113, and 115, respectively) or a single cysteine residue a the carboxy-terminus (SEQ ID NOS: 72, 114, and 116, respectively).

Substitutions may be conservative (i.e., wherein a residue is replaced by another of the same general type or group) or non-conservative (i.e., wherein a residue is replaced by an amino acid of another type). In addition, a non-naturally occurring amino acid may be substituted for a naturally occurring amino acid (i.e., non-naturally occurring conservative amino acid substitution or a non-naturally occurring non-conservative amino acid substitution).

Polypeptides made synthetically may include substitutions of amino acids not naturally encoded by DNA (e.g., non-naturally occurring or unnatural amino acid). Examples of non-naturally occurring amino acids include D-amino acids, an amino acid having an acetylaminomethyl group attached to a sulfur atom of a cysteine, a pegylated amino acid, the omega amino acids of the formula $NH_2(CH_2)_nCOOH$ wherein n is 2-6, neutral nonpolar amino acids, such as sarcosine, t-butyl alanine, t-butyl glycine, N-methyl isoleucine, and norleucine. Phenylglycine may substitute for Trp, Tyr, or Phe; citrulline and methionine sulfoxide are neutral nonpolar, cysteic acid is acidic, and ornithine is basic. Proline may be substituted with hydroxyproline and retain the conformation conferring properties.

Analogues may be generated by substitutional mutagenesis and retain the biological activity of the original polypeptide. Examples of substitutions identified as "conservative substitutions" are shown in Table 3. If such substitutions result in a change not desired, then other type of substitutions, denominated "exemplary substitutions" in Table 3, or as further described herein in reference to amino acid classes, are introduced and the products screened.

Substantial modifications in function or immunological identity are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation. (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

(1) hydrophobic: norleucine, methionine (Met), Alanine (Ala), Valine (Val), Leucine (Leu), Isoleucine (Ile), Histidine (His), Tryptophan (Trp), Tyrosine (Tyr), Phenylalanine (Phe),

(2) neutral hydrophilic: Cysteine (Cys), Serine (Ser), Threonine (Thr)

(3) acidic/negatively charged: Aspartic acid (Asp), Glutamic acid (Glu)

(4) basic: Asparagine (Asn), Glutamine (Gln), Histidine (His), Lysine (Lys), Arginine (Arg)

(5) residues that influence chain orientation: Glycine (Gly), Proline (Pro);

(6) aromatic: Tryptophan (Trp), Tyrosine (Tyr), Phenylalanine (Phe), Histidine (His),

(7) polar: Ser, Thr, Asn, Gln

(8) basic positively charged: Arg, Lys, His, and;

(9) charged: Asp, Glu, Arg, Lys, His

Other conservative amino acid substitutions are listed in Table 3.

Table 3

Original residue	Exemplary substitution	Conservative substitution
Ala (A)	Val, Leu, Ile	Val
Arg (R)	Lys, Gln, Asn	Lys
Asn (N)	Gln, His, Lys, Arg	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Asn, Gln, Lys, Arg	Arg
Ile (I)	Leu, Val, Met, Ala, Phe, norleucine	Leu
Leu (L)	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys (K)	Arg, Gln, Asn	Arg
Met (M)	Leu, Phe, Ile	Leu
Phe (F)	Leu, Val, Ile, Ala	Leu
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser	Phe
Val (V)	Ile, Leu, Met, Phe, Ala, norleucine	Leu

Additional polypeptide analogues

The compounds of the invention may include polypeptide analogs of aprotinin known in the art where the analogs include an amino acid that is covalently bonded to a podophyllotoxin derivative (e.g., a compound of Formula (I)) or to doxorubicin or a doxorubicin derivative (e.g., a compound of Formula (II)). For example, U.S. Patent No. 5,807,980 describes Bovine Pancreatic Trypsin Inhibitor (aprotinin)-derived inhibitors as well as a method

for their preparation and therapeutic use, including the polypeptide of SEQ ID NO:102. These polypeptides have been used for the treatment of a condition characterized by an abnormal appearance or amount of tissue factor and/or factor VIIIa such as abnormal thrombosis. U.S. Patent No. 5,780,265 describes serine protease inhibitors capable of inhibiting plasma kallikrein, including SEQ ID NO:103. U.S. Patent No. 5,118,668 describes Bovine Pancreatic Trypsin Inhibitor variants, including SEQ ID NO:105. The aprotinin amino acid sequence (SEQ ID NO:98), the Angiopep-1 amino acid sequence (SEQ ID NO:67), and SEQ ID NO:104, as well as some sequences of biologically active analogs may be found in International Application Publication No. WO 2004/060403.

An exemplary nucleotide sequence encoding an aprotinin analogue is illustrated in SEQ ID NO:106 (atgagaccag atttctgcct cgagccgccg tacactgggc cctgcaaagc tcgtatcatc cgttacttct acaatgcaaa ggcaggcctg tgtcagacct tcgtatacgg cggctgcaga gctaagcgta acaacttcaa atccgcggaa gactgcatgc gtacttgcgg tggtgcttag; Genbank accession No. X04666). This sequence encodes a lysine at position 16 instead of a valine, as found in SEQ ID NO:98. A mutation in the nucleotide sequence of SEQ ID NO:106 may be introduced by methods known in the art to change the produce the polypeptide of SEQ ID NO:98 having a valine in position 16. Additional mutations or fragments may be obtained using any technique known in the art.

Other examples of aprotinin analogs may be found by performing a protein BLAST (Genebank: www.ncbi.nlm.nih.gov/BLAST/) using the synthetic aprotinin sequence (or portion thereof) disclosed in International Application No. PCT/CA2004/000011. Exemplary aprotinin analogs are found under accession Nos. CAA37967 (GI:58005) and 1405218C (GI:3604747).

Preparation of polypeptide derivatives and peptidomimetics

In addition to polypeptides consisting only of naturally occurring amino acids, peptidomimetics or polypeptide analogs are also encompassed by the

present invention. Polypeptide analogs are commonly used in the pharmaceutical industry as non-polypeptide drugs with properties analogous to those of the template polypeptide. The non-polypeptide compounds are termed "polypeptide mimetics" or peptidomimetics (Fauchere et al., *Infect. Immun.* 54:283-287,1986; Evans et al., *J. Med. Chem.* 30:1229-1239, 1987). Polypeptide mimetics that are structurally related to therapeutically useful polypeptides may be used to produce an equivalent or enhanced therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to the paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity) such as naturally-occurring receptor-binding polypeptides, but have one or more peptide linkages optionally replaced by linkages such as —CH_2NH—, —CH_2S—, —CH_2—CH_2—, —CH=CH— (cis and trans), —CH_2SO—, —$CH(OH)CH_2$—, —$COCH_2$— etc., by methods well known in the art (Spatola, *Peptide Backbone Modifications, Vega Data,* 1(3):267, 1983); Spatola et al. (*Life Sci.* 38:1243-1249, 1986); Hudson et al. (*Int. J. Pept. Res.* 14:177-185, 1979); and Weinstein. B., 1983, Chemistry and Biochemistry, of Amino Acids, Peptides and Proteins, Weinstein eds, Marcel Dekker, New York). Such polypeptide mimetics may have significant advantages over naturally-occurring polypeptides including more economical production, greater chemical stability, enhanced pharmacological properties (e.g., half-life, absorption, potency, efficiency), reduced antigenicity and others.

While the polypeptides of the invention may be effective in entering particular cell types (e.g., those described herein), their effectiveness may be reduced by the presence of proteases. Serum proteases have specific substrate requirements. The substrate must have both L-amino acids and peptide bonds for cleavage. Furthermore, exopeptidases, which represent the most prominent component of the protease activity in serum, usually act on the first peptide bond of the polypeptide and require a free N-terminus (Powell et al., *Pharm. Res.* 10:1268-1273, 1993). In light of this, it is often advantageous to use modified versions of polypeptides. The modified polypeptides retain the

structural characteristics of the original L-amino acid polypeptides that confer biological activity with regard to IGF-1, but are advantageously not readily susceptible to cleavage by protease and/or exopeptidases.

Systematic substitution of one or more amino acids of a consensus sequence with D-amino acid of the same type (e.g., D-lysine in place of L-lysine) may be used to generate more stable polypeptides. Thus, a polypeptide derivative or peptidomimetic of the present invention may be all L, all D or mixed D, L polypeptide. The presence of an N-terminal or C-terminal D-amino acid increases the *in vivo* stability of a polypeptide because peptidases cannot utilize a D-amino acid as a substrate (Powell et al., *Pharm. Res.* 10:1268-1273, 1993). Reverse-D polypeptides are polypeptides containing D-amino acids, arranged in a reverse sequence relative to a polypeptide containing L-amino acids. Thus, the C-terminal residue of an L-amino acid polypeptide becomes N-terminal for the D-amino acid polypeptide, and so forth. Reverse D-polypeptides retain the same tertiary conformation and therefore the same activity, as the L-amino acid polypeptides, but are more stable to enzymatic degradation *in vitro* and *in vivo*, and thus have greater therapeutic efficacy than the original polypeptide (Brady and Dodson, *Nature* 368:692-693, 1994; Jameson et al., *Nature* 368:744-746, 1994). In addition to reverse-D-polypeptides, constrained polypeptides comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods well known in the art (Rizo and Gierasch, *Ann. Rev. Biochem.* 61:387-418, 1992). For example, constrained polypeptides may be generated by adding cysteine residues capable of forming disulfide bridges and, thereby, resulting in a cyclic polypeptide. Cyclic polypeptides have no free N- or C-termini. Accordingly, they are not susceptible to proteolysis by exopeptidases, although they are, of course, susceptible to endopeptidases, which do not cleave at peptide termini. The amino acid sequences of the polypeptides with N-terminal or C-terminal D-amino acids and of the cyclic polypeptides are usually identical to the sequences of the polypeptides to which they correspond, except for the

presence of N-terminal or C-terminal D-amino acid residue, or their circular structure, respectively.

A cyclic derivative containing an intramolecular disulfide bond may be prepared by conventional solid phase synthesis while incorporating suitable S-protected cysteine or homocysteine residues at the positions selected for cyclization such as the amino and carboxy termini (Sah et al., *J. Pharm. Pharmacol.* 48:197, 1996). Following completion of the chain assembly, cyclization can be performed either (1) by selective removal of the S-protecting group with a consequent on-support oxidation of the corresponding two free SH-functions, to form a S-S bonds, followed by conventional removal of the product from the support and appropriate purification procedure or (2) by removal of the polypeptide from the support along with complete side chain de-protection, followed by oxidation of the free SH-functions in highly dilute aqueous solution.

The cyclic derivative containing an intramolecular amide bond may be prepared by conventional solid phase synthesis while incorporating suitable amino and carboxyl side chain protected amino acid derivatives, at the position selected for cyclization. The cyclic derivatives containing intramolecular -S-alkyl bonds can be prepared by conventional solid phase chemistry while incorporating an amino acid residue with a suitable amino-protected side chain, and a suitable S-protected cysteine or homocysteine residue at the position selected for cyclization.

Another effective approach to confer resistance to peptidases acting on the N-terminal or C-terminal residues of a polypeptide is to add chemical groups at the polypeptide termini, such that the modified polypeptide is no longer a substrate for the peptidase. One such chemical modification is glycosylation of the polypeptides at either or both termini. Certain chemical modifications, in particular N-terminal glycosylation, have been shown to increase the stability of polypeptides in human serum (Powell et al., *Pharm. Res.* 10:1268-1273, 1993). Other chemical modifications which enhance serum

stability include, but are not limited to, the addition of an N-terminal alkyl group, consisting of a lower alkyl of from one to twenty carbons, such as an acetyl group, and/or the addition of a C-terminal amide or substituted amide group. In particular, the present invention includes modified polypeptides consisting of polypeptides bearing an N-terminal acetyl group and/or a C-terminal amide group.

Also included by the present invention are other types of polypeptide derivatives containing additional chemical moieties not normally part of the polypeptide, provided that the derivative retains the desired functional activity of the polypeptide. Examples of such derivatives include (1) N-acyl derivatives of the amino terminal or of another free amino group, wherein the acyl group may be an alkanoyl group (e.g., acetyl, hexanoyl, octanoyl) an aroyl group (e.g., benzoyl) or a blocking group such as F-moc (fluorenylmethyl-O—CO—); (2) esters of the carboxy terminal or of another free carboxy or hydroxyl group; (3) amide of the carboxy-terminal or of another free carboxyl group produced by reaction with ammonia or with a suitable amine; (4) phosphorylated derivatives; (5) derivatives conjugated to an antibody or other biological ligand and other types of derivatives.

Longer polypeptide sequences which result from the addition of additional amino acid residues to the polypeptides of the invention are also encompassed in the present invention. Such longer polypeptide sequences would be expected to have the same biological activity (e.g., entering particular cell types) as the polypeptides described above. While polypeptides having a substantial number of additional amino acids are not excluded, it is recognized that some large polypeptides may assume a configuration that masks the effective sequence, thereby preventing binding to a target (e.g., a member of the LRP receptor family such as LRP or LRP2). These derivatives could act as competitive antagonists. Thus, while the present invention encompasses polypeptides or derivatives of the polypeptides described herein having an

extension, desirably the extension does not destroy the cell targeting activity of the polypeptide or derivative.

Other derivatives included in the present invention are dual polypeptides consisting of two of the same, or two different polypeptides of the present invention covalently linked to one another either directly or through a spacer, such as by a short stretch of alanine residues or by a putative site for proteolysis (e.g., by cathepsin, see e.g., U.S. Patent No. 5,126,249 and European Patent No. 495 049). Multimers of the polypeptides of the present invention consist of polymer of molecules formed from the same or different polypeptides or derivatives thereof.

The present invention also features polypeptide derivatives that are chimeric or fusion proteins containing a polypeptide described herein, or fragment thereof, linked at its amino- or carboxy-terminal end, or both, to an amino acid sequence of a different protein. Such a chimeric or fusion protein may be produced by recombinant expression of a nucleic acid encoding the protein. For example, a chimeric or fusion protein may contain at least 6 amino acids of a polypeptide of the present invention and desirably has a functional activity equivalent or greater than a polypeptide of the invention.

Polypeptide derivatives of the present invention can be made by altering the amino acid sequences by substitution, addition, or deletion or an amino acid residue to provide a functionally equivalent molecule, or functionally enhanced or diminished molecule, as desired. The derivative of the present invention include, but are not limited to, those containing, as primary amino acid sequence, all or part of the amino acid sequence of the polypeptides described herein (e.g., any one of SEQ ID NOS:1-105 and 107-112) including altered sequences containing substitutions of functionally equivalent amino acid residues. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity that acts as a functional equivalent, resulting in a silent alteration. Substitution for an amino acid within the sequence may be selected from other members of the class to

which the amino acid belongs. For example, the positively charged (basic) amino acids include, arginine, lysine and histidine. The nonpolar (hydrophobic) amino acids include, leucine, isoleucine, alanine, phenylalanine, valine, proline, tryptophan and methionine. The uncharged polar amino acids include serine, threonine, cysteine, tyrosine, asparagine and glutamine. The negatively charged (acid) amino acids include glutamic acid and aspartic acid. The amino acid glycine may be included in either the nonpolar amino acid family or the uncharged (neutral) polar amino acid family. Substitutions made within a family of amino acids are generally understood to be conservative substitutions.

Assays to identify peptidomimetics

As described above, non-peptidyl compounds generated to replicate the backbone geometry and pharmacophore display (peptidomimetics) of the polypeptides identified by the methods of the present invention often possess attributes of greater metabolic stability, higher potency, longer duration of action and better bioavailability.

The peptidomimetics compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries, while the other four approaches are applicable to polypeptide, non-peptide oligomer or small molecule libraries of compounds (Lam, *Anticancer Drug Des.* 12:145, 1997). Examples of methods for the synthesis of molecular libraries can be found in the art, for example, in: DeWitt et al. (*Proc. Natl. Acad. Sci. USA* 90:6909, 1993); Erb et al. (*Proc. Natl. Acad. Sci. USA* 91:11422, 1994); Zuckermann et al., *J. Med. Chem.* 37:2678, 1994); Cho et al. (*Science* 261:1303, 1993); Carell

et al. (*Angew. Chem, Int. Ed. Engl.* 33:2059, 1994 and ibid 2061); and in Gallop et al. (*Med. Chem.* 37:1233, 1994). Libraries of compounds may be presented in solution (e.g., Houghten, *Biotechniques* 13:412-421, 1992) or on beads (Lam, *Nature* 354:82-84, 1991), chips (Fodor, *Nature* 364:555-556, 1993), bacteria or spores (U.S. Patent No. 5,223,409), plasmids (Cull et al., *Proc. Natl. Acad. Sci. USA* 89:1865-1869, 1992) or on phage (Scott and Smith, *Science* 249:386-390, 1990), or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

Once a polypeptide of the present invention is identified, it may be isolated and purified by any number of standard methods including, but not limited to, differential solubility (e.g., precipitation), centrifugation, chromatography (e.g., affinity, ion exchange, size exclusion, and the like) or by any other standard techniques used for the purification of polypeptides, peptidomimetics or proteins. The functional properties of an identified polypeptide of interest may be evaluated using any functional assay known in the art. Desirably, assays for evaluating downstream receptor function in intracellular signaling are used (e.g., cell proliferation).

For example, the peptidomimetics compounds of the present invention may be obtained using the following three-phase process: (1) scanning the polypeptides of the present invention to identify regions of secondary structure necessary for targeting the particular cell types described herein; (2) using conformationally constrained dipeptide surrogates to refine the backbone geometry and provide organic platforms corresponding to these surrogates; and (3) using the best organic platforms to display organic pharmacophores in libraries of candidates designed to mimic the desired activity of the native polypeptide. In more detail the three phases are as follows. In phase 1, the lead candidate polypeptides are scanned and their structure abridged to identify the requirements for their activity. A series of polypeptide analogs of the original are synthesized. In phase 2, the best polypeptide analogs are investigated using the conformationally constrained dipeptide surrogates. Indolizidin-2-one,

indolizidin-9-one and quinolizidinone amino acids (I^2aa, I^9aa and Qaa respectively) are used as platforms for studying backbone geometry of the best polypeptide candidates. These and related platforms (reviewed in Halab et al., *Biopolymers* 55:101-122, 2000; and Hanessian et al. *Tetrahedron* 53:12789-12854, 1997) may be introduced at specific regions of the polypeptide to orient the pharmacophores in different directions. Biological evaluation of these analogs identifies improved lead polypeptides that mimic the geometric requirements for activity. In phase 3, the platforms from the most active lead polypeptides are used to display organic surrogates of the pharmacophores responsible for activity of the native polypeptide. The pharmacophores and scaffolds are combined in a parallel synthesis format. Derivation of polypeptides and the above phases can be accomplished by other means using methods known in the art.

Structure function relationships determined from the polypeptides, polypeptide derivatives, peptidomimetics or other small molecules of the present invention may be used to refine and prepare analogous molecular structures having similar or better properties. Accordingly, the compounds of the present invention also include molecules that share the structure, polarity, charge characteristics and side chain properties of the polypeptides described herein.

In summary, based on the disclosure herein, those skilled in the art can develop polypeptides and peptidomimetics screening assays which are useful for identifying compounds for targeting an agent to particular cell types (e.g., those described herein). The assays of this invention may be developed for low-throughput, high-throughput, or ultra-high throughput screening formats. Assays of the present invention include assays that are amenable to automation.

Polypeptide Conjugates Covalently Bonded to Additional Agents

The compounds described herein, or functional derivatives thereof, in addition to including an amino acid sequence that is covalently bonded through

an amino acid to a podophyllotoxin derivative (e.g., a compound having a structure according to Formula (I)) or to doxorubicin or a doxorubicin derivative (e.g., a compound of Formula (II)), may also include a covalent bond to another agent (e.g., another therapeutic agent, a diagnostic agent, or to a label). In certain embodiments, the amino acid sequence is also linked to or labeled with a detectable label, such as a radioimaging agent, for diagnosis of a disease or condition. Examples of these agents include a radioimaging agent-antibody-vector conjugate, where the antibody binds to a disease or condition-specific antigen (e.g., for diagnosis or therapy). Other binding molecules are also contemplated by the invention. In other cases, the compound of the invention, or a functional derivative thereof, is linked to another therapeutic agent, to treat a disease or condition, or may be linked to or labeled with mixtures thereof. The disease or condition may be treated by administering a vector-agent conjugate to an individual under conditions which allow transport of the agent across the BBB or into a particular cell type. Each polypeptide may include at least 1, 2, 3, 4, 5, 6, or 7 additional agents. In other embodiments, each agent has at least 1, 2, 3, 4, 5,6 7, 10, 15, 20, or more polypeptides attached thereto. The conjugates of the invention may be able to promote accumulation (e.g., due to increased uptake or reduced removal) of the agent in a particular cell type or tissue such as the brain, ovary, liver, lung, kidney, spleen or muscle of a subject.

An agent (e.g., a podophyllotoxin derivative such as a compound of Formula (I) or doxorubicin or a doxorubicin derivative (e.g., a compound of Formula (II)), another therapeutic agent, a diagnostic agent, or a label) that has a covalent bond to an amino acid in any of the amino acid sequences described herein (e.g., those listed in Table 1, or functional derivatives thereof) may be releasable from the vector after transport into a particular cell type or across the BBB. The agent can be released, for example, by enzymatic cleavage or other breakage of a chemical bond between the vector and the agent. The released agent may then function in its intended capacity in the absence of the vector.

Other methods and cross-linkers can be used to conjoin the polypeptides and RNAi agents of the invention. For example, a 5' or 3' thiol-containing siRNA sense strand can be linked by a disulfide bond to a cysteine residue placed at either the amino or carboxy terminus of the polypeptide. Muratovska *et al.* (*FEBS Letters* 558:63-68, 2004) and Turner *et al.* (*Blood Cells, Molecules, and Diseases* 38:1-7, 2007) provide exemplary chemical bonding methods for conjugating polypeptides to RNA molecules and are hereby incorporated by reference.

Therapeutic Agents

A therapeutic agent may be any biologically active agent. For example, a therapeutic may be a drug, a medicine, an agent emitting radiation, a cellular toxin (for example, a chemotherapeutic agent), a biologically active fragment thereof, or a mixture thereof to treat a disease (e.g., to killing cancer cells) or it may be an agent to treat a disease or condition in an individual. Podophyllotoxin derivatives (e.g., the compounds of Formula (I)) and doxorubicin and doxorubicin derivatives (e.g., compounds of Formula (II)) are exemplary useful classes of therapeutic agents. A therapeutic agent may be a synthetic product or a product of fungal, bacterial or other microorganism (e.g., mycoplasma or virus), animal, such as reptile, or plant origin. A therapeutic agent and/or biologically active fragment thereof may be an enzymatically active agent and/or fragment thereof, or may act by inhibiting or blocking an important and/or essential cellular pathway or by competing with an important and/or essential naturally occurring cellular component. Other therapeutic agents include antibodies and antibody fragments.

Any anticancer agent known in the art may be part of a conjugate of the invention. Podophyllotoxin derivatives (e.g., the compounds of Formula (I)) and doxorubicin and doxorubicin derivatives (e.g., compounds of Formula (II)) can be anticancer agents. Additional anticancer agents may also be conjugated to the compounds of the invention as described herein. Cancers of the brain

may be treated with a conjugate containing a vector that is efficiently transported across the BBB (e.g., AngioPep-2, AngioPep-3, AngioPep-4a, AngioPep-4b, AngioPep-5, or AngioPep-6). Ovary, liver, lung, kidney, or spleen cancers may be treated with an anticancer agent conjugated to a vector that is transported efficiently into the appropriate cell type (e.g., AngioPep-7).

Conjugate activities

Compounds, or a pharmaceutically acceptable salt thereof, that include an amino acid sequence, where the amino acid sequence is covalently bonded through an amino acid to a podophyllotoxin derivative (e.g., a compound having a structure according to Formula (I)) or to doxorubicin or doxorubicin derivatives (e.g., compounds of Formula (II)) can achieve desirable properties, such as altered pharmacokinetics or altered tissue distribution (e.g., increased delivery to particular tissues or cell types such as ovary, liver, brain, lung, spleen, or kidney) relative to the unconjugated biologically active agent. Accordingly, the compounds of the invention can be used as vectors. Polypeptides such as AngioPep-3, AngioPep-4a, AngioPep-4b, AngioPep-5, and AngioPep-6 efficiently transport agents across the BBB. Like AngioPep-2, these polypeptides may also be capable of targeting agents to other cell types or tissues (e.g., ovary, liver, lung, kidney, spleen, or muscle). The AngioPep-7 polypeptide, which is not efficiently transported across the BBB, is transported to particular tissues (e.g., ovary, liver, lung, kidney, spleen, or muscle). This activity may be useful where transport across the BBB is not desired. For example, the use of a compound of the invention can increase the concentration of the therapeutic agent in the target tissue by anywhere from 10%-20,000% relative to that observed with the unconjugated biologically active agent (for example, etoposide, etoposide phosphate, $etoposide_{DMG}$, teniposide, doxorubicin, or epirubicin).

Because the compounds of the invention can transport agents to specific tissues, conjugated agents may result in lower toxicity (e.g., fewer side effects),

higher efficacy (e.g., because the agent is concentrated into a target tissue due to increased uptake or decreased efflux from the tissue or because the agent has greater stability when conjugated), or a combination thereof. Such activities are described below and in International Publication No. WO 2007/009229, hereby incorporated by reference.

In some cases, conjugation of an agent to a vector allows the agent to escape the action of P-glycoprotein (P-gp), an efflux pump capable of expelling certain agents from a cell. By decreasing the ability of P-gp to expel an agent from a cell, the potency of that agent in a cell can be increased. These conjugates can thus actively inhibit cancer cell proliferation. Moreover, results obtained for in vivo tumor growth indicate that the vectors of the invention may target the receptor LRP. Also, conjugation may modify the pharmacokinetics or biodistribution of the unconjugated agent.

Taken together, conjugates can be used against primary tumors including ovary, breast, lung, and skin cancers as well as metastasis originating from primary tumors.

Methods of treatment

The invention also features methods of treatment using the compounds of the invention, or pharmaceutical compositions thereof, described herein. The compounds of the invention (e.g., compounds that include an amino acid sequence that is covalently bonded through an amino acid to a podophyllotoxin derivative such as a compound of Formula (I) or to doxorubicin or doxorubicin derivatives (e.g., compounds of Formula (II)) that are efficiently transported across the BBB (e.g., AngioPep-2, AngioPep-3, AngioPep-4a, AngioPep-4b, AngioPep-5, and AngioPep-6) may be used to treat any brain or central nervous system disease. Exemplary neurological diseases include, but are not limited to, brain cancers such as a brain tumor, a spinal cord tumor (e.g., chordoma), and a brain metastasis

Brain tumors may be primary metastatic brain tumors. Brain tumors that originate in the brain are primary brain tumors. Brain tumors caused by the spread of cancer elsewhere in the body (e.g., lung, breast, melanoma, colon, kidney, and other cancers) are metastatic brain tumors. Exemplary categories of tumors, as described by their location in the brain, include brain stem tumors, cerebellopontine angle tumors (e.g., acoustic nerve tumors), cerebral hemisphere tumors, frontal lobe tumors, parietal lobe tumors, pineal region tumors, occipital lobe tumors, temporal lobe tumors, subcortical tumors, meningeal brain tumors, midline tumors (e.g., craniopharyngioma, optic nerve glioma, and tumors of the thalamus and sellar areas), posterior fossa tumors (e.g., tumors of the fourth ventricle, andcerebellar tumors).

Exemplary brain tumors include acoustic neuroma (neurilemmoma, schwannoma, neurinoma), adenoma, astrocytoma (e.g., juvenile pilocytic astrocytomas, subependymal giant cell astrocytomas, gemistocytic astrocytoma, anaplastic astrocytoma, malignant astrocytoma, glioblastoma multiforme, and gliosarcoma), brain stem glioma which may be anastrocytoma, anaplastic astrocytoma, glioblastoma multiforme, or a mixed tumor, choroid plexus papilloma, cns lymphoma, ependymoma (e.g., anaplastic ependymoma), gangliocytoma, ganglioglioma, glioma, glioblastoma multiforme, medulloblastoma (mdl), anaplastic (malignant) meningioma, mixed glioma, neurofibromatosis (von Recklinghausen's Disease), oligodendroglioma, and optic nerve glioma (e.g., pilocytic astrocytoma).

Conjugates can also be efficiently transported to the liver, ovary, lung, kidney, spleen or muscle and therefore may also be used, in conjunction with an appropriate therapeutic agent, to treat a disease associated with these tissues (e.g., a cancer). Because AngioPep-7 is not efficiently transported to the brain, but is transported efficiently to tissues and cells such as liver, lung, kidney, spleen and muscle, compounds of the invention that include AngioPep-7 may be especially well suited as a vector treatment of diseases associated with these tissues when targeting the agent to the brain is not desired. Exemplary diseases

of the liver include hepatocellular carcinoma (hepatoma) and liver cancer. Exemplary lung diseases include lung cancers such as small cell carcinoma (e.g., oat cell cancer), mixed small cell/large cell carcinoma, combined small cell carcinoma, and metastatic tumors. Metastatic tumors can originate from cancer of any tissue, including breast cancer (e.g., metastatic breast carcinoma), colon cancer, prostate cancer (e.g., metastatic prostate carcinoma), sarcoma, bladder cancer, neuroblastoma, and Wilms' tumor (nephroblastoma). Spleen diseases include cancers such as lymphoma, non-Hodgkin's lymphoma, and certain T-cell lymphomas.

Additional exemplary cancers that may be treated using a conjugate or composition of the invention include breast cancer, cancers of the head and neck including various lymphomas such as mantle cell lymphoma, adenoma, squamous cell carcinoma, laryngeal carcinoma, cancers of the retina, cancers of the esophagus, multiple myeloma, ovarian cancer (e.g., ovarian germ-cell tumors and ovarian carcinoma), uterine cancer, melanoma, colorectal cancer, bladder cancer, prostate cancer, lung cancer (including small cell lung carcinoma and non-small cell lung carcinoma), pancreatic cancer, cervical cancer, head and neck cancer, skin cancers, nasopharyngeal carcinoma, liposarcoma, epithelial carcinoma, renal cell carcinoma, gallbladder adenocarcinoma, parotid adenocarcinoma, endometrial sarcoma, multidrug resistant cancers; and proliferative diseases and conditions, such as neovascularization associated with tumor angiogenesis, macular degeneration (e.g., wet/dry AMD), comeal neovascularization, diabetic retinopathy, neovascular glaucoma, myopic degeneration and other proliferative diseases and conditions such as restenosis and polycystic kidney disease.

As described herein, brain cancers that can be treated with the compounds or the compositions of the invention that are transported efficiently across the BBB include astrocytoma, pilocytic astrocytoma, dysembryoplastic neuroepithelial tumor, oligodendrogliomas, ependymoma, glioblastoma multiforme, mixed gliomas, oligoastrocytomas, medulloblastoma,

retinoblastoma, neuroblastoma, germinoma, and teratoma. Other exemplary cancers that may be treated with the compounds or compositions of the invention include mycosis fungoides (also known as Alibert-Bazin syndrome or granuloma fungoides), Hodgkin's disease (Hodgkin's lymphoma), acute myelogenous leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, Kaposi's sarcoma related to acquired immune deficiency syndrome (AIDS), AIDS-related non-Hodgkin's lymphoma, gestational trophoblastic tumors, Ewing's sarcoma, rhabdomyosarcoma, refractory advanced breast cancer, testicular cancer (e.g., malignant tumor of testis, refractory testicular neoplasm, and testicular germ cell tumor carcinoma), refractory advanced malignant neoplasms, diffuse large B-cell lymphoma, osteosarcoma, Burkitt's lymphoma, adult acute lymphocytic leukemia, Burkitt's leukemia, mediastinal neoplasms, lymphoblastic lymphoma, large cell anaplastic lymphoma, plasma cell neoplasm.

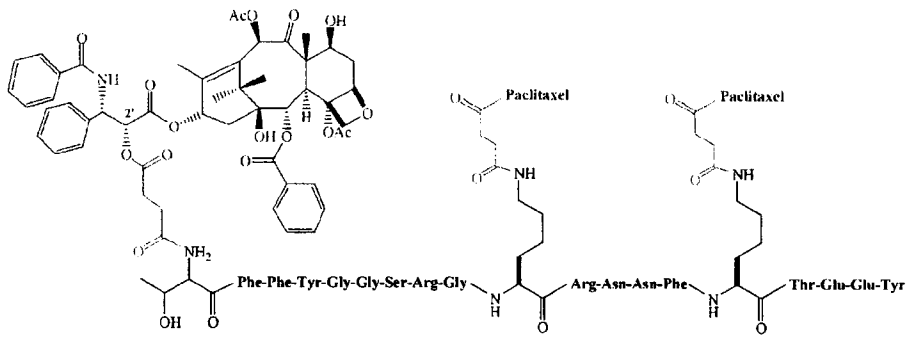
A compound or composition of the invention may be administered by any means known in the art; e.g., orally, intraarterially, intranasally, intraperitoneally, intravenously, intramuscularly, subcutaneously, transdermally or *per os* to the subject. The agent may be, for example, an anti-angiogenic compound.

Combination Therapies

The compounds of the invention may be administered concurrently with other therapeutic agents or other therapeutic regimes. In some embodiments, the additional therapeutic agent or agents may also have a covalent bond to the polypeptides, or derivatives thereof, described herein (e.g., the polypeptides of Table 1 and derivatives thereof). In other embodiments, the additional therapeutic agent or agents are not covalently bound to the polypeptides described herein. Exemplary therapeutic regimes and therapeutic agents that can be used in combination therapy with the compounds of the invention include, but are not limited to: radiation therapy, chemotherapy, high dose

chemotherapy, stem cell transplant (e.g., autologous stem cell transplant), bone marrow transplant, surgery, surgery to remove tumors, hyperthermia treatment, cisplatin, irinotecan, irinotecan hydrochloride, carboplatin, chlorambucil (Leukeran ®), tositumomab (Bexxar®), rituximab (Rituxan® and MabThera®), bleomycin, vincristine, vinblastine, cyclophosphamide, procarbazine, mitoxantrone, prednisone, prednisolone, gemcitabine (Gemzar®), paclitaxel (Taxol®), ifosfamide, methotrexate, doxorubicin, (Adriamycin®), dexamethasone, cyclosporin, Rad-001 (Certican), cytarabine (Ara-C), daunorubicin, fludarabine, idarubicin, vorinostat (SAHA), niacinamide, AZD2171, mitotane, Gemtuzumab ozogamicin (Mylotarg®), mitoxantrone, clofarabine, asparaginase, mercaptopurine, granulocyte colony-stimulating factor (G-CSF or GCSF), vindesine, thioguanine, VM26, VP16, dacarbazine, dactinomycin, temozolomide, thiotepa, epirubicin hydrochloride, carmustine, filgrastim, docetaxel, gefitinib, or pharmaceutically acceptable salts thereof, or any combination thereof.

The second therapeutic agents used in the methods described herein can also be a polypeptide that includes or that consists of the sequence of Angiopep-2 (SEQ ID NO:97), preferably where the Angiopep-2 is conjugated to an anticancer agent (e.g., paclitaxel). An exemplary therapeutic agent that can be used in combination with any of the compounds described herein is ANG1005, which has the following structure:



ANG1005:
TxlAn2 (3:1 conjugate)

Still other exemplary second therapeutic agents are described in U.S. Patent No. 7,557,182, herein incorporated by reference.

Pharmaceutical compositions

Pharmaceutical compositions of the invention include a compound of the invention as described herein, in association with a pharmaceutically acceptable carrier. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts). Solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance. Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines). Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal, oral, vaginal, rectal routes. In one embodiment the pharmaceutical composition is administered parenterally, paracancerally, transmucosally, transdermally, intramuscularly, intravenously, intradermally,

subcutaneously, intraperitonealy, intraventricularly, intracranially, and intratumorally.

Pharmaceutically acceptable carriers further include 0.01-0.1 M or 0.05 M phosphate buffer or 0.8 % saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, collating agents, inert gases and the like.

Other formulations include poly-oxyethylene esters of a fatty acid (e.g., 12-hydroxystearic acid) such as Solutol® HS15. Thus, in some embodiments, a pharmaceutical composition may comprise a) a conjugate described herein, b) Solutol® HS15 and c) an aqueous solution or buffer (e.g., Ringer/Hepes solution at a pH of 5 to 7). The concentration of Solutol® HS15 in the formulation may be at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, or 60% (e.g., 30%) or within any range between any two of these numbers. The concentration of conjugate may be determined based upon the dose required for efficiently treating a subject, or the amount the ester required for solubility of the conjugate being administered. The use of Solutol in a formulation for administration of a Taxol conjugate is described, for example, in International Publication No. WO 2007/009229, hereby incorporated by reference.

Parenteral compositions

The pharmaceutical composition may be administered parenterally by injection, infusion, or implantation (subcutaneous, intravenous, intramuscular, intraperitoneal, or the like) in dosage forms, formulations, or via suitable delivery devices or implants containing conventional, non-toxic pharmaceutically acceptable carriers and adjuvants. The formulation and preparation of such compositions are well known to those skilled in the art of pharmaceutical formulation.

Compositions for parenteral use may be provided in unit dosage forms (e.g., in single-dose ampoules), or in vials containing several doses and in which a suitable preservative may be added (see below). The composition may be in form of a solution, a suspension, an emulsion, an infusion device, or a delivery device for implantation, or it may be presented as a dry powder to be reconstituted with water or another suitable vehicle before use. Apart from the active agent(s), the composition may include suitable parenterally acceptable carriers and/or excipients. The active agent(s) may be incorporated into microspheres, microcapsules, nanoparticles, liposomes, or the like for controlled release. Furthermore, the composition may include suspending, solubilizing, stabilizing, pH-adjusting agents, tonicity adjusting agents, and/or dispersing agents.

As indicated above, the pharmaceutical compositions according to the invention may be in a form suitable for sterile injection. To prepare such a composition, the suitable active agent(s) are dissolved or suspended in a parenterally acceptable liquid vehicle. Among acceptable vehicles and solvents that may be employed are water, water adjusted to a suitable pH by addition of an appropriate amount of hydrochloric acid, sodium hydroxide or a suitable buffer, 1,3-butanediol, Ringer's solution, dextrose solution, and isotonic sodium chloride solution. The aqueous formulation may also contain one or more preservatives (e.g., methyl, ethyl or n-propyl p-hydroxybenzoate). In cases where one of the compounds is only sparingly or slightly soluble in water,

a dissolution enhancing or solubilizing agent can be added, or the solvent may include 10-60% w/w of propylene glycol or the like.

The administration of a parenteral composition or formulation that includes a compound of the invention may be administered to a patient over a time period that is, for example, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, or 120 minutes or, for example, over 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0 hours.

Controlled release parenteral compositions

Controlled release parenteral compositions may be in form of aqueous suspensions, microspheres, microcapsules, magnetic microspheres, oil solutions, oil suspensions, or emulsions. The composition may also be incorporated in biocompatible carriers, liposomes, nanoparticles, implants, or infusion devices.

Materials for use in the preparation of microspheres and/or microcapsules are, e.g., biodegradable/bioerodible polymers such as polygalactin, poly-(isobutyl cyanoacrylate), poly(2-hydroxyethyl-L-glutamnine), poly(lactic acid), polyglycolic acid, and mixtures thereof. Biocompatible carriers that may be used when formulating a controlled release parenteral formulation are carbohydrates (e.g., dextrans), proteins (e.g., albumin), lipoproteins, or antibodies. Materials for use in implants can be non-biodegradable (e.g., polydimethyl siloxane) or biodegradable (e.g., poly(caprolactone), poly(lactic acid), poly(glycolic acid) or poly(ortho esters)) or combinations thereof.

Solid Dosage Forms for Oral Use

Formulations for oral use include tablets containing the active ingredient(s) in a mixture with non-toxic pharmaceutically acceptable excipients, and such formulations are known to the skilled artisan (e.g., U.S. Patent Nos.: 5,817,307, 5,824,300, 5,830,456, 5,846,526, 5,882,640, 5,910,304,

6,036,949, 6,036,949, 6,372,218, hereby incorporated by reference). These excipients may be, for example, inert diluents or fillers (e.g., sucrose, sorbitol, sugar, mannitol, microcrystalline cellulose, starches including potato starch, calcium carbonate, sodium chloride, lactose, calcium phosphate, calcium sulfate, or sodium phosphate); granulating and disintegrating agents (e.g., cellulose derivatives including microcrystalline cellulose, starches including potato starch, croscarmellose sodium, alginates, or alginic acid); binding agents (e.g., sucrose, glucose, sorbitol, acacia, alginic acid, sodium alginate, gelatin, starch, pregelatinized starch, microcrystalline cellulose, magnesium aluminum silicate, carboxymethylcellulose sodium, methylcellulose, hydroxypropyl methylcellulose, ethylcellulose, polyvinylpyrrolidone, or polyethylene glycol); and lubricating agents, glidants, and anti-adhesives (e.g., magnesium stearate, zinc stearate, stearic acid, silicas, hydrogenated vegetable oils, or talc). Other pharmaceutically acceptable excipients can be colorants, flavoring agents, plasticizers, humectants, buffering agents, and the like.

The tablets may be uncoated or they may be coated by known techniques, optionally to delay disintegration and absorption in the gastrointestinal tract and thereby providing a sustained action over a longer period. The coating may be adapted to release the agent in a predetermined pattern (e.g., in order to achieve a controlled release formulation) or it may be adapted not to release the agent(s) until after passage of the stomach (enteric coating). The coating may be a sugar coating, a film coating (e.g., based on hydroxypropyl methylcellulose, methylcellulose, methyl hydroxyethylcellulose, hydroxypropylcellulose, carboxymethylcellulose, acrylate copolymers, polyethylene glycols, and/or polyvinylpyrrolidone), or an enteric coating (e.g., based on methacrylic acid copolymer, cellulose acetate phthalate, hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, polyvinyl acetate phthalate, shellac, and/or ethylcellulose). Furthermore, a time delay material such as, e.g., glyceryl monostearate or glyceryl distearate, may be employed.

The solid tablet compositions may include a coating adapted to protect the composition from unwanted chemical changes, (e.g., chemical degradation prior to the release of the active substances). The coating may be applied on the solid dosage form in a similar manner as that described in *Encyclopedia of Pharmaceutical Technology*, supra.

The compositions of the invention may be mixed together in the tablet, or may be partitioned. In one example, a first agent is contained on the inside of the tablet, and a second agent is on the outside, such that a substantial portion of the second agent is released prior to the release of the first agent.

Formulations for oral use may also be presented as chewable tablets, or as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent (e.g., potato starch, lactose, microcrystalline cellulose, calcium carbonate, calcium phosphate, or kaolin), or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil. Powders and granulates may be prepared using the ingredients mentioned above under tablets and capsules in a conventional manner using, e.g., a mixer, a fluid bed apparatus, or spray drying equipment.

Dosage Regimens

The dosage of any compound, conjugate, or composition described herein or identified using the methods described herein depends on several factors, including: the administration method, the disease (e.g., cancer) to be treated, the severity of the disease, whether the cancer is to be treated or prevented, and the age, weight, and health of the subject to be treated.

With respect to the treatment methods of the invention, it is not intended that the administration of a vector, conjugate, or composition to a subject be limited to a particular mode of administration, dosage, or frequency of dosing; the invention contemplates all modes of administration. The conjugate, or composition may be administered to the subject in a single dose or in multiple doses. For example, a compound described herein or identified using screening

methods of the invention may conjugate be administered, for example, once a week for, e.g., 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, or more weeks. A compound of the invention may also be administered, for example, daily for 1, 2, 3, 4, 5, 6, or 7 days or for 1, 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, or more weeks.

Time periods during which the compound of the invention are administered may be followed or preceded by time periods during which the compound is not administered. For example, following administration of the compound as described herein, the compound of the invention is not administered to the patient for 1, 2, 3, 4, 5, 6, or 7 days or for 1, 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, or more weeks. In some embodiments, the patient can receive other therapeutic agents during said time period. These cycles of chemotherapy that include a period of time during which the compound of the invention is administered to a patient that is followed by a period of time during which the compound of the invention is not administered to said patient can be repeated as medically necessary (for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 times).

It is to be understood that, for any particular subject, specific dosage regimes should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the vector, conjugate, or composition. For example, the dosage of a conjugate can be increased if the lower dose does not provide sufficient activity in the treatment of a disease or condition described herein (e.g., cancer). Conversely, the dosage of the compound can be decreased if the disease (e.g., cancer) is reduced or eliminated.

While the attending physician ultimately will decide the appropriate amount and dosage regimen, a therapeutically effective amount of a compound, vector, conjugate, or composition described herein, may be, for example, in the range of 0.0035 μg to 20 μg/kg body weight/day or 0.010 μg to 140 μg/kg body weight/week. Desirably a therapeutically effective amount is in the range of 0.025 μg to 10 μg/kg, for example, at least 0.025, 0.035, 0.05, 0.075, 0.1, 0.25,

0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, or 9.0 μg/kg body weight administered daily, every other day, or twice a week. In addition, a therapeutically effective amount may be in the range of 0.05 μg to 20 μg/kg, for example, at least 0.05, 0.7, 0.15, 0.2, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, 14.0, 16.0, or 18.0 μg/kg body weight administered weekly, every other week, or once a month. Furthermore, a therapeutically effective amount of a compound may be, for example, in the range of 0.100 mg/m^2 to 2000 mg/m^2 administered every other day, once weekly, or every other week. In a desirable embodiment, the therapeutically effective amount is in the range of 1 mg/m^2 to 1000 mg/m^2, for example, at least 100, 150, 400, or 800 mg/m^2 of the compound administered daily, every other day, twice weekly, weekly, or every other week.

For example, the compounds of the invention (e.g., Compound (1)) can be used to administer podophyllotoxin derivatives (e.g., a compound of Formula (I) such as etoposide, etoposide phosphate, $etoposide_{DMG}$, and teniposide) or to administer doxorubicin or doxorubicin derivatives (e.g., Compound (2) or a compound of the invention that includes a compound of Formula (II))) to a patient using any of the methods of administration, dosage amounts, and dosage schedules described herein. Because the compounds of the invention can include covalent bonds to, for example, 1, 2, 3, 4, or 5 molecules of a podophyllotoxin derivative such as those of Formula (I) (e.g., etoposide, etoposide phosphate, $etoposide_{DMG}$, and teniposide) or of Formula (II) (e.g., doxorubicin or epirubicin), this stoichiometry can be used to calculate and to adjust the dosage amount to be administered ("equivalent dose"). For example, the Etoposide:Angiopep-2 (3:1) conjugate ("Etop-An2(3:1)") has a molecular weight of 4354 g/mol, with the etoposide content accounting for 40% of the molecular weight. Similarly, the Doxorubicin:Angiopep-2 (3:1) conjugate has a molecular weight of 4178 g/mol, with the doxorubicin accounting for 40% of the molecular weight. Using this information, the amount of a compound of the invention that is included in a composition for

administration (e.g., parenteral or oral) may be calculated in order to administer 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 mgs/m^2/day of a podophyllotoxin derivative (e.g., etoposide, etoposide phosphate, $etoposide_{DMG}$, or etoposide) to a patient. This dosage can be administered to a patient daily for 2, 3, 4, 5, 6, or 7 days. The administration period can be followed by 1, 2, 3, 4, 5, 6, or 7 days or by 2, 3, 4, 5, 6, 7, or 8 weeks of rest without administration of a compound of the invention, with the cycle of administration periods/rest periods repeated for, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 additional times.

The compounds of the invention (e.g., Compounds (1) and (2), or a pharmaceutically acceptable salt thereof) can show improved physicochemical and pharmaceutical properties relative to the respective unconjugated therapeutic agent (e.g., etoposide, etoposide 4-dimethylglycine, or doxorubicin). For example, increased targeting of exemplary cell types, tissues, or organs as described herein (e.g., brain, ovary, liver, lung, kidney, spleen, or muscle) allows for subtherapeutic doses of the compound (e.g., Compounds (1) or (2)) to be administered to a patient. Compounds of the invention can also exhibit reduced toxicity relative to the respective unconjugated therapeutic agent and thus allow for supertherapeutic doses of the compound to be administered to a patient. For example, unconjugated doxorubicin is typically administered in dose schedules that range from 60-75 mg/m^2 when administered alone as a single intravenous injection or from 40-50 mg/m^2 when administered in combination with another chemotherapeutic agent. Typical dose schedules for unconjugated etoposide or etoposide phosphate can range from 1-5 mg/m^2/day, 1-50 mg/m^2/day, 35-50 mg/m^2/day, or 50-100 mg/m^2/day. Improved targeting of cells types, tissues, or organs by the compounds of the invention (e.g., Compounds (1) or (2)) can allow for reduced doses of the therapeutic agent relative to those used for the corresponding unconjugated

therapeutic agent ("subtherapeutic doses," i.e., an effective dose that is, for example, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 2000, 3000, 4000, or 5000 times less than the minimum effective dose of the corresponding unconjugated therapeutic agent). Reduced toxicity that can be associated with the compounds of the invention (e.g., Compounds (1) or (2)) can allow for increased doses to be safely administered to a patient relative to those used for the corresponding unconjugated therapeutic agent ("supertherapeutic doses," i.e., an effective dose that is, for example, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 2000, 3000, 4000, or 5000 times more than the maximum allowed dose of the unconjugated therapeutic agent). Similarly, improved physicochemical properties can also affect the doses administered to a patient. For example, improved solubility can allow for the administration of subtherapeutic doses. These improved physicochemical pharmaceutical characteristics can allow for the safe administration of the compounds of the invention (e.g., Compounds (1) or (2)) to, for example, pediatric or geriatric patient populations.

The following examples are intended to illustrate, rather than limit the invention.

EXAMPLES

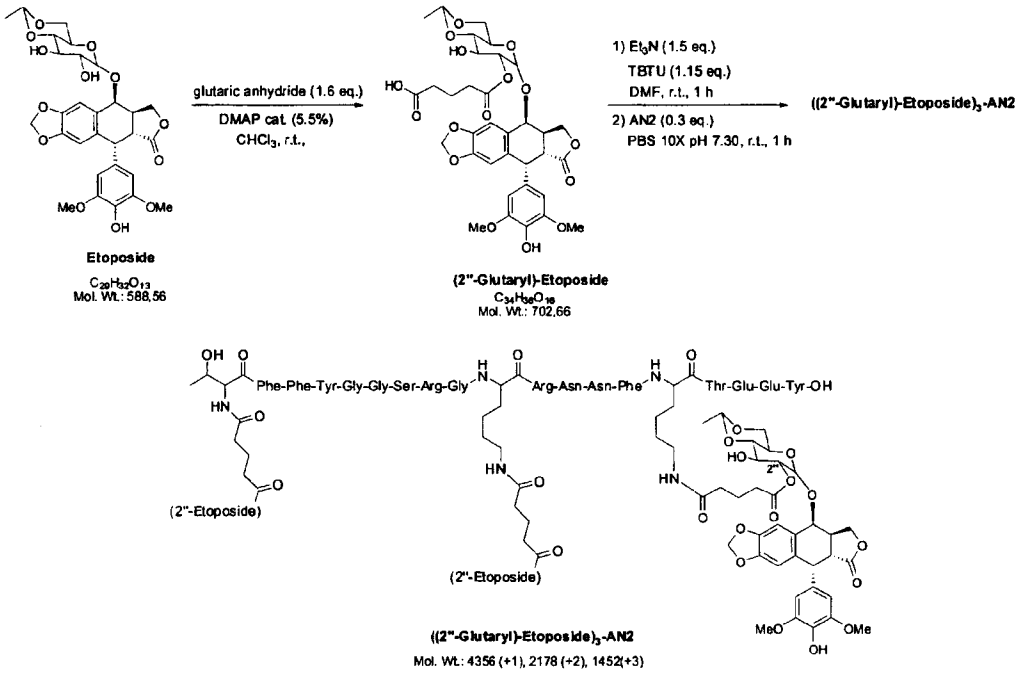
Example 1: Synthesis of a 3:1 Etoposide:Angiopep-2 Conjugate

Synthesis of the compounds of the invention can be accomplished by the combination of a podophyllotoxin derivative (e.g., a compound of Formula (I) such as etoposide, etoposide phosphate, etoposide$_{DMG}$, or teniposide), a difunctional hydrolyzable linking group (e.g., a dicarboxylic acid or a diisocyanate), and any amino acid sequence described herein, or a functional derivative thereof. Variation of the equivalents of the derivatized

podophyllotoxin intermediate (e.g., 2"-glutaryl etoposide) relative to the polypeptide can allow the synthesis of polypeptide conjugates with different stoichiometries (for example, "Etop-An2(1:1)" or "Eto-An2(1:1)", where one molecule of etoposide is bound to the Angiopep-2 polypeptide).

Scheme 5 shows the synthesis of a compound that includes an Angiopep-2 polypeptide covalently bonded to three etoposide molecules ("Etop-An2(3:1)"or ("Eto-An2(3:1)").

Scheme 5



(2''-Glutaryl)-Etoposide. To a solution of Etoposide (10g, 17 mmol) in $CHCl_3$ (120 mL) were added anhydride glutaric (3.13g, 27.4 mmol) and DMAP (115 mg, 0.942 mmol). After 72 hours at room temperature, the mixture was evaporated and purified by reverse phase chromatography using a polystyrene/DVB column (15 to 35 % acetonitrile (ACN) in H_2O, no trifluoroacetic acid (TFA)). HPLC of the crude product showed a mixture of regioisomers 2''-Glutaryl Etoposide (2''-glu-Etop) and 3''-Glutaryl Etoposide (3''-glu-Etop) in a 2:1 ratio. After evaporation and lyophilisation, (2''-Glutaryl)-Etoposide was obtained as a white powder (4.1g, 34 %). The

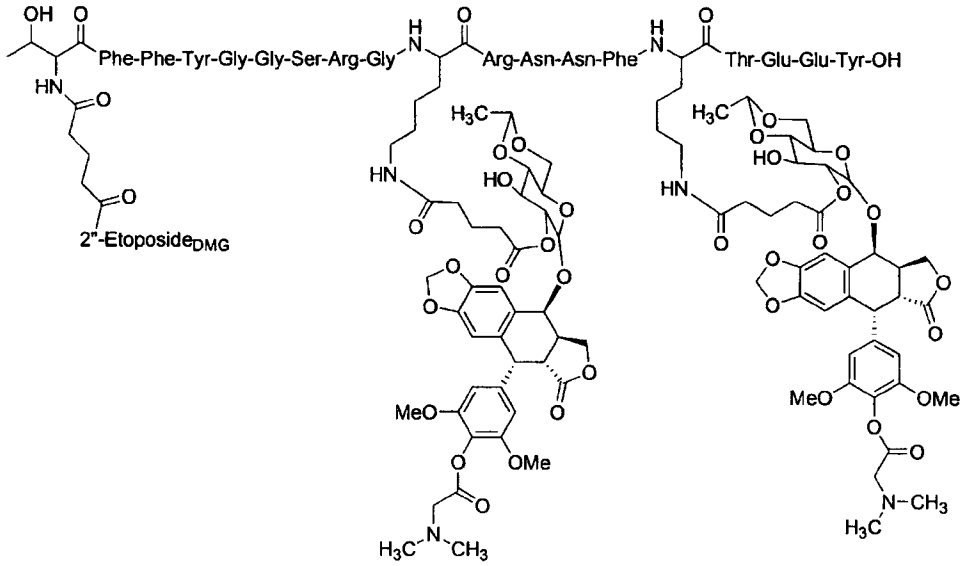
regioisomeric (3''-glutaryl)-etoposide was also isolated as a white solid (2.4 g, 20 %). The purities of 2''-glu-Etop and 3''-glu-Etop were determined by RP-HPLC. Using a MetaChem Taxsil-3 column and gradient elution (1 mL/min; 10% to 65% (0.05% TFA in H_2O):(0.05% TFA in ACN) over 15 minutes), 2''-glu-Etop has a retention time of 9.00 minutes and 3''-glu-Etop has a retention time of 9.42 minutes. The purity of 2''-glu-Etop (retention time = 9.00 minutes) was >98% (99.4%) and the purity of 3''-glu-Etop was >98% (99.6%).

((2''-Glutaryl)-Etop)$_3$-(Angiopep2) (Etop-An2(3:1)). To a solution of (2''-Glutaryl)-Etoposide (2.83 g, 4.025 mmol) in anhydrous DMF (400 mL) were added triethylamine (Et_3N; 0.84 mL, 6.037 mmol) and *N,N,N',N'*-Tetramethyl-O-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU; 1.32 g, 4.628 mmol). The reaction mixture was stirred at room temperature for 1 hour (pH = 9.3). A solution of AN2 (Angiopep-2, 75% in content, 3.68 g, 1.207 mmol) in PBS 10X buffer (pH 7.3; 200 mL) was prepared by adjusting pH with 10N NaOH (to pH 7.3). After cooling with dry iced bath, the previously activated acid was added (4 x 100 mL) to Angiopep-2, and the pH of the mixture was adjusted to 7.2 by addition of 10N NaOH. After 1 hour at room temperature, the reaction was filtered to eliminate phosphate salts and evaporated to obtain 35 mL of the crude mixture. HPLC of the crude product showed a mixture of (3:1)- and (2:1) conjugates in a 3:1 ratio. The residue was purified by reverse phase chromatography using a polystyrene/DVB column (15 to 37.5 % ACN in H_2O). After purification, 0.1 % acetic acid was added to the combined fractions in order to increase the solubility. Evaporation and lyophilisation of the mixture afforded the Etop-An2(3:1) product as a white solid (596 mg, 12 %). Using a MetaChem Taxsil-3 column and gradient elution (1 mL/min; 10% to 65% (0.05% TFA in H_2O):(0.05% TFA in ACN) over 15 minutes), the isolated product was found to have a retention time of 9.36 minutes and to be >95% (97.3%) pure. *m/z* (ESI-TOF) : 2178 (+2), 1452 (+3).

Example 2: Synthesis of of a 3:1 Etoposide$_{DMG}$:Angiopep-2 Conjugate

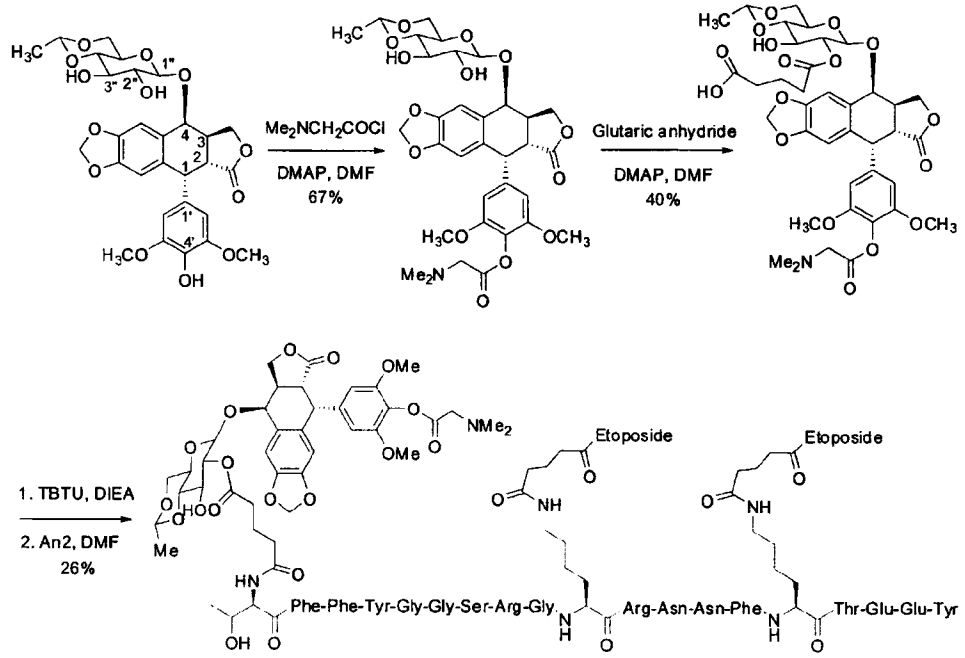
The procedure described in Example 1 can be used to prepare compounds that include peptides conjugated to other podophyllotoxin derivatives. For example, Etoposide$_{DMG}$ can be used to prepare the conjugate shown in **Scheme 7** ("Etop$_{DMG}$-An2 (3:1)") using the synthetic procedure shown in **Scheme 6**.

Scheme 6



((2"-Glutaryl)-Etoposide$_{DMG}$)$_3$-AN2

Scheme 7



Etoposide 4'-Dimethylglycine: A mixture of etoposide (235 mg, 0.4 mmol) and DMAP (73 mg, 0.6 mmol) in DMF (4 mL) was stirred at room temperature for 20 minutes, and then N,N-dimethylacetyl chloride (96 mg, 0.52 mmol) was added in one pot with stirring. After 30 minutes, the reaction was complete according to HPLC. Formic acid (1M in DMF, 0.5 mL) was added, and the solvent was concentrated to 1 mL. The resulting solution was loaded onto an AKTA RPC column for purification (gradient 10% to 30% MeCN in H_2O with 0.1% Formic acid). After lyophilization, etoposide 4'-dimethylglycine ("Etoposide$_{DMG}$" or "Etop$_{DMG}$"; 180 mg, 67%) was obtained as a colorless powder. ^{1}H NMR (CD_3OD) δ 7.01 (1H, s), 6.56 (1H, s), 6.39 (2H, s), 5.98 (2H, d, J = 2.9 Hz), 5.05 (1H, d, J = 3.4 Hz), 4.77 (1H, q, J = 4.9 Hz), 4.68 (1H, d, J = 5.4 Hz), 4.66 (1H, d, J = 7.8 Hz), 4.46 (2H, s), 4.45 (1H, dd, J = 10.3, 8.8 Hz), 4.31 (1H, t, J = 8.0 Hz), 4.17 (1H, dd, J = 10.3, 4.9 Hz), 3.68 (6H, s), 3.56 (1H, q, J = 10 Hz), 3.54 (1H, t, J = 9.3 Hz), 3.52 (1H, dd, J = 14.2, 5.6 Hz), 3.32 (1H, m), 3.26 (1H, dd, J = 9.1, 4.1 Hz), 3.24 (1H, dd, J = 9.2, 5.4 Hz), 3.02 (6H, s), 2.96 (1H, m), 1.33 (3H, d, J = 4.9 Hz). ^{13}C NMR

(DMSO) δ 175.26, 168.68, 151.35, 148.49, 147.01, 139.39, 132.74, 129.6, 127.28, 110.65, 110.45, 108.02, 102.19, 102.02, 99.25, 80.78, 75.06, 73.39, 72.41, 68.43, 68.01, 66.44, 59.73, 56.63, 56.47, 45.03, 43.86, 37.89, 20.99; HRMS (MicroTOF) calcd. for $C_{33}H_{39}NO_{14}$ 673.2371, found 274.2534 (M+1).

Etoposide 4'-Dimethylglycine 2''-Glutaric acid: A mixture of etoposide 4'-dimethylglycine (655 mg, 0.97 mmol) and DMAP (18 mg, 0.15 mmol) in chloroform (11 mL) was cooled to 0 °C. DMF (3 mL) and N,N-diisopropylethylamine (DIEA; 0.25 mL, 1.46 mmol) were added consecutively, followed by glutaric anhydride (222 mg, 1.94 mmol). The reaction mixture was stirred at room temperature, monitored by HPLC. After 2 days, the solvent was concentrated to 3 mL. The resulting solution was loaded to an AKTA RPC column for purification (gradient elution, 10% to 30% MeCN in H_2O), and Etoposide 4'-Dimethylglycine 2''-Glutaric acid (305 mg, 40%) was obtained as a white powder after lyophilization. ^{1}H NMR (CD_3OD) δ 7.0 (1H, s), 6.53 (1H, s), 6.39 (2H, s), 5.99 (2H, d, J = 4.6 Hz), 4.97 (1H, q, J = 7.9 Hz), 4.78 (1H, q, J = 4.75 Hz), 4.74 (1H, d, J = 7.9 Hz), 4.68 (1H, d, J = 5.6 Hz), 4.45 (2H, s), 4.41 (1H, dd, J = 9.6, 8.8 Hz), 4.29 (1H, t. J = 8.2 Hz), 4.15 (1H, dd, J = 10.0, 4.5 Hz), 3.78 (1H, t, J = 9.4 Hz), 3.69 (6H, s), 3.61 (1H, t, J = 10.2 Hz), 3.42 (1H, td, J = 9.6, 5.2 Hz), 3.33 (1H, dd, J = 8.7, 8.2 Hz), 3.3 (1H, dd, J = 13.4, 5.3 Hz), 3.02 (6H, s), 2.93 (1H, m), 2.26 (1H, m), 2.16 (2H, m), 2.02 (1H, m), 1.64 (2H, m), 1.32 (3H, d, J = 4.9 Hz). ^{13}C NMR (DMSO) δ 175.96, 175.33, 172.46, 163.74, 151.14, 148.96, 147.43, 139.53, 131.90, 129.83, 126.42, 110.20, 109.18, 107.40, 101.91, 100.65, 99.63, 80.39, 74.55, 73.95, 71.55, 71.29, 68.43, 67.82, 66.46, 56.43, 55.35, 43.90, 43.15, 40.82, 38.0, 32.86, 32.59, 19.93, 19.39. HRMS (MicroTOF) calcd. for $C_{38}H_{45}NO_{17}$ 787.2687, found 788.2432 (M+1).

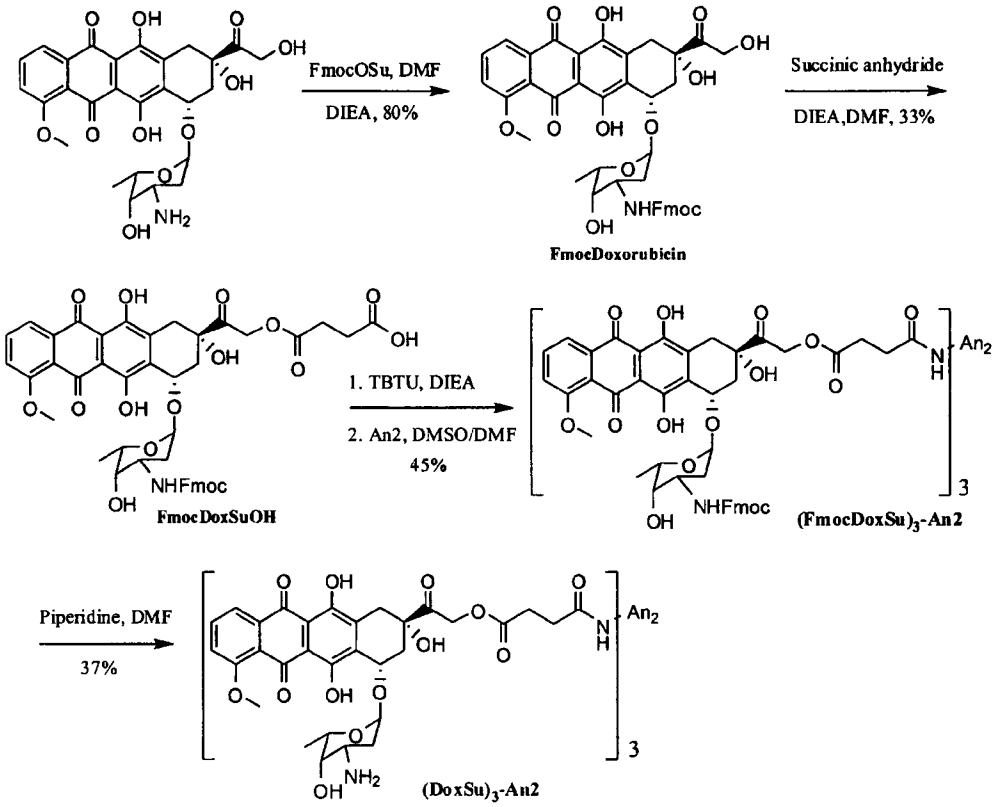
(Etoposide-4'-Dimethylglycine-2''-Glutaric)$_3$-Angiopep-2 Conjugate ("Etop-4'-DMGly-2''-Glu)$_3$-An2" or "Etop$_{DMG}$-An2(3:1)"): DIEA (0.17 mL, 0.98 mmol) was added dropwise to a mixture of Etoposide 4'-Dimethylglycine 2''-Glutaric acid (330 mg, 0.42 mmol) and TBTU (145 mg,

0.46 mmol) in DMF (24 mL). The mixture was stirred at room temperature for 50 minutes. A solution of Angpep-2 (422 mg, 0.14 mmol) in DMSO (1.5 mL) and DMF (9 mL) was then added, followed by DIEA (0.084 mL, 0.48 mmol). The mixture was stirred at room temperature for 20 minutes. An aliquot (10 mL) was taken for UPLC analysis, and it showed the reaction was complete. After stirring for another 10 minutes, the reaction solution was concentrated to 3 mL and purified using AKTA RPC column (gradient elution, 10% to 25% MeCN in H_2O with 0.05% formic acid). (Etop-4'-DMGly-2''-Glu)$_3$-An2 (172 mg, 26%) was yielded as a colorless powder after lyophilization. MS (MicroTOF), m/z, 2305.9327 (2+), 1537.6443 (3+), 1153.7463 (4+), 922.7970 (5+).

Example 3: Synthesis of of a 3:1 Doxorubicin:Angiopep-2 Conjugate ("(DoxSu)$_3$-An2")

A 3:1 doxorubicin:angiopep-2 conjugate be prepared according to the synthetic scheme shown in **Scheme 8** and described herein.

Scheme 8



FmocDoxorubicin: DIEA (1.5 mL, 8.63 mmol) was added dropwise to a solution of doxorubicin (2.0 g, 3.45 mmol) and 9-fluorenylmethyl N-succinimidyl carbonate (FmocOSu; 2.32 g, 6.9 mmol) in DMF (35 mL) with stirring. The mixture was stirred at room temperature for 3 hours and concentrated. The resulting residue was triturated with 0.1% TFA in H_2O (3 × 20 mL), washed with Et_2O (8 × 20 mL). The resulting red solid was collected and dried over vacuum to give FmocDoxorubicin as a red powder (2.1 g, 80% yield). UPLC purity, 98%. MS (ESI, MicroTOF), 788. 2411 (M + Na).

FmocDoxSuOH: DIEA (0.17 mL, 1.0 mmol) was added dropwise to a solution of FmocDoxorubicin (0.28 g, 0.366 mmol) and succinic anhydride (0.11 g, 1.1 mmol) in DMF (20 mL) under stirring. The mixture was stirred at room temperature and monitored by UPLC. After two days, the solvent was removed and the resulting residue was purified using a Biotage column (silica gel, 2% to 9% MeOH in DCM) to give FmocDoxSuOH as a red powder (100

mg, 33% yield). UPLC purity: 95%. MS (ESI, MicroTOF), 888. 2577 (M + Na).

(FmocDoxSu)$_3$-An2: DIEA (0.25 mL, 1.44 mmol) was added dropwise to a solution of FmocDoxSuOH (599 mg, 0.692 mmol) and TBTU (231 mg, 0.72 mmol) in DMF (21 mL) under stirring. The mixture was stirred at room temperature for 50 minutes and then a solution of Angpep-2 (671 mg, 0.229 mmol) in DMSO (2 mL) and DMF (12 mL) was added. The mixture was stirred at room temperature for 20 minutes, at which time HPLC showed the reaction was complete. After stirring for another 10 minutes, the solvent was removed and the residue was purified using a Biotage C18 column (40% to 80% MeCN in water and 0.05% TFA) to give (FmocDoxSu)$_3$An-2 as a red powder (500 mg, 45% yield). UPLC purity, 95%. MS (ESI, MicroTOF), *m/z* 2423.4239 (2+), 1615.6190 (3+).

(DoxSu)$_3$-An2: Piperidine (20% in DMF, 1.5 mL) was added to a solution of (FmocDoxSu)$_3$An-2 (260 mg, 0.053 mmol) in DMSO (1 mL) and DMF (12 mL). The solution became blue. After stirring for 10 minues, the solution was cooled to 0 °C and treated with formic acid (0.5 M in DMF, 6 mL) to get a clear red solution. The solvent was removed using vacuum pump, and the resulting residue was triturated with Et_2O (3 × 10 ml) and AcOEt (3 × 10 ml). The resulting red solid was purified using AKTA RPC 30 column (10% to 40% MeCN in water and 0.15% formic acid) to give (DoxSu)$_3$An-2 as a red powder (82 mg, 37% yield). UPLC purity: 95%. MS (ESI, MicroTOF), *m/z* 2089.9674 (2+), 1393.2419 (3+), 1045.4395 (4+).

Example 3: Effect of etoposide, etoposide-Angiopep conjugates, doxorubicin, and doxorubicin-Angiopep conjugates on cell proliferation

For the in vitro cell proliferation assay, between 2.5 and 5 x 10^4 of U87 or SK-HEP-1 cells were seeded in a 24 well tissue culture microplate in a final volume of 1 mL of medium with 10% serum and incubated for 24 hours at 37 °C and 5% CO_2. The medium was then replaced with serum-free medium and

incubated overnight. The next morning the agent was freshly dissolved in dimethyl sulfoxide (DMSO) and the medium was replaced with complete medium containing the agent at different concentrations in triplicate. The final concentration of DMSO was 0.1%. The control used was a microplate well with cells and without agent. The cells were incubated for 48 to 72 hrs at 37 °C and 5% CO_2. After the incubation, the medium was changed and replaced with 1 mL of complete medium containing [^{3}H]-thymidine (1 pCi/assay). The plate was incubated at 37 °C and 5% CO_2 for 4 hrs. The medium as removed, and the cells were washed with PBS at 37 °C. The cells were fixed with a mix of ethanol:acetic acid (3:1), washed with water, and precipitated 3 times with 10% of ice-cold TCA (trichloroacetic acid). Finally 500 µL of PCA (perchloric acid) were added to the wells and the microplates were heated for 30 min at 65 °C and 30 min at 75 °C. The contents of each well were then transferred to a scintillation vial with 10 mL of scintillation cocktail and the activity was measured in CPM (count per minute) on a liquid scintillation counter Tri-Carb from Packard. The results of the cell proliferation assay using unconjugated etoposide, Etop-An2(1:1), and Etop-An2(3:1) are shown in **Table 4**. **Table 5** shows the results obtained for $Etop_{DMG}$-An2(3:1), unconjugated $etoposide_{DMG}$, the doxorubicin/Angiopep-2(3:1) conjugate ("Doxorubicin-An2 (3:1)"), and unconjugated doxorubicin.

In addition to the in vitro studies, the inhibition of cell proliferation has been studied in xenograft tumor models and these results are shown in **Figure 1**. U87 glioblastoma cells (2.5 × 106) were subcutaneously implanted in the right flank of nude mice. Treatments started on day 15 after implantation (corresponding to day 0 on the graph shown in Figure 1) when tumor volume reached about 150-200 mm^3. The mice were treated once a week for three weeks by i.v. bolus injection with doxorubicin (6 mg/kg) and doxorubicin-An2 conjugate (20 and 40 mg/kg). The doxorubicin-An2 conjugate was diluted in acidified D5W (5% dextrose in water) at 5 mg/ml.

Table 4

Drug	IC_{50} (nM)		
	24 hours	48 hours	72 hours
	U-87 Cells		
Etoposide	160	221	145
Eto-An2 (1:1)	1313	722	550
Eto-An2 (3:1)	453	151	164
(Etoposide equiv.)	(1359)	(453)	(492)
	SK-HEP-1		
Etoposide	116	56	50
Eto-An2 (1:1)	679	245	153
Eto-An2 (3:1)	160	59	52
(Etoposide equiv.)	(480)	(168)	(156)

Table 5

Drug	Glioblastoma (U87)	IC_{50} (nM) Hepatocarcinoma (SK-Hep-1)	Lung Carcinoma (NCI-H460)
$Etop_{DMG}$	145	62	90
$Etop_{DMG}$-An2(3:1)	330	48	148
Doxorubicin	18	10	11
Doxorubicin-An2 (3:1)	6.0	4.6	7.3

Example 3: In situ brain perfusion studies

The procedures described in U.S. Patent Publication 20060189515, herein incorporated by reference, were used for the in situ brain perfusion studies. These procedures are further described herein.

Example 3a: Etop-An2(3:1)

The brain uptake of the compounds of the invention (e.g., Etop-An2(3:1), $Etop_{DMG}$-An2(3:1), and Doxorubicin-An2 (3:1)) relative to the corresponding unconjugated drugs were measured using in situ brain perfusion techniques described herein and in Dagenais et al., *J. Cereb. Blood Flow Metab.* 20(2):381-386 (2000). The uptake of [^{125}I]-polypeptides to the luminal side of mouse brain capillaries was measured using the in situ brain perfusion

method adapted in our laboratory for the study of agent uptake in the mouse brain.

Polypeptides were iodinated with standard procedures using iodo-beads from Sigma. Briefly polypeptides were diluted in 0.1 M phosphate buffer, pH 6.5 (PB). Two iodo-beads were used for each protein. These beads were washed twice with 3 mL of PB on a Whatman filter and re-suspended in 60 µL of PB. ^{125}I (1 mCi) from Amersham-Pharmacia biotech was added to the bead suspension for 5 min at room temperature. Each iodination was initiated by the addition of the polypeptide (100 µg). After an incubation of 10 min at room temperature, the free iodine was removed by HPLC.

Briefly, the right common carotid of ketamine/xylazine (140/8 mg/kg i.p.) anesthetized mice was exposed and ligated at the level of the bifurcation of the common carotid, rostral to the occipital artery. The common carotid was then catheterized rostrally with polyethylene tubing filled with heparin (25 U/mL) and mounted on a 26-gauge needle. The syringe containing the perfusion fluid ([^{125}I] -polypeptides or [^{14}C]-inulin in Krebs/bicarbonate buffer at a pH7.4 gassed with 95% O_2 and 5% CO_2) was placed in an infusion pump (Harvard pump PHD 2000; Harvard Apparatus) and connected to the catheter. Prior to the perfusion, the contralateral blood flow contribution was eliminated by severing heart ventricles. The brain was perfused for the indicated times at a flow rate of 1.15 mL/min. After 14.5 min of perfusion, the brain was further perfused for 60 seconds with Krebs buffer to wash the excess of [^{125}I]-proteins. Mice were then decapitated to terminate perfusion and the right hemisphere was isolated on ice before being subjected to capillary depletion. Aliquots of homogenates, supernatants, pellets and perfusates were taken to measure their contents in [^{125}I]-conjugates by TCA precipitation and to evaluate the apparent volume of distribution.

The results of these experiments are illustrated in **Figures 2A-D** and **3**. In **Figure 2A,** in situ brain perfusion shows that the Vd is higher for Etop-An2(3:1) than for the unconjugated etoposide (i.e., the observed slope for Etop-

An2(3:1) is greater than that observed for unconjugated etoposide). Similar trends are observed for $Etop_{DMG}$-An2(3:1) (**Figure 2B**) relative to unconjugated $Etoposide_{DMG}$ and for doxorubicin-An2(3:1) relative to unconjugated doxorubicin (**Figure 2C**). As can be see from **Figure 2C**, the ratio of the K_{in} for Doxorubicin-An2(3:1):unconjugated doxorubicin is 15. This procedure also distinguishes between compounds remaining in the brain vascular compartment from those having crossed the abluminal endothelial membrane to enter the brain parenchyma. **Figure 3** shows the in situ perfusion of Etop-An2(3:1). In each grouping in this graph, the left bar represents unconjugated etoposide, the middle bar represents $Etop_{DMG}$-An2(3:1) (Batch 1), and the right bar represents $Etop_{DMG}$-An2(3:1) (Batch 2). The brain repartition of Etop-An2(3:1) following brain capillary depletion is illustrated in **Figures 4A** and **4B**. Moreover, in contrast to etoposide the brain uptake of Etop-An2(3:1) is similar in wild-type and P-gp knock-out mice, indicating that Etop-An2(3:1) is not a P-gp substrate (**Figure 5**). In this figure, the left bar of each groups shows the results obtained using CD-1 mice and the right bar shows the results obtained using P-gp knockout mice. The brain uptake of Etop-An2(3:1) can be inhibited by the coadministration of an unconjugated polypeptide. **Figure 6** shows that the co-perfusion of [^{125}I]- Etop-An2(3:1) with a two-fold excess of unconjugated Angiopep-2 reduces the parenchyma Vd by 27%.

Example 3b: $Etop_{DMG}$-An2 (3:1) and Doxorubicin-An2(3:1)

The brain uptake of $Etop_{DMG}$-An2 (3:1) relative to the unconjugated $Etop_{DMG}$ was measured using the methods described for Example 3a, and these results are shown in Table 6 and in **Figure 7**. Table 6 also includes the corresponding data for Doxorubicin-An2(3:1) and doxorubicin. In contrast to unconjugated $Etop_{DMG}$, the brain uptake of doxorubicin-An2(3:1) is similar in wild-type and P-gp knock-out mice, indicating that doxorubicin-An2(3:1) is not a P-gp substrate.

Table 6

Drug	Brain K_{in} (ml/s/g)
$Etop_{DMG}$-An2	1.4×10^{-3}
$Etop_{DMG}$	9.0×10^{-5}
Doxorubicin-An2(3:1)	3.7×10^{-3}
Doxorubicin	2.8×10^{-4}

Example 4: Plasma kinetics of the 3:1 etoposide-Angiopep-2 conjugate

Figure 8 describes the plasma kinetics of Etop-An2(3:1)following administration as a bolus. Radiolabeled (^{125}I)Etop-An2 (20 mg/kg) was injected in bolus by intravenous (i.v.) or intraperitoneal (i.p.) routes in CD-1 mice weighing about 25-30 g. The injection solution was composed of 12.5% dimethylsulfoxide (DMSO), 12.5% anhydrous ethanol 25% polyethylene glycol 400 (PEG400) and 50 % NaCl/Glycine buffer. At several time intervals (0.5, 1, 2, and 6 hours) the blood was collected by cardiac puncture and animals were sacrificed. After blood centrifugation, the plasma radioactivity was measured in a gamma counter (Wizard 1470 Automatic Gamma Counter). The radioactivity was interpreted as % of injected dose per gram of plasma. Results were plotted using GraphPad prism software and the area under the curve (AUC) for each injection mode was calculated. The bioavailability of intraperitoneal Etop-An2 conjugate was then estimated by dividing the AUC after i.p. injection by the AUC after i.v. injection. The estimated bioavailability following i.p. administration is calculated as 46%. Pharmacokinetic parameters of Etop-An2(3:1)following IV bolus administration in mice are shown in **Table 7**. Literature data for unconjugated etoposide shows a $T_{1/2\alpha}$ = 0.13 hour (Reddy et al., *Journal of drug targeting*, 13(10): 543-553 (2005)).

Table 7

Molecule	Dose (mg/kg)	T_{max} (min)	C_{max} (µg/mL)	$T_{1/2\alpha}$ (hr)	$AUC_{0-\infty}$ (hr°µg/mL)	Elim. Rate Const. (hr^{-1})
Etop-An2 (3:1)	20	5	46	0.43	82	1.6

Example 5: Tissue distribution of 3:1 etoposide$_{DMG}$-Angiopep-2 conjugate and 3:1 etoposide-Angiopep-2 conjugate

The effect of conjugation of an agent to a vector on distribution of the agent or the pharmacokinetics of a polypeptide that is conjugated to an agent was evaluated by administering a labeled polypeptide or conjugate to an animal and measuring distribution of the polypeptide or conjugate to organs (e.g., using ^{3}H or ^{125}I labeled conjugates) to mice. Similar experiments can be performed with compounds that include any of polypeptides described herein (e.g., the polypeptides described in Table 1 such as AngioPep-3, AngioPep-4a, AngioPep-4b, AngioPep-5, AngioPep-6, and AngioPep-7, or analogs thereof). Here, the unconjugated anticancer agent and the conjugates were injected intravenously to mice as a bolus. Tissues were collected at different times (0.25, 0.5, 1, and 4 hrs) and homogenized. To quantify the amount of ^{3}H-labeled conjugate, tissue homogenates were digested with tissue solubilizer, and 10 mL of liquid scintillator was added to samples. The amount of the ^{125}I labeled conjugate, in the different tissues is measured after TCA precipitation. Radioactivity associated with the tissues is quantified. The area under the curve (AUC 0-4) is estimated using the Prism software and is plotted for the different tissues.

Figure 9 shows the brain distribution of Etop$_{DMG}$-An2 following IV bolus administration in mice. In this figure, the left bar of each grouping shows results obtained with unconjugated etoposide and the right bar shows results obtained with Etop-An2(3:1). **Figure 10** shows the brain distribution of Etop-An2(3:1) following IV or IP bolus administration in mice. **Figure 11** compares the brain distribution of Etop-An2(3:1) versus unconjugated etoposide thirty minutes after IV bolus administration to mice. **Figure 12** shows the tissue distribution of Etop-An2(3:1) compared to unconjugated etoposide. In this figure, the left bar of each grouping shows results obtained with unconjugated etoposide and the right bar shows results obtained with

Etop-An2(3:1); use of the Etop-An2(3:1) leads to increased concentration in the tissues studied.

Example 6: Anti-Tumor Effect of Doxorubicin-An2(3:1) Conjugate in a Mouse Model of Human Brain Tumor

All animals used in these studies were handled and maintained in accordance to the Guidelines of the Canadian Council on Animal Care (CCAC). Animal protocols were approved by the Institutional Animal Care and Use Committee of Université du Québec à Montréal.

The intracerebral human brain tumor model was established by stereotactic inoculation of $5x10^5$ U87 cells in nude mice brain. Female athymic nude mice (Crl:Nu/Nu-*nu*BR; 20-25g, 4-6 weeks old; Charles River Canada, St-Constant, QC) were used for tumor models and were maintained in a pathogen-free environment. One hour before surgery, mice received a subcutaneous injection of buprenorphine (0.1 mg kg^{-1}). For tumor cell inoculation, mice were anesthetized by i.p. injection of ketamine/xylazine (120/10 mg kg^{-1}) and placed in a stereotactic apparatus (Kopf; Tujunga, CA). A burr hole was drilled 1.5 mm anterior and 2.5 mm lateral to the bregma. The cell suspension in 5 µL of serum free cell culture medium was injected over a 5 minute period using a Hamilton syringe at a depth of 3.5 mm.

Drug treatment started 3 days post-inoculation (**Table 8**). The therapeutic compound (e.g., doxorubicin or doxorubicin-An2(3:1) conjugate) was given intravenously by bolus tail-vein injection (once per week). Drug solutions were prepared in dextrose 5% water (D5W). Injection solutions were freshly prepared before each administration. Clinical signs of disease progression and body weights were monitored everyday. When mice reached terminal endpoints (20% of decrease in body weight), they were sacrificed by carbon dioxide asphyxiation.

Table 8

Compound	Dose (mg/kg)	Administration	Survival (days)		
			Median		Mean
			Trial 1	Trial 2	Trial 1
Control	0	i.v.	18	22	18.5
Doxorubicin	6	i.v. (1× per week)	21	23	20.6
$(DoxSu)_3$-An2	40	i.v. (1× per week)	22	28	21.1
$(DoxSu)_3$-An2 + Angiopep2+paclitaxel conjugate	60 + 40	i.p. (2× per week) + i.v. (1× per week)	22	--	22.4

Each of **Figures 13A** and **13B** show the efficacy of the $(DoxSu)_3$-An2 conjugate when administered alone or in combination with a paclitaxel-Angiopep2 conjugate. **Figure 13A** shows results obtained in one trial and **Figure 13B** shows results obtained in a second set of experiments. Statistical analysis of the data obtained from Trial 2 (**Fig. 13B**) showed that the observed 27% improvement was statistically significant ($p < 0.007$).

Other embodiments

The content of each publication, patent, and patent application mentioned in the present application is incorporated by reference. Although the invention has been described in details herein and illustrated in the accompanying drawings, it is to be understood that the invention is not limited to the embodiments described herein and that various changes and modifications may be effected without departing from the scope or spirit of the invention.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come

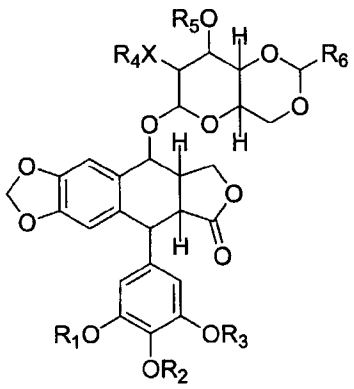
within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

What is claimed is:

5

CLAIMS

1. A compound, or a pharmaceutically acceptable salt thereof, comprising an amino acid sequence substantially identical to an amino acid sequence selected from the group consisting of SEQ ID NOS:1-105 and 107-116, or a functional derivative thereof, wherein said amino acid sequence comprises a covalent bond from an amino acid of said amino acid sequence to a podophyllotoxin derivative, and wherein said podophyllotoxin derivative is a compound having a structure according to Formula (I)



(I),

or a stereoisomer or a pharmaceutically acceptable salt thereof, wherein

each R_1, R_2, and R_3 is selected, independently, from H, optionally substituted C_{1-6} alkyl, $C(O)R_8$, $P(O)(OR_9)(OR_{10})$, $S(O)_2(OR_9)$, or a hydrolyzable linker Y that comprises a covalent bond to an amino acid of the polypeptide;

X is O or NR_7;

each R_4, R_5, and R_7 is selected, independently, from H, optionally substituted C_{1-6} alkyl, $C(O)R_8$, or a hydrolyzable linker Y that comprises a covalent bond to an amino acid of the polypeptide;

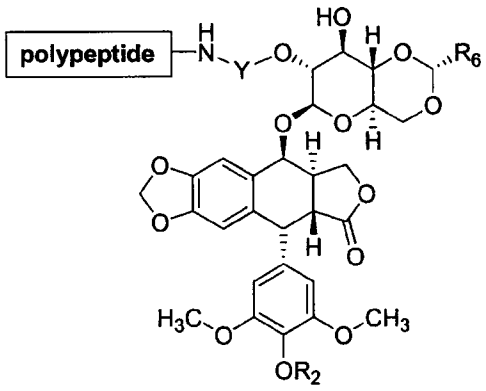
R_6 is H, optionally substituted C_{1-6} alkyl, optionally substituted aryl, optionally substituted heteroaryl,

R_8 is selected from optionally substituted C_{1-6} alkyl or optionally substituted aryl;

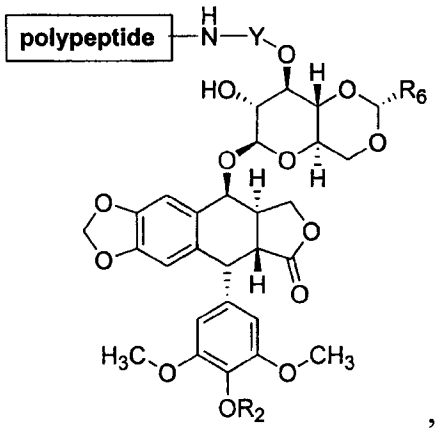
each R_9 and R_{10} is selected, independently, from H, optionally substituted C_{1-6} alkyl, or optionally substituted aryl; and

wherein one and only one of R_1, R_2, R_3, R_4, R_5, and R_7 is Y.

2. The compound of claim 1, wherein each compound of Formula (I) is selected, independently, from



and



,

wherein

each R_2 is, independently, H, $P(O)(OH)_2$, or $-C(O)R_8$;

each R_6 is, independently, CH_3 or 2-thiophene;

each R_8 is, independently, optionally substituted C_{1-6} alkyl;

each Y is $-C(O)(CH_2)_nC(O)-$; and

each n is, independently, 2, 3, or 4; and

wherein each Y is covalently bound to an amino acid.

3. The compound of claim 1 or 2, wherein Y is $-C(O)(CH_2)_nC(O)-$ and n is 2, 3, or 4.

4. The compound of claim 1 or 2, wherein each R_2 is $-C(O)CH_2N(CH_3)_2$.

5. The compound of claim 1 or 2, wherein said amino acid sequence is covalently bonded to a compound having a structure according to Formula (I) through a second amino acid of said amino acid sequence.

6. The compound of claim 5, wherein said amino acid sequence is covalently bonded to a compound having a structure according to Formula (I) through a third amino acid of said amino acid sequence.

7. The compound of claim 1, wherein each amino acid covalently bonded to said hydrolyzable linker Y is attached via an amino-, a guanidino-, a hydroxyl-, a phenol-, or a thiol functional group of said amino acid.

8. The compound of claim 7, wherein each amino acid covalently bonded to said hydrolyzable linker Y is attached via an amino functional group.

9. The compound of claim 7, wherein said amino acid is lysine or threonine.

10. The compound of claim 1 or 2, wherein said amino acid sequence has at least 50% identity to an amino acid sequence selected from the group consisting of Angiopep-2 (SEQ ID NO:97), Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7 (SEQ ID NOS:109-112).

11. The compound of claim 10, wherein said amino acid sequence has at least 70% identity to the amino acid sequence of Angiopep-2 (SEQ ID NO:97).

12. The compound of claim 10, wherein said amino acid sequence has at least 90% identity to the amino acid sequence of Angiopep-2 (SEQ ID NO:97).

13. The compound of claim 1 or 2, wherein said amino acid sequence comprises the amino acid sequence of Angiopep-2 (SEQ ID NO:97), Angiopep-4b, Angiopep-5, Angiopep-6, or Angiopep-7 (SEQ ID NOS:109-112).

14. The compound of claim 13, wherein said amino acid sequences consists of the amino acid sequence of Angiopep-2 (SEQ ID NO:97), Angiopep-4b, Angiopep-5, Angiopep-6, or Angiopep-7 (SEQ ID NOS:109-112).

15. The compound of claim 14, wherein said amino acid sequence consists of the amino acid sequence of Angiopep-2 (SEQ ID NO:97).

16. The compound of claim 12, having the following structure

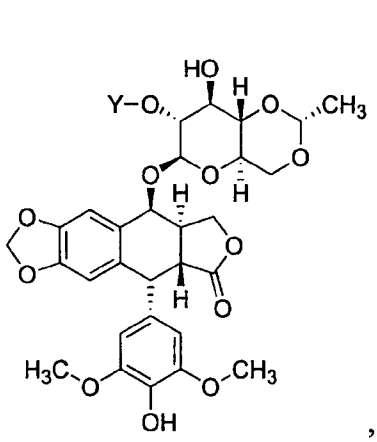
T F F Y G G S R G K R N N F K T E E Y
| | |
Formula (I) Formula (I) Formula (I)

wherein each (—(Formula(I)) represents an optional covalent bond between the indicated amino acid and a compound of Formula (I), or a pharmaceutically acceptable salt thereof, and wherein there is at least one covalent bond between an amino acid of the polypeptide and said compound of Formula (I).

17. The compound of claim 16, wherein the threonine at position 1 and the lysines at positions 10 and 15 of the polypeptide each comprise a covalent bond to a compound having a structure according to Formula (I).

18. The compound of claim 16, wherein each R_2 is, independently, H or $P(O)(OH)_2$.

19. The compound of claim 16, wherein each compound of Formula (I) has the following structure



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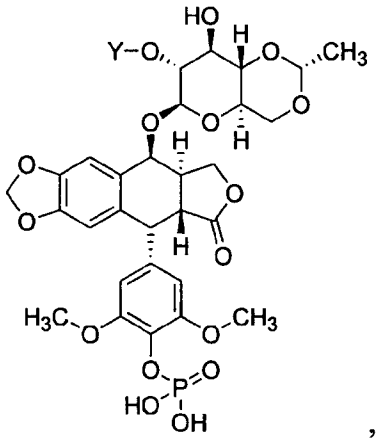
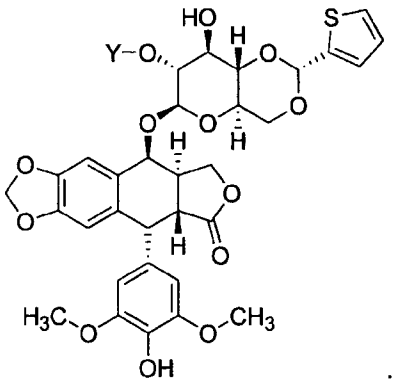


, or



.

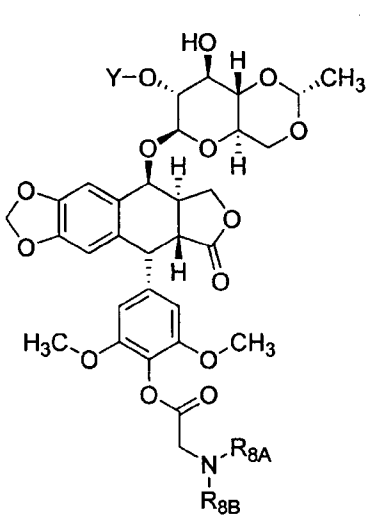
20. The compound of claim 19, wherein n is 3.

21. The compound of claim 16, wherein each R_2 is a C-linked α-amino acid.

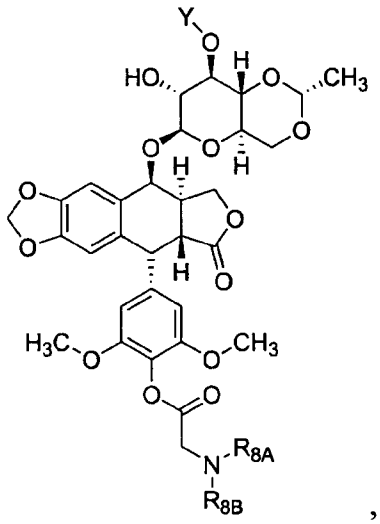
22. The compound of claim 16, wherein R_8 is a C_{1-6} alkyl comprising an amino substituent.

23. The compound of claim 16, wherein $-C(O)R_8$ is dimethylglycine.

24. The compound of claim 16, wherein each compound of Formula (I) is selected, independently, from



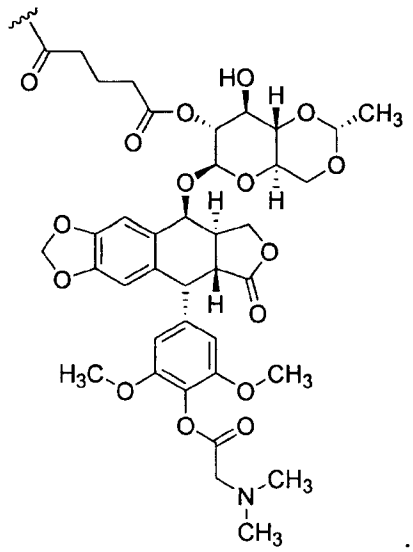
or



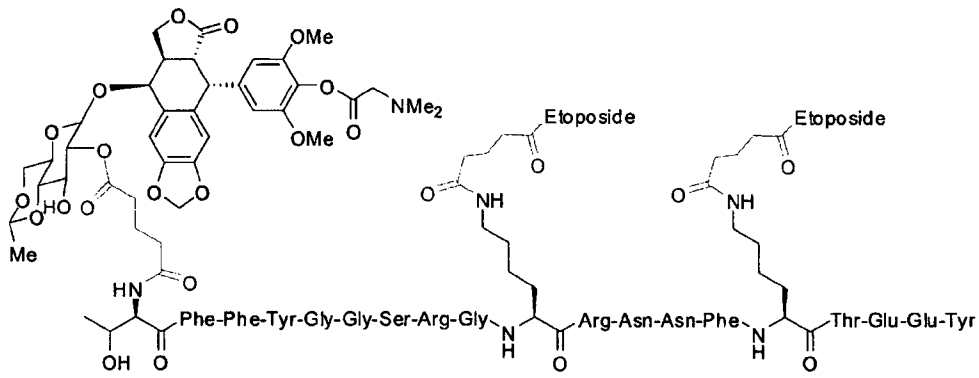
, wherein each R_{8A} and R_{8B} is, independently, H, optionally substituted C_{1-6} alkyl, or R_{8A} and R_{8B} combine to form an optionally substituted 3-7 membered ring.

25. The compound of claim 24, wherein each R_{8A} and R_{8B} is optionally substituted C_{1-6} alkyl.

26. The compound of claim 16, wherein each compound of Formula (I) is



27. The compound of claim 1, wherein said compound has the following structure:



(1),

wherein each "Etoposide" represents etoposide 4'-dimethylglycine, or a pharmaceutically acceptable salt thereof, attached to the carbonyl group at the 2" hydroxyl.

28. A pharmaceutical composition comprising the compound of any of claims 1-27, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

29. A method of treating a cancer, said method comprising administering to a patient a therapeutically effective amount of a compound of any of claims 1-27, or a pharmaceutically acceptable salt thereof.

30. The method of claim 29, further comprising the administration of a second therapeutic agent.

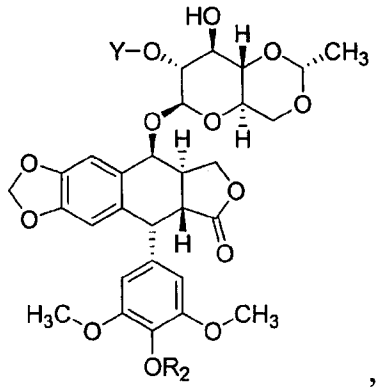
31. The method of claim 30, wherein said administering is concurrent with another therapeutic regime.

32. The method of claim 31, wherein said therapeutic regime is radiation therapy, chemotherapy, stem cell transplantation, bone marrow transplant, surgery, or hyperthermia treatment.

33. The method of any of claims 29-32, wherein said compound, or a pharmaceutically acceptable salt thereof, has the structure

T F F Y G G S R G K R N N F K T E E Y
| | | |
Formula (I) Formula (I) Formula (I) Formula (I) ,

wherein each (—Formula(I)) group represents a covalent bond between the indicated amino acid and a compound of Formula (I) that has the following structure



, wherein

each R_2 is, independently, H, $P(O)(OH)_2$, or $-C(O)R_8$;

each R_8 is, independently, optionally substituted C_{1-6} alkyl;

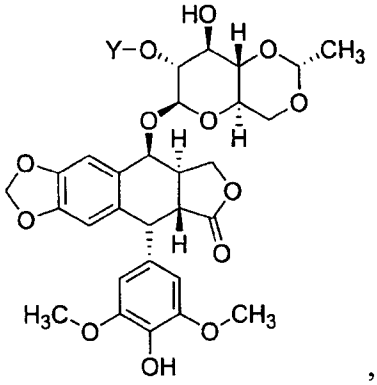
each Y is $-C(O)(CH_2)_nC(O)-$; and

each n is, independently, 2, 3, or 4.

34. The method of claim 33, wherein each R_2 is, independently, H or $P(O)(OH)_2$.

35. The method of claim 33, wherein each R_2 is $-C(O)R_8$ and R_8 is C_{1-6} alkyl comprising an optionally substituted amino group.

36. The method of claim 35, wherein each compound of Formula (I) is selected from:



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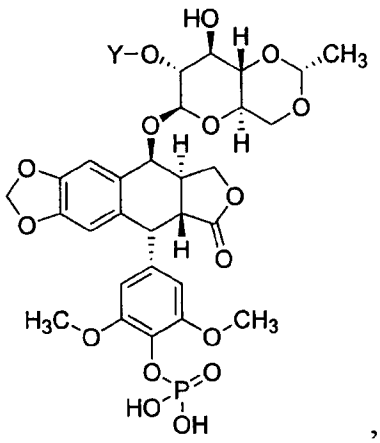
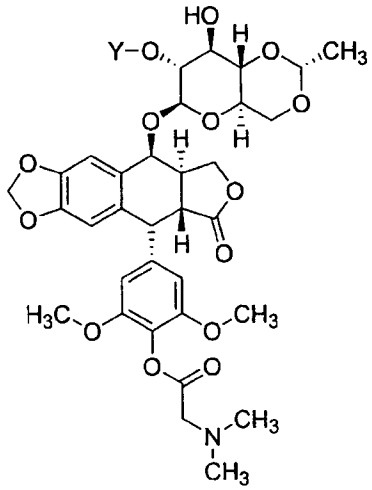


, and



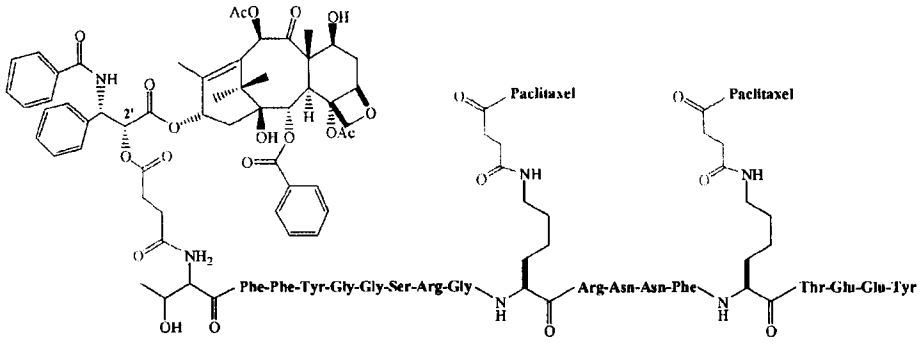
.

37. The method of claim 36, wherein n is 3.

38. The method of any of claims 29-37, wherein said second therapeutic agent is a polypeptide comprising the sequence of Angiopep-2 (SEQ ID NO:97), and wherein said Angiopep-2 is conjugated to an anticancer agent.

39. The method of claim 38, wherein said anticancer agent is paclitaxel.

40. The method of claim 38 or 39, wherein said second therapeutic agent is ANG1005,



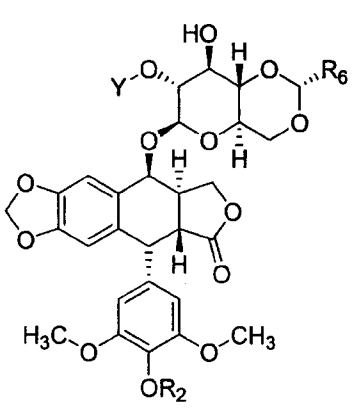
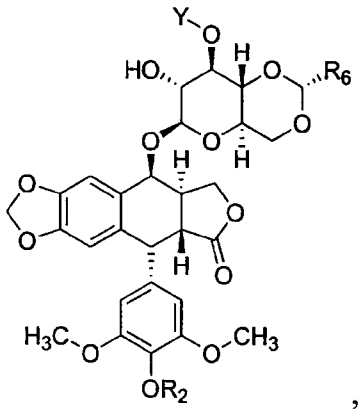
41. The method of claim 29, wherein said cancer is cancer of the brain.

42. The method of claim 41, wherein said cancer is a glioblastoma, a glioma, an acoustic neuroma, an adenoma, an astrocytoma, a choroid plexus papilloma, CNS lymphoma, ependymoma, a gangliocytoma, a ganglioglioma, a medulloblastoma (mdl), an anaplastic (malignant) meningioma, or neurofibromatosis.

43. The method of claim 42, wherein said cancer is glioblastoma.

44. A method of making the compound of any of claims 1-27, said method comprising the step of covalently binding a podophyllotoxin derivative to an amino acid sequence selected from SEQ ID NOS:1-105 and 107-116, or a functional derivative thereof, using a difunctional hydrolyzable linking group.

45. The method of claim 44, wherein said podophyllotoxin derivative is selected from

and , wherein

Y is H;

each R_2 is, independently, H, $P(O)(OH)_2$, or $-C(O)R_8$;

each R_6 is, independently, CH_3 or 2-thiophene; and

each R_8 is, independently, optionally substituted C_{1-6} alkyl.

46. The method of claim 45, wherein each R_2 is, independently, H, $P(O)(OH)_2$, or $-C(O)R_8$, where R_8 is C_{1-6} alkyl comprising an amino substituent.

47. The method of claim 46, wherein said difunctional hydrolyzable linking group is selected from succinic acid, glutaric acid, glutaric anhydride, or butaric acid.

48. The method of claim 47, wherein said amino acid sequence comprises the amino acid sequence of Angiopep-2 (SEQ ID NO:97), Angiopep-4b, Angiopep-5, Angiopep-6, or Angiopep-7 (SEQ ID NOS:109-112).

49. The method of claim 44, wherein

(a) said compound of Formula (I) is first combined with said difunctional hydrolyzable linking group to form a covalent adduct; and

(b) the adduct of (a) is then combined with said amino acid sequence;

wherein the adduct of (a) may be optionally purified prior to use in (b).

50. The method of claim 44, wherein 1.1-3.0 equivalents of said difunctional hydrolyzable linking group is used relative to the compound of Formula (I).

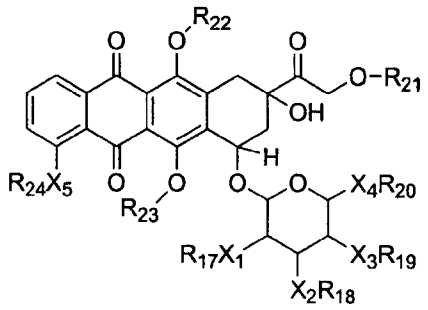
51. The method of claim 44, wherein the difunctional hydrolyzable linking group of (a) is glutaric anhydride.

52. The method of claim 44, wherein said compound of Formula (I) is etoposide, etoposide phosphate, etoposide-4'-dimethylglycine, or teniposide, or a pharmaceutically acceptable salt thereof.

53. The method of claim 44or 49, further comprising the use of a peptide coupling agent.

54. The method of claim 53, wherein said peptide coupling agent is *N,N,N',N'*-tetramethyl-O-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU).

55. A compound, or a pharmaceutically acceptable salt thereof, comprising an amino acid sequence substantially identical to an amino acid sequence selected from the group consisting of SEQ ID NOS:1-105 and 107-116, or a functional derivative thereof, wherein said amino acid sequence comprises a covalent bond from an amino acid of said amino acid sequence to a doxorubicin derivative, and wherein said doxorubicin derivative is a compound having a structure according to Formula (II):



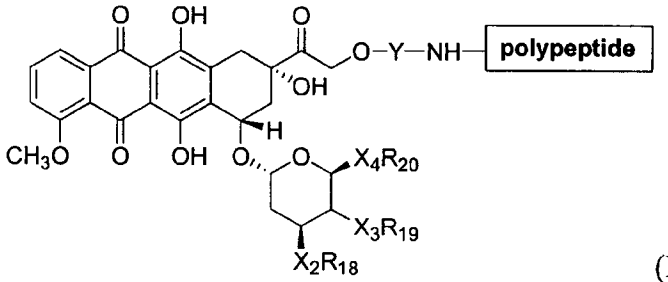
(II), or a stereoisomer or pharmaceutically acceptable salt thereof, wherein

each X_1, X_2, X_3, X_4, and X_5 is selected, independently, from a covalent bond, O, or NR_{25};

each R_{17}, R_{18}, R_{19}, R_{20}, R_{20}, R_{21}, R_{22}, R_{23}, R_{24}, and R_{25}, is selected, independently, from H, optionally substituted C_{1-6} alkyl, optionally substituted C_{2-6} alkenyl, optionally substituted C_{2-6} alkynyl, optionally substituted cycloalkyl, optionally substituted heterocyclyl, or is a hydrolyzable linker Y; and

wherein one and only one of R_{17}, R_{18}, R_{19}, R_{20}, R_{20}, R_{21}, R_{22}, R_{23}, R_{24}, and R_{25} is Y.

56. The compound of claim 55, wherein the compound of Formula (II) has the following structure:



(II-A), wherein

X_2R_{18} is H or NH_2;

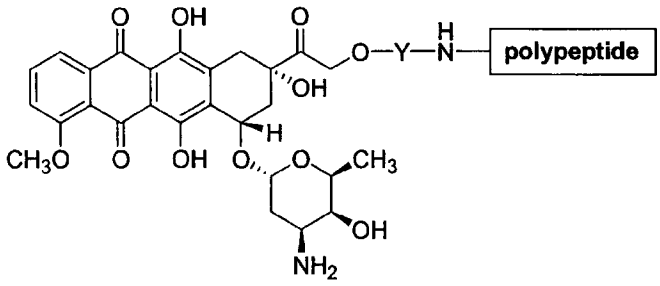
X_3R_{19} is H or OH;

X_4R_{20} is H or optionally substituted C_{1-3} alkyl;

Y is $-C(O)(CH_2)_nC(O)-$;

and n is 2, 3, or 4.

57. The compound of claim 56, wherein said compound has the following structure:



58. The compound of any of claims 55-57, wherein wherein Y is –$C(O)(CH_2)_nC(O)$– and n is 2, 3, or 4.

59. The compound of claim of any of claims 55-57, wherein said amino acid sequence is covalently bonded to a compound having a structure according to Formula (II) through a second amino acid of said amino acid sequence.

60. The compound of claim of any of claims 55-57, wherein said amino acid sequence is covalently bonded to a compound having a structure according to Formula (I) through a third amino acid of said amino acid sequence.

61. The compound of claim 60, wherein each amino acid covalently bonded to said hydrolyzable linker Y is attached via an amino-, a guanidino-, a hydroxyl-, a phenol-, or a thiol functional group of said amino acid.

62. The compound of claim 61, wherein each amino acid covalently bonded to said hydrolyzable linker Y is attached via an amino functional group.

63. The compound of claim 61, wherein said amino acid is lysine or threonine.

64. The compound of any of claims 55-57, wherein said amino acid sequence has at least 50% identity to an amino acid sequence selected from the group

consisting of Angiopep-2 (SEQ ID NO:97), Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7 (SEQ ID NOS:109-112).

65. The compound of claim 64, wherein said amino acid sequence has at least 70% identity to the amino acid sequence of Angiopep-2 (SEQ ID NO:97).

66. The compound of claim 65, wherein said amino acid sequence has at least 90% identity to the amino acid sequence of Angiopep-2 (SEQ ID NO:97).

67. The compound of claim 66, wherein said amino acid sequence comprises the amino acid sequence of Angiopep-2 (SEQ ID NO:97), Angiopep-4b, Angiopep-5, Angiopep-6, or Angiopep-7 (SEQ ID NOS:109-112).

68. The compound of claim 67, wherein said amino acid sequences consists of the amino acid sequence of Angiopep-2 (SEQ ID NO:97), Angiopep-4b, Angiopep-5, Angiopep-6, or Angiopep-7 (SEQ ID NOS:109-112).

69. The compound of claim 68, wherein said amino acid sequence consists of the amino acid sequence of Angiopep-2 (SEQ ID NO:97).

70. The compound of any of claims 55-57, having the following structure

T F F Y G G S R G K R N N F K T E E Y

| | |

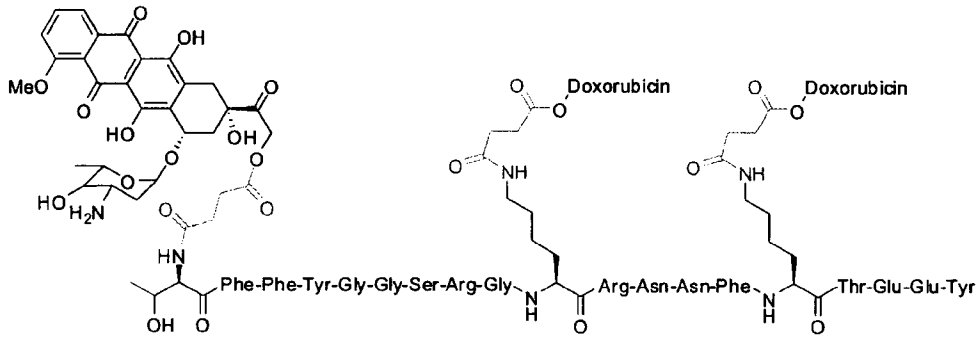
Formula (II) Formula (II) Formula (II)

wherein each (—(Formula(II)) represents an optional covalent bond between the indicated amino acid and a compound of Formula (II), or a pharmaceutically acceptable salt thereof, and wherein there is at least one covalent bond between an amino acid of the polypeptide and said compound of Formula (II).

71. The compound of claim 70, wherein the threonine at position 1 and the lysines at positions 10 and 15 of the polypeptide each comprise a covalent bond to a compound having a structure according to Formula (II).

72. The compound of claim 70, wherein Y is $-C(O)(CH_2)_nC(O)-$ and n is 2.

73. The compound of claim 55, wherein said compound has the following structure:



(2), wherein each doxorubicin, or a pharmaceutically acceptable salt thereof, is attached by the succinic group at the C14 hydroxyl group.

74. The compound of claim 73, wherein said compound comprises the hydrochloride salt of 1, 2, or 3 of the covalently attached doxorubicin moieties.

75. The compound of claim 73 or 74, wherein said compound is the trihydrochloride salt of compound (2).

76. A pharmaceutical composition comprising the compound of any of claims 55-75 and a pharmaceutically acceptable carrier.

77. A method of treating a cancer, said method comprising administering to a patient a therapeutically effective amount of a compound of any of claims 55-75.

78. The method of claim 77, further comprising the administration of a second therapeutic agent.

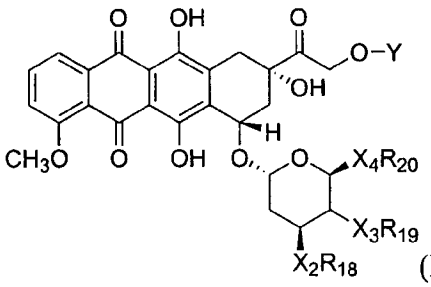
79. The method of claim 78, wherein said administering is concurrent with another therapeutic regime.

80. The method of claim 79, wherein said therapeutic regime is radiation therapy, chemotherapy, stem cell transplantation, bone marrow transplant, surgery, or hyperthermia treatment.

81. The method of any of claims 77-80, wherein said compound has the structure

T F F Y G G S R G K R N N F K T E E Y
| | |
Formula (II) Formula (II) Formula (II) ,

wherein each (—Formula(II)) group represents a covalent bond between the indicated amino acid and a compound of Formula (II), or a pharmaceutically acceptable salt thereof, that has the following structure



(II-A), wherein

X_2R_{18} is H or NH_2;

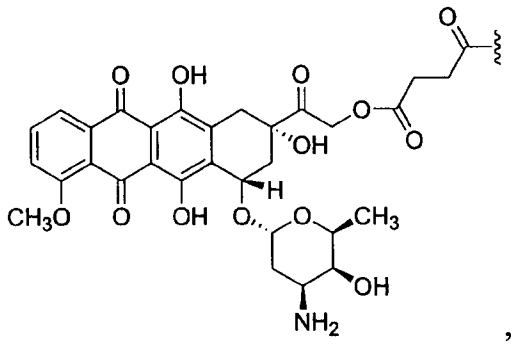
X_3R_{19} is H or OH;

X_4R_{20} is H or optionally substituted C_{1-3} alkyl;

Y is $-C(O)(CH_2)_nC(O)-$;

and n is 2, 3, or 4.

82. The method of claim 81, wherein said compound has the following structure:

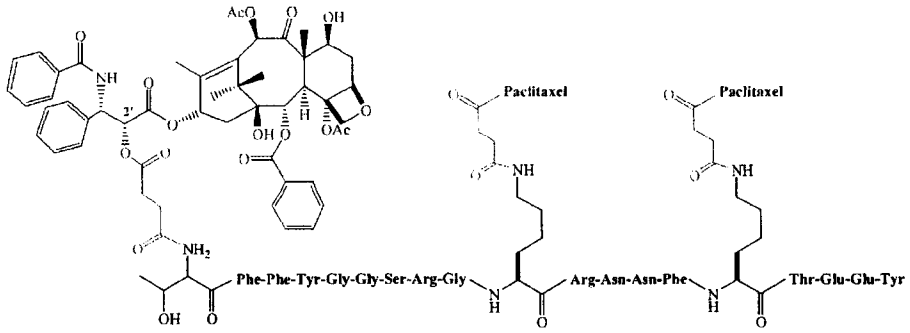


, or a pharmaceutically acceptable salt thereof.

83. The method of any of claims 77-82, wherein said second therapeutic agent is a polypeptide comprising the sequence of Angiopep-2 (SEQ ID NO:97), and wherein said Angiopep-2 is conjugated to an anticancer agent.

84. The method of claim 83, wherein said anticancer agent is paclitaxel.

85. The method of claim 83 or 84, wherein said second therapeutic agent is ANG1005, which has the following structure



86. The method of claim 77, wherein said cancer is acute lymphocytic leukemia, acute myeloblastic leukemia, adrenocortical cancer, intravenous and

intravesical bladder cancer, bone sarcoma, breast cancer, carcinoid syndrome (small bowel), endometrial cancer, Ewing's sarcoma, gynecological sarcoma, head and neck cancer (squamous cell), hepatic cancer, Hodgkin's disease, islet cell cancer, leukemia, lung cancer, malignant lymphoma, multiple myeloma, neuroblastoma, non-Hodgkin's lymphoma, pancreatic cancer, prostate cancer, osteogenic sarcoma, ovarian cancer, retinoblastoma, rhabdomyosarcoma, stomach cancer, testicular cancer, thyroid cancer, transitional cell bladder carcinoma, soft tissue sarcoma, or Wilms' tumor.

87. The method of claim 29, wherein said method comprises subtherapeutic dosing of the podophyllotoxin derivative, or a pharmaceutically acceptable salt thereof, or claim 77, wherein said method comprises subtherapeutic dosing of the doxorubicin derivative, or a pharmaceutically acceptable salt thereof.

88. The method of claim 87, wherein said method comprises subtherapeutic dosing of etoposide or etoposide 4'-dimethylglycine.

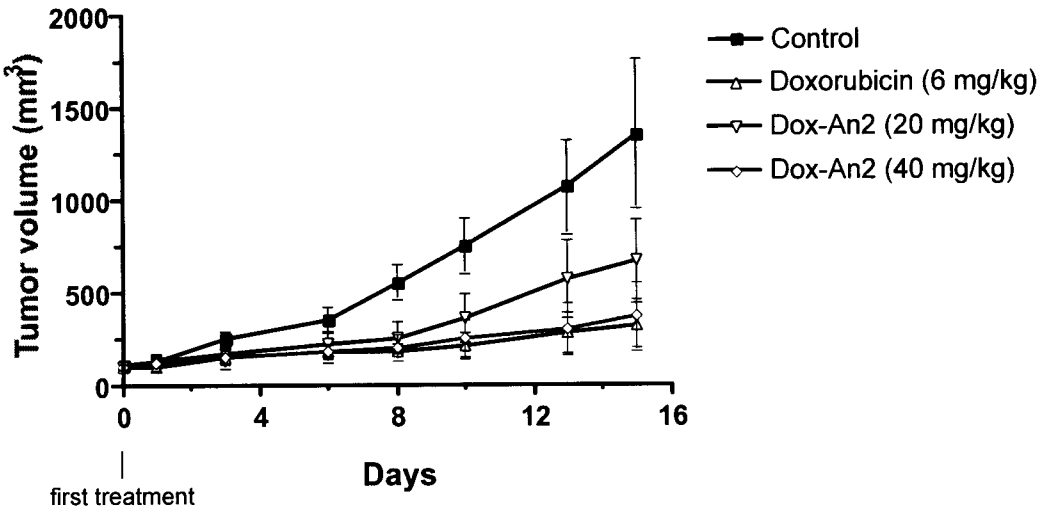
89. The method of claim 87, wherein said method comprises subtherapeutic dosing of doxorubicin.

90. The method of claim 29, wherein said method comprises supertherapeutic dosing of the podophyllotoxin derivative or claim 77, wherein said method comprises supertherapeutic dosing of the doxorubicin derivative.

91. The method of claim 90, wherein said method comprises supertherapeutic dosing of etoposide or etoposide 4'-dimethylglycine.

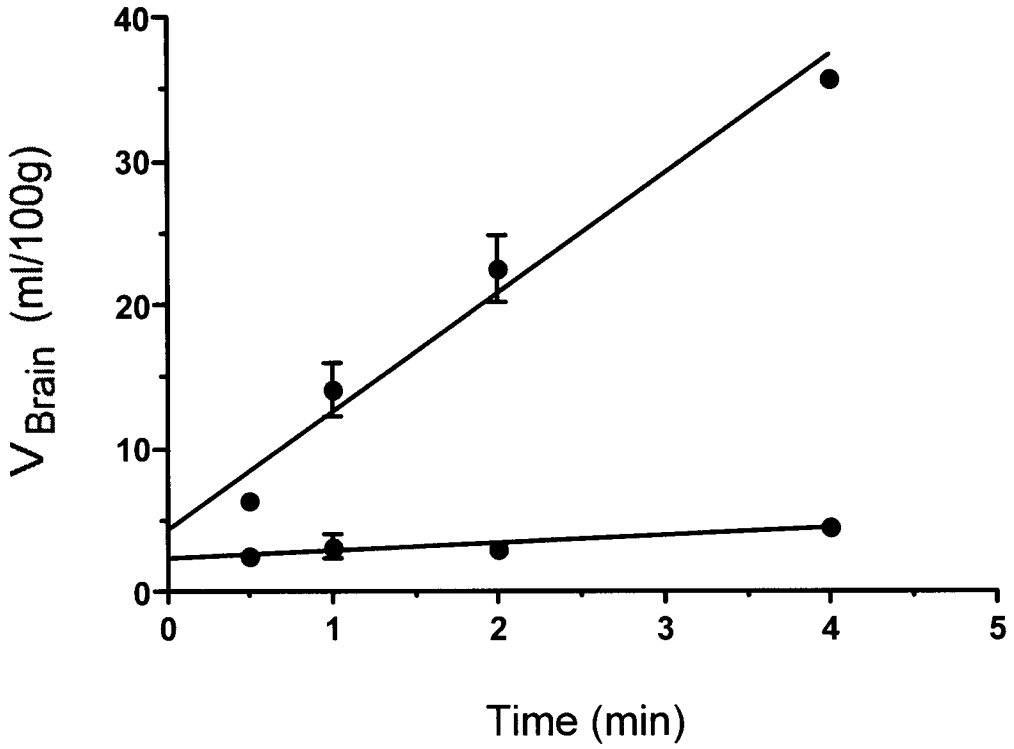
92. The method of claim 90, wherein said method comprises supertherapeutic dosing of doxorubicin.

Fig. 1



Mice (n=5 per group) were treated once a week for 3 weeks

Fig. 2A

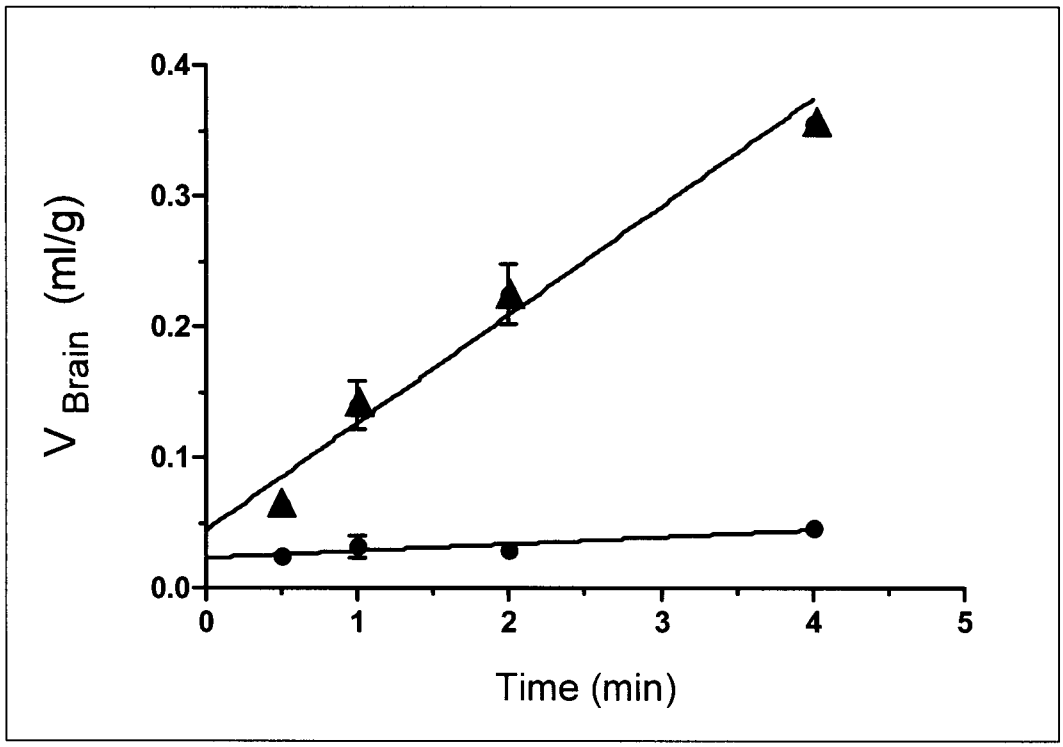


	K_{in} (ml/100g/min)
Etoposide-Angiopep-2:	8.26
Etoposide:	0.54
K_{in} ratio :	15.3

——— Etoposide-Angiopep-2(3:1)

——— Etoposide (unconjugated)

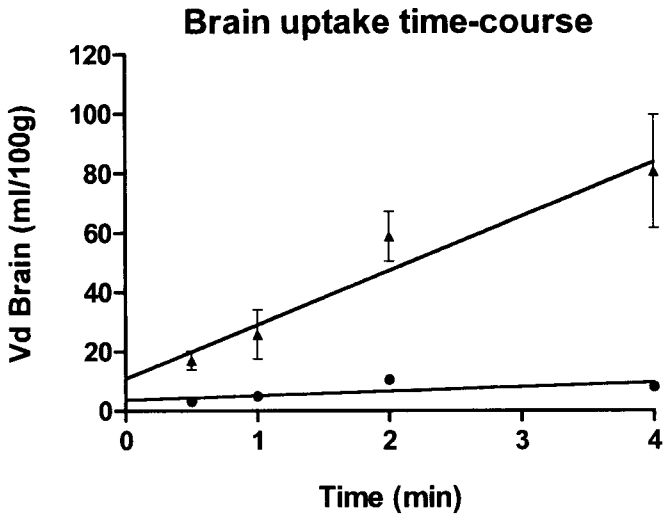
FIG. 2B



Etoposide$_{DMG}$-Angiopep-2(3:1)

Etoposide$_{DMG}$ (unconjugated)

Fig. 2C

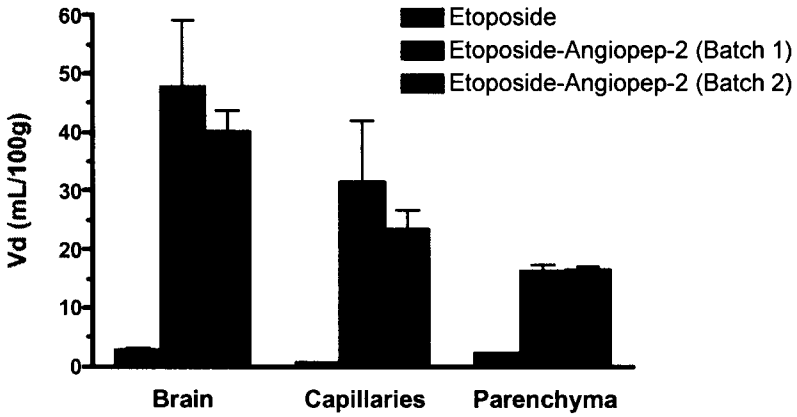


▲ Doxorubicin-An2 Kin= 3.7×10^{-3} mL/g/s

◆ Doxorubicin Kin= 2.5×10^{-4} mL/g/s

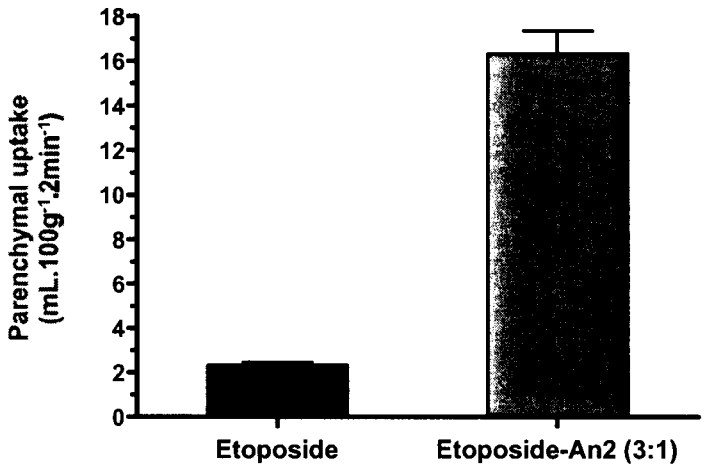
Ratio 15

Fig. 3


Etoposide
Etoposide-Angiopep-2 (Batch 1)
Etoposide-Angiopep-2 (Batch 2)
Vd (mL/100g)
60
50
40
30
20
10
0
Brain
Capillaries
Parenchyma

Figs. 4A and 4B

(4A)



(4B)

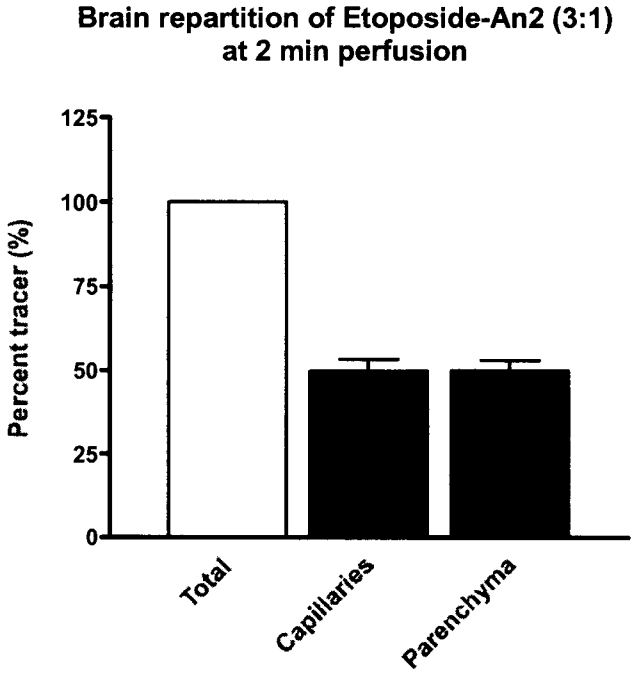


Fig. 5

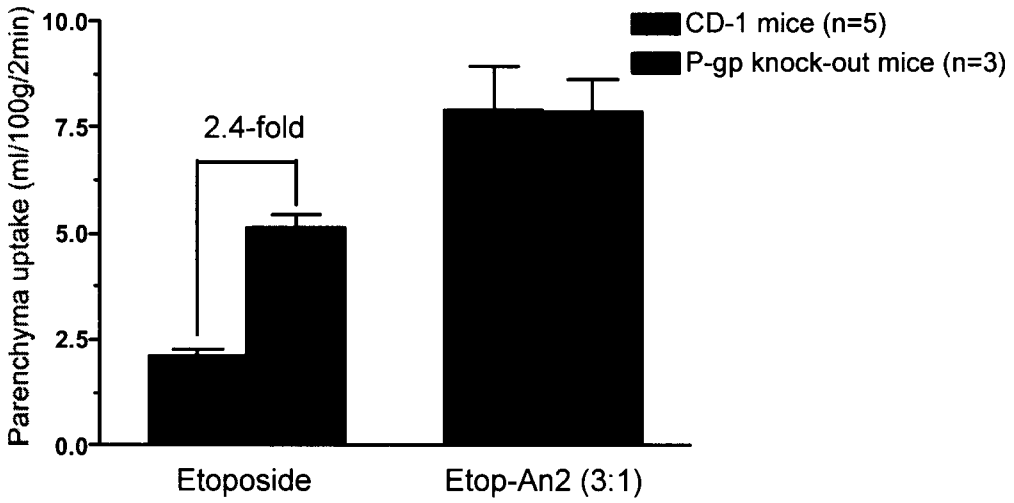

CD-1 mice (n=5)
P-gp knock-out mice (n=3)
Parenchyma uptake (ml/100g/2min)
10.0
7.5
5.0
2.5
0.0
2.4-fold
Etoposide
Etop-An2 (3:1)

Fig. 6

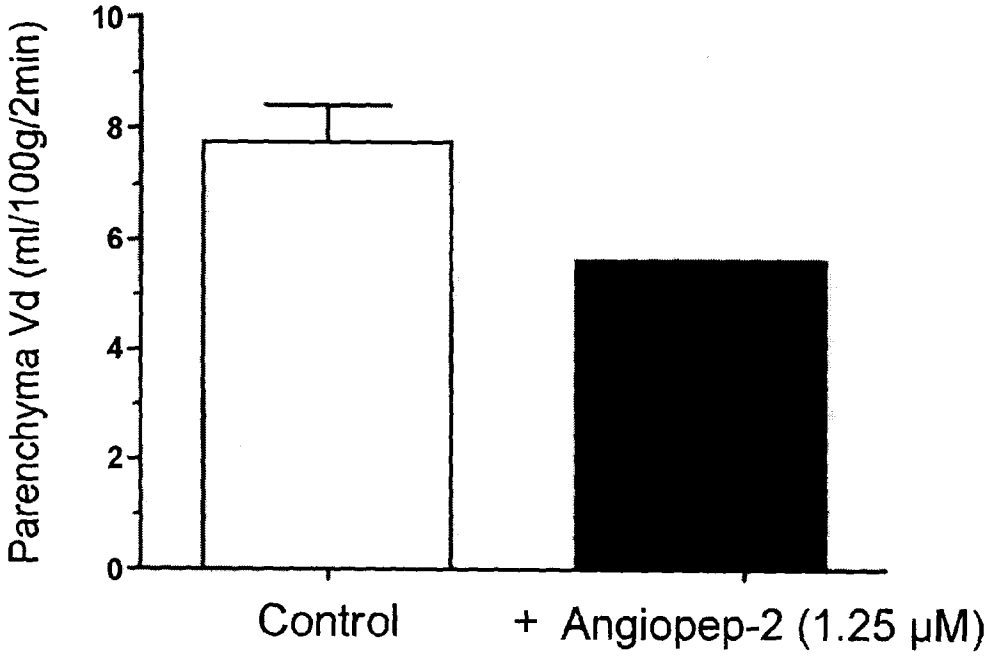

Parenchyma Vd (ml/100g/2min)
10
8
6
4
2
0
Control
+ Angiopep-2 (1.25 µM)

Fig. 7

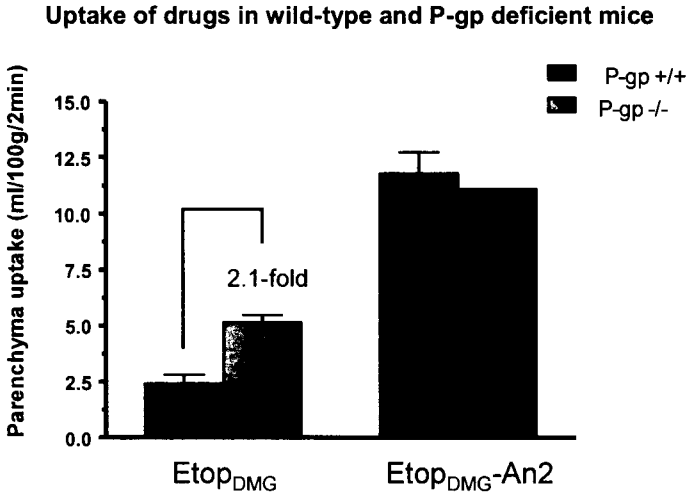
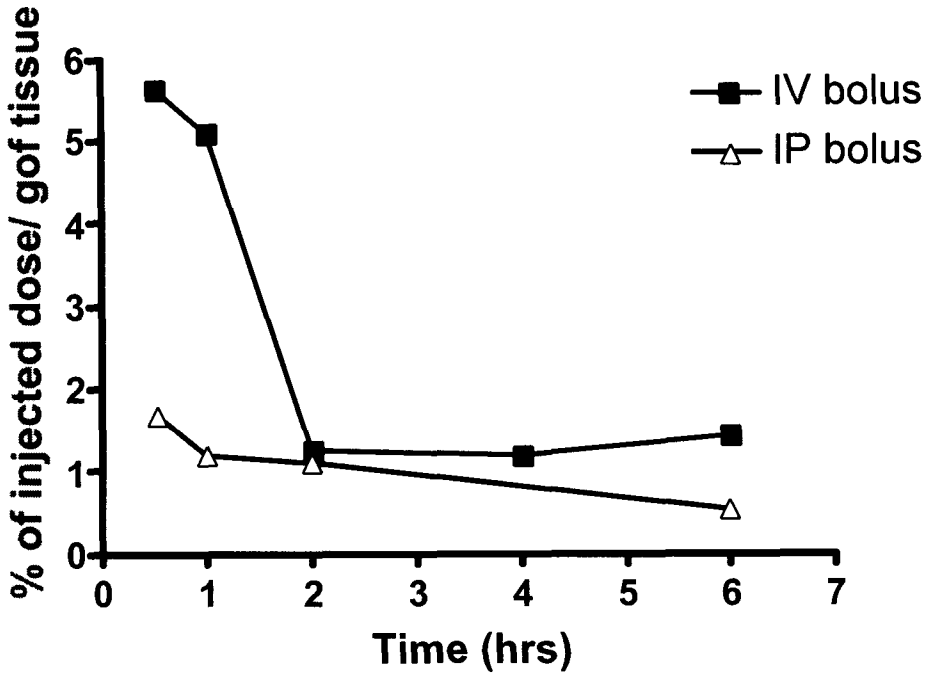

Uptake of drugs in wild-type and P-gp deficient mice
P-gp +/+
P-gp -/-
Parenchyma uptake (ml/100g/2min)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
2.1-fold
Etop$_{DMG}$
Etop$_{DMG}$-An2

Fig. 8



Estimated bioavailability after i.p. administration

$(AUCi.p./AUC_{i.v.})x100=(5.1/11)x100=46\%$

Fig. 9

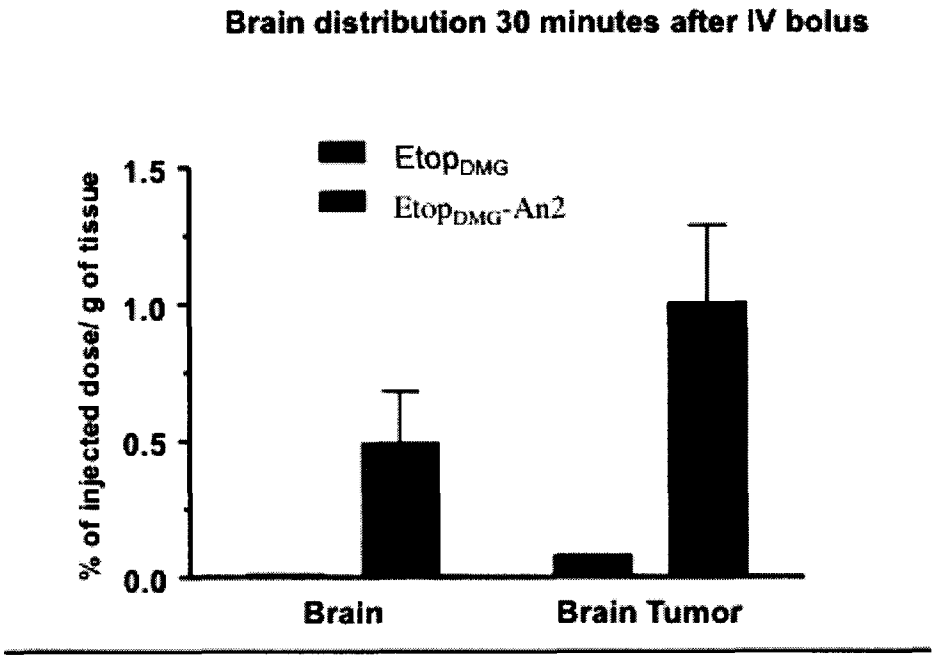

Brain distribution 30 minutes after IV bolus
Etop$_{DMG}$
Etop$_{DMG}$-An2
% of injected dose/ g of tissue
1.5
1.0
0.5
0.0
Brain
Brain Tumor

Fig. 10

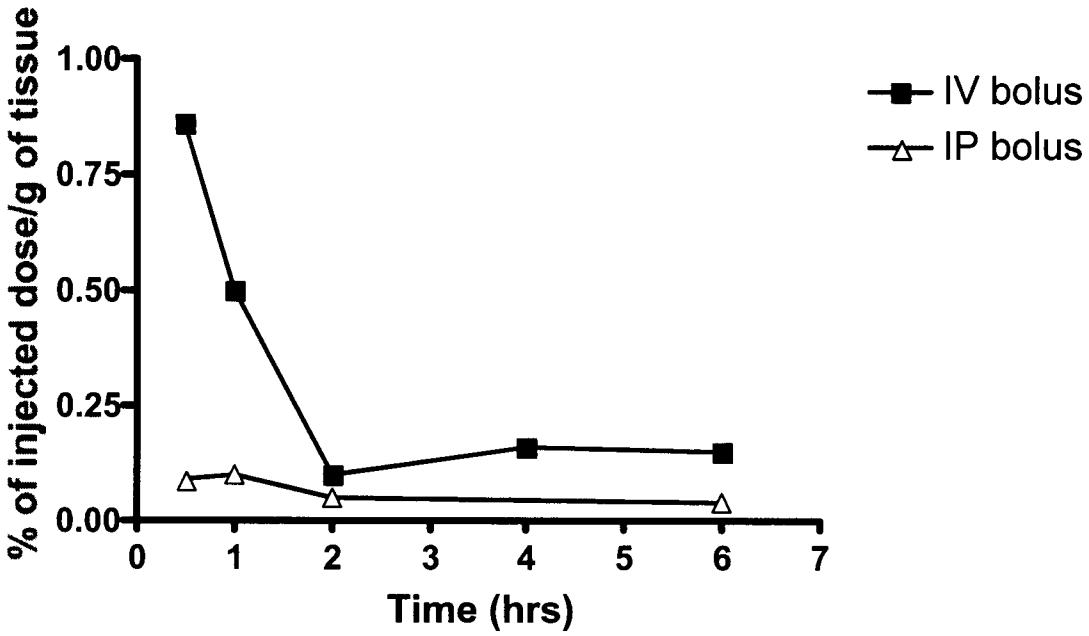

% of injected dose/g of tissue
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
Time (hrs)
IV bolus
IP bolus

Fig. 11

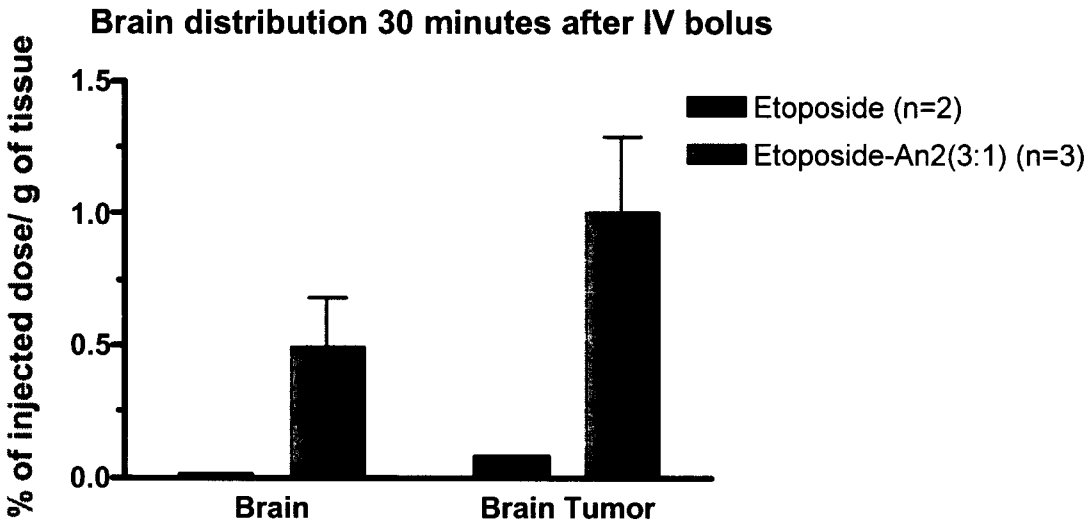

Brain distribution 30 minutes after IV bolus
% of injected dose/ g of tissue
1.5
1.0
0.5
0.0
Brain
Brain Tumor
Etoposide (n=2)
Etoposide-An2(3:1) (n=3)

Fig. 12

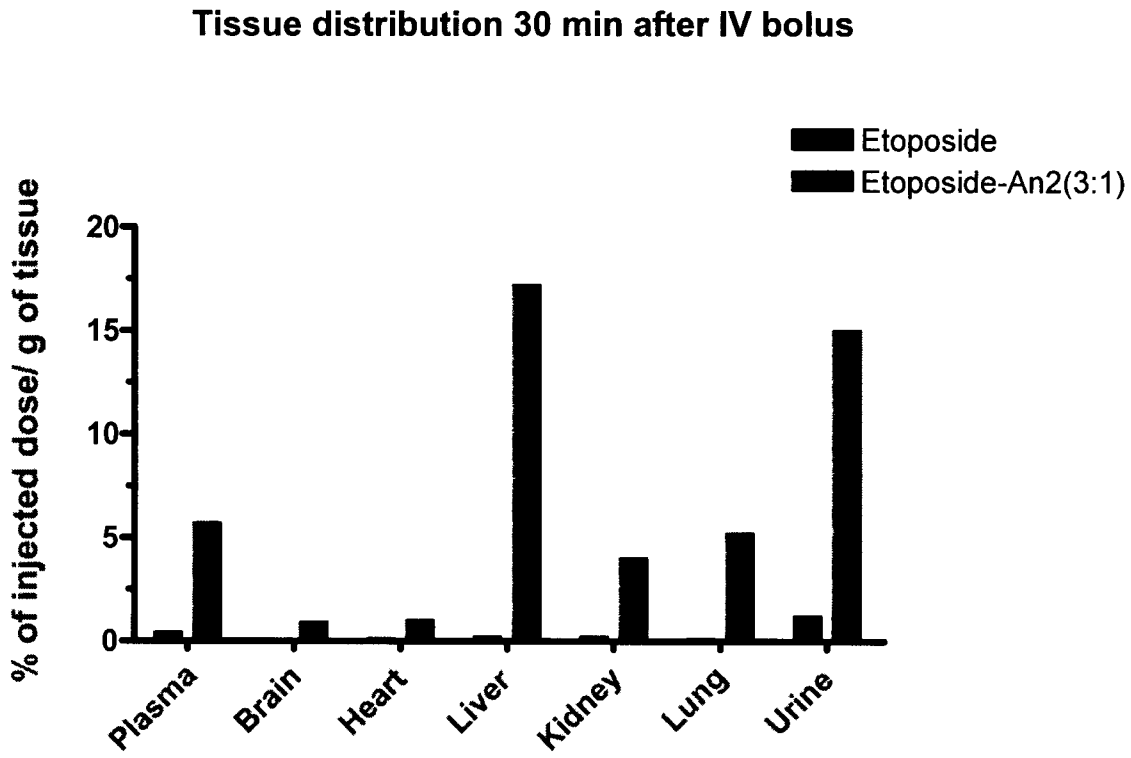

Tissue distribution 30 min after IV bolus
Etoposide
Etoposide-An2(3:1)
% of injected dose/ g of tissue
20
15
10
5
0
Plasma
Brain
Heart
Liver
Kidney
Lung
Urine

Fig. 13A

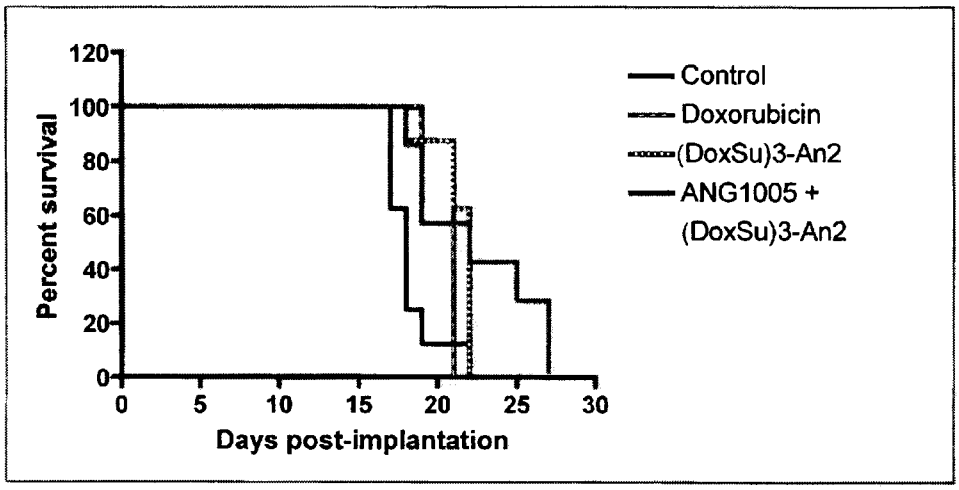
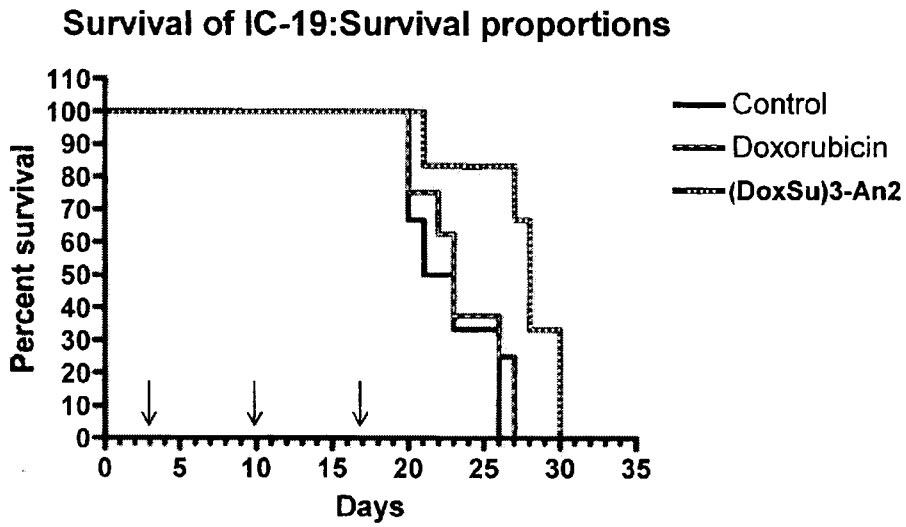


Fig. 13B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2009/001481

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C07K 14/81*** (2006.01), ***A61K 31/704*** (2006.01), ***A61K 31/7048*** (2006.01), ***A61K 47/42*** (2006.01), ***A61K 47/48*** (2006.01), ***A61P 35/00*** (2006.01), ***C07H 15/252*** (2006.01), ***C07H 17/04*** (2006.01), ***C07K 14/00*** (2006.01), ***C07K 7/08*** (2006.01), *C07K 14/705* (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K, A61K, A61P, C07H

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Databases: PubMed, Scopus, Delphion, Canadian Patent Database; Search terms: aprotinin, angiopep, etoposide, pdodphyllotoxin, doxorubicin, conjugate, coupl*, peptide, polypeptide, blood brain barrier, brain tumor, anti-cancer, anti-tumor (in various combinations).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/009229 A1 (BELIVEAU, R. et al) 25 January 2007 (25-01-2007)	1-28, 44-72, 76
X	WO 2006/086870 A1 (BELIVEAU, R. et al) 24 August 2006 (24-08-2006)	1-28, 44-72, 76
Y	GABIUS, H.J. et al. Targeting of Neoglycoprotein-Drug Conjugates to Cultured Human Embryonal Carcinoma Cells. J. Cancer Res. Clin. Oncol. 1987, Vol.113, No.2, pp126-130, ISSN: 0171-5216.	1-28, 44-54

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 December 2009 (18-12-2009)	5 January 2010 (05-01-2010)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Riad Qanbar (819) 934-7937

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   [X] on paper

   [X] in electronic form

   b. (time)

   [ ] in the international application as filed

   [ ] together with the international application in electronic form

   [X] subsequently to this Authority for the purposes of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments :

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2009/001481

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [X] Claim Nos. : 29-43 and 77-92
because they relate to subject matter not required to be searched by this Authority, namely :

Claims 29-43 and 77-92 are directed to a method for treatment of the human or animal body by surgery or therapy, which the International Search Authority is not required to search under Rule of the PCT 39.1 (iv). However, this Authority has carried out a search based on the alleged effects or purposes/uses of the product defined in claims 1-27 and 55-75.

2. [X] Claim Nos. : 1-14, 28-32, 38-68, 76-80, 83-92 insofar as they relate to the peptides of SEQ ID NOS 1-96, 98-105 and 107-116
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

These claims are so inadequately supported by the description that no meaningful opinion could be formed.

3. [ ] Claim Nos. :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

The claims are directed to a plurality of inventions as follows:

Group 1 - Claims 1-14, 28-32, 38-54, 87-92 (partially) and claims 15-27, 33-37 (completely) are directed to a targeting polypeptide of SEQ ID NO: 97, also known as angiopep-2, conjugated to a podophyllotoxin derivative of formula I and methods for its manufacture and use;

Continued in Supplemental Box.

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RÉGINA A, et al. Antitumour Activity of Ang1005, a Conjugate Between Paclitaxel and the New Brain Delivery Vector Angiopep-2. Br. J. Pharmacol. September 2008, Vol.155, No.2, pp185-197, ISSN:0007-1188.	1-28, 44-72, 76
X,P --- X	ANONYMOUS. Blood-Brain Barrier Tackled. *ecancermedicalscience*, 22 October 2008, [retrieved on 2009-12-18]. Retrieved from the Internet: <URL:http://www.ecancermedicalscience.com/news-insider-news.asp?itemId=326>	1-26, 28 55-72, 76
Y	TRAIL, P.A. et al. Cure of Xenografted Human Carcinomas by Br96-doxorubicin Immunoconjugates. Science. 1993, Vol.261, pp.212-215, ISSN:0036-8075.	55-72, 76
Y	ROUSSELLE. C. et al. New Advances in the Transport of Doxorubicin Through the Blood-Brain Barrier by a Peptide Vector-Mediated Strategy. Mol. Pharmacol. 2000, Vol.57, No.4, pp679-686, ISSN:0026:895X.	55-72, 76
Y	GARSKY, V.M. et Al. The Synthesis of a Prodrug of Doxorubicin Designed to Provide Reduced Systemic Toxicity and Greater Target Efficacy. J. Med. Chem. 2001, Vol.44, No.24, pp4216-4224, ISSN:0022-2623.	55-72, 76
A	CHARI, R.V. Targeted Cancer Therapy: Conferring Specificity to Cytotoxic Drugs. Acc. Chem. Res. January 2008, Vol.41, No.1, pp98-107, ISSN:0001-4842.	1-28, 44-76

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2007009229A1	25-01-2007	AU2005327497A1	24-08-2006
		AU2006272405A1	25-01-2007
		BRPI0520032A2	14-04-2009
		CA2597958A1	24-08-2006
		CA2614687A1	25-01-2007
		CN101160403A	09-04-2008
		CN101262890A	10-09-2008
		EP1859041A1	28-11-2007
		EP1859041A4	08-10-2008
		EP1907009A1	09-04-2008
		EP1907009A4	02-09-2009
		JP2008529539T	07-08-2008
		JP2009500431T	08-01-2009
		MX2007010113A	07-12-2007
		RU2007134566A	27-03-2009
		RU2008105677A	20-08-2009
		US7557182B2	07-07-2009
		US2006189515A1	24-08-2006
		US2008299039A1	04-12-2008
		US2009016959A1	15-01-2009
		US2009082277A1	26-03-2009
		WO2006086870A1	24-08-2006
		WO2008144919A1	04-12-2008
		ZA200706917A	29-10-2008
WO2006086870A1	24-08-2006	AU2005327497A1	24-08-2006
		AU2006272405A1	25-01-2007
		BRPI0520032A2	14-04-2009
		CA2597958A1	24-08-2006
		CA2614687A1	25-01-2007
		CN101160403A	09-04-2008
		CN101262890A	10-09-2008
		EP1859041A1	28-11-2007
		EP1859041A4	08-10-2008
		EP1907009A1	09-04-2008
		EP1907009A4	02-09-2009
		JP2008529539T	07-08-2008
		JP2009500431T	08-01-2009
		MX2007010113A	07-12-2007
		RU2007134566A	27-03-2009
		RU2008105677A	20-08-2009
		US7557182B2	07-07-2009
		US2006189515A1	24-08-2006
		US2008299039A1	04-12-2008
		US2009016959A1	15-01-2009
		US2009082277A1	26-03-2009
		WO2007009229A1	25-01-2007
		WO2008144919A1	04-12-2008
		ZA200706917A	29-10-2008

Continuation of Box No. III Observations where unity of invention is lacking

Groups 2-115 - Claims 1-14, 28-32, 38-54, 87-92 (all partially) are directed to a targeting polypeptide/anti-cancer-drug conjugate, wherein the polypeptide is of SEQ ID NO: 1-96, 98-105, 107-116, respectively, and the anti-cancer drug is a podophyllotoxin derivative of formula I, as well as methods for the manufacture and use of said conjugate;

Group 116 - Claims 55-68, 76-80, 83-92 (partially) and claims 69-75, 81, 82 (completely) are directed to a targeting polypeptide of SEQ ID NO: 97, also known as angiopep-2, conjugated to a doxorubicin derivative of formula II and methods for its manufacture and use;

Groups 117-230 - Claims 55-68, 76-80, 83-92 (all partially) are directed to a targeting polypeptide/anti-cancer-drug conjugate, wherein the polypeptide is of SEQ ID NO: 1-96, 98-105, 107-116, respectively, and the anti-cancer drug is a doxorubicin derivative of formula II, as well as methods for the manufacture and use of said conjugate.

The subject matter of groups 1 to 230 relates to conjugates between targeting polypeptides and anti-cancer drugs. However, conjugation of targeting polypeptide to anti-cancer drugs was known in the art on the relevant date (see, for example, the documents cited on pages 4 and 5 of this International Search Report). Accordingly, target polypeptide conjugation to an anti-cancer drug cannot serve as a unifying feature for the subject matter of groups 1 to 230.

Even though the subject matter of groups 1 to 115 relates to targeting polypeptides conjugated to a podophyllotoxin derivative of formula I, the common feature of these groups is not novel. Targeting polypeptides covalently linked to a podophyllotoxin derivative of formula I, such as etoposide, were described in the art before the relevant date (see, for example, GABIUS et al, cited on page 4 of this International Search Report). The same argument applies to the subject matter of groups 116-230, which relate to conjugates of targeting polypeptides with doxorubicin (see, for example, any one of TRAIL et al, ROUSSELLE, et al, and GARSKY et al; cited on page 5 of this report).

The subject matter of group 1 and group 116 –which are the only groups for which there is substantive support in the description– relates to angiopep-2 (SEQ ID NO: 97) conjugated to an anti-cancer drug. However, angiopep-2 conjugates with an anti-cancer drug were known in the art before the relevant date (see for example, RÉGINA et al, cited on page 5 of this International Search Report). Therefore, anti-cancer drug conjugation to angiopep-2 cannot serve as a unifying feature for the subject matter of groups 1 and 116.

In view of the above, each of groups 1 to 230 is considered a separate alleged invention.

The claims must be limited to one inventive concept as set out in Rule 13 of the PCT.

This International Search Report was established on the basis of the subject matter of claims 1-92 insofar as they relate to groups 1 and 116.